PATHOLOGICAL STUDIES ON THE FOWL WITH SPECIAL REFERENCE TO NEOPLASIA

by

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Thesis presented for the Degree of Doctor of Science to the University of Edinburgh.

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INTRODUCTION

The following 25 papers represent studies on a number of different aspects of disease in the domesticated fowl. As the majority are on the subject of neoplasia, they will be summarised first. They are divisible into studies from the aspects of comparative pathology (Nos. 1, 2, 3, 6, 13); the classification of the leucoses (Nos. 9, 12, 18); the tumour viruses (Nos. 7, 8); chemical induction experiments (Nos. 10, 11); enzyme histochemistry (No.5.); and endocrine studies (Nos. 14, 15, 16, 17).

In addition, there are five papers of joint authorship on miscellaneous subjects. The earlier papers are based on work done while the author was in charge of the Poultry Diseases Department at the (then) Royal (Dick) Veterinary College, whilst the later papers are the result of work undertaken subsequent to appointment as Research Officer in the British Empire Cancer Campaign Unit at the A.R.C. Poultry Research Centre, Edinburgh. The paper on Bangkok Haemorrhagic Disease (No.20) is based on material collected while working in an advisory capacity to the Government of Thailand, for the Food and Agricultural Organisation of the United Nations.

LIST OF PAPERS

1. Myelocytoma in the domestic fowl. 1942. Vet. J. 96 (2).

2. Embryonal nephroma in the domestic fowl. 1942. Vet. J. 98 (5).

3. Histiocytic and Fibroplastic Sarcoma (Mixed-cell Sarcoma) in the domestic fowl. 1943. J. Comp. Path. Therap. 53 (4).

4. Neoplastic disease of the fowl, with special reference to its history, incidence, and seasonal variation. 1945. J. Comp. Path. Therap. 55 (4).

5. The intracellular localisation of β-Glucuronidase. 1950. Brit. J. exp. Path. 30, 548.

6. Some unusual gonadal tumours of the fowl. 1951. Brit. J. Cancer, 5 (1), 69.

7. The occurrence of blood filaments (pseudospirochaetes) in certain neoplastic conditions. 1952. Brit. Vet. J. 108 (6), 191.

8. Observations on the "Eclipse Phase" in a Virus-Associated Leukaemia of the Chicken. 1954. Brit. J. Cancer, 8, 737.

9. Avian Leucosis: A Plea for Clarification. 1954. Off. Rep. Xth World's Poult. Congr. Section C. p. 193.

10. Induction of Multiple Primary Tumours in Fowls with 2-Acetamidofluorene. 1954. Brit. J. Cancer, 9, 163.

11. An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort (Senecio jacobaea Linn.) with special reference to the Induction of Liver Tumours with Seneciphylline. 1956. Proc. Roy. Soc. Edin. B. 66, 111.

12. Leucosis and Fowl Paralysis Compared and Contrasted. 1956. Vet. Rec. 68, 527.

13. Two cases of Rhabdomyosarcoma in the Fowl. 1956. Brit. J. Cancer, 10, 700.

14. Studies on the influence of sex hormones on the avian liver. I. Sexual differences in Avian Liver Clearance Curves. 1957. J. Endocrinol. 15, 339.

15. Studies on the influence of sex hormones on the avian liver. II. Acute liver damage in the male Fowl, and the protective effect of oestrogen, as determined by a liver function test. 1957. J. Endocrinol. 15, 346.

16. Studies on the influence of sex hormones on the avian liver. III. Oestrogen-induced Regeneration of the Chronically Damaged Liver. 1957. J. Endocrinol. 15, 351.

17. The Detection and Identification of Avian Gonadal Tumours by means of a Liver Clearance Test. 1958. In Press.

18. Recent Investigations on Avian Leucosis and Fowl Paralysis. 1958. Vet. Rec. 70, 64.

19. An Infectious Enteritis of Young Turkeys associated with Cochlosoma sp. 1945. Vet. J. 101. (12)

20. Bangkok Haemorrhagic Disease. An Unusual Condition associated with an organism of uncertain Taxonomy. 1953. J. Path. Bact. 68 (2), 423.

21. (with SLOAN, J. E. N.) A possible new Species of Trematode Parasite in the kidneys of the King Penguin (Aptenodytes longirostris). 1943 Vet. J. 99 (2).

22. (with ROBERTSON, A. & GRAVES, D. N.) Experimental Zinc Phosphide Poisoning in Fowls. 1945. J. Comp. Path. Therap. 55 (4).

23. (with LEVVY, G. A. & KERR, L. M. H.) β-Glucuronidase and Cell Proliferation. 1948. Biochem. J. 42 (3),462.

24. (with KERR, L. M. H. & LEVVY, G. A.) β-Glucuronidase as an Index to Growth in the Uterus and Other Organs. 1949. Biochem. J. 44 (4) : 487.

25. (with KERR, L. M. H & LEVVY, G. A.) Further Observations on Changes in β-Glucuronidase Activity in the Mouse. 1950. Biochem. J. 46 (3), 278.

MYELOCYTOMA IN THE DOMESTIC FOWL

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In the course of over a thousand post-mortems, extending over a period of two and a half years, two, possibly three, cases of myelocytoma in the fowl have been examined, two in adult birds (both Buff Rocks) and what was probably a case in a young pullet. Unfortunately, as the infrequency of the condition was not appreciated, a full record of findings was not kept, these post-mortems only being represented now by an odd histological preparation or two and a very brief description of the macroscopic appearance.

Recently, however, a third case was examined, and the findings are described in some detail below.

Post-mortem No. 7772

The subject was a 4½ lb. Buff Rock hen, aged two years, which was seen by Mr. Hillock, veterinary surgeon, Haddington. Symptoms of extreme weakness suddenly appeared and the bird was destroyed on the third day of illness, after a period of paralysis.

It was in moderately good condition. As soon as the pectoral muscles and sternum were reflected a large soft white mass (7 x 4½ x 3 cms.), resembling brain tissue, was found growing from the internal aspect of the sternum. Examination of this bone showed that the medullary cavity was greatly enlarged and filled with similar soft white tissue, which appeared to extend diffusely into the overlying pectoral muscles.

The visceral pericardium showed several flattish discoidal growths (0.5 cm.) on its surface, and the parietal pericardium was slightly adherent to these. Transverse section of the heart showed the inter-ventricular septum to be almost entirely replaced by the tumour. The blood was decidedly anæmic and watery.

The lungs were firmly attached to the ribs and intercostal muscles by a thick (1.0 cm.) white mass covering their parietal aspect. Section of the organs showed whitish masses in the interior.

The ovary was enlarged (6 x 6 x 4 cm.), firm, and presented a pinkish-white cauliflower-like appearance.

The kidneys were both involved. The left organ had only a small portion of normal substance left in the posterior lobe, the rest being a soft, white mass of neoplastic tissue, continuous with similar tissue which extended into the depths of the pelvic cavity. The right organ had a considerable part of the upper and middle lobes intact, but the rest were tumorous.

The liver and spleen were not enlarged, but the former had many small whitish areas shining through the capsule.

In the neck region, a string of small growths was found in the site of the thymus, which was, of course, almost completely atrophied. The thyroid and parathyroids were unaffected, although a mass of tumour tissue lay in close proximity to the left glands.

The skeleton was carefully examined after boiling, and evidence of fresh

bone formation found, in the form of masses of small delicate spicules growing from the periosteum of the following bones: Between the fused transverse processes of the lumbo-sacral vertebræ, on the internal aspect of the ileum, and in the bodies of the fused thoracic vertebræ. There was no sign of ulceration or necrosis of the bones and the shaft walls of long bones were slightly thicker than normal. The bone marrow in the long bones had, when fresh, a dirty pinkish appearance and completely filled the medullary cavity as a compact but soft mass.

Histological Characters of the Tumour

The cell type combines myeloblastic and myelocytic characters. When closely packed, as in the centre of the neoplasm, each cell is polyhedral from mutual pressure. Towards the periphery, however, the cells usually tend to be spherical, and when diffusely distributed, as when infiltrating between muscle fibres, etc., are quite spherical or oval. Their average size is 7.2 x 5.7μ. The nucleus is large, vesicular, and has one or two prominent nucleoli. It is generally situated eccentrically in the cytoplasm, in this respect resembling a plasma cell. Mitotic figures are common. (Fig. 1.)

Sections stained with hæmatoxylin and eosin show the majority of cells to have coarse spherical eosinophilic granules lying in the cytoplasm. (If stained with giemsa, however, they are seen to be metachromatic, purple granules lying interspersed with the eosinophilic ones.) Occasional groups of cells show particularly vivid scarlet granules very closely packed in the cytoplasm.

There is but little inter-cellular connective tissue, and blood vessels are few. Circumscribed areas of necrosis are fairly common. The cells tend to be arranged in long strands or columns and occasionally several such columns lie side by side to form broad sheets of cells. (Fig. 2.)

Argyrophil Reticulin Fibrils

These are present as an irregular network enclosing groups of cells in some parts of the tumour (Fig. 3); in other parts they are scanty or not present at all.

The following parts were subjected to a microscopical examination :—

Sternum: A transverse section shows the bone to be badly eroded, with greatly distended medullary spaces packed with cells. The overlying great pectoral muscle is almost completely replaced by these cells, leaving a thin superficial layer of muscle, the fibres of which are widely separated by infiltrating cells. (Fig. 4.)

Femur: Transverse section of this bone shows the medullary cavity filled with tumour cells, scattered amongst which are islands of normoblasts appearing as densely staining nuclei. Evidence of both erosion and new bone formation in the form of spicules is detected in the spongiosa of the shaft. The Haversian and Volkmann canals contain groups of tumour cells, many showing the usual mitotic activity. On the whole, the shaft wall appears slightly thicker than normal and shows a concentric arrangement as though layers of new bone have been laid down internally. (Fig. 5.)

Heart: The cells appear to have infiltrated beneath the pericardium, and, proliferating there, grown inwards in strands and columns between the heart

muscle fibres, separating these widely. Pericardial fat spaces are outlined by masses of cells.

The blood: Unfortunately proved unsatisfactory for microscopical examination, owing to the extreme degeneration of the leucocytes, but the leucocytic " smudges " did not appear to be excessive.

Lungs: Masses of actively proliferating cells have infiltrated along the interlobular septa and ensheathed the pulmonary vessels at the angles of the lobules. They also invade the actual wall of the lobule. (Fig. 6). The cells have extended beneath the parietal pleura and proliferated to a considerable thickness, thus fixing the lung to the ribs and intercostal muscles. Beneath this thick sheet, the pulmonary alveoli are compressed, and many obliterated. The alveolar capillaries in the more normal lobules are seen, under high magnification, to contain many tumour cells, most of which have a greater breadth than the containing capillary, and so distend and block it.

Liver: Tumour cells are present wherever there is connective tissue, burrowing and infiltrating especially round the blood vessels and bile ducts. Adjacent to such areas, the hepatic cells show signs of pressure atrophy, and necrobiosis. Isolated tumour cells are seen lying in the liver sinusoids amongst the erythrocytes.

Ovary: The stroma is almost completely replaced by tumour cells, and only a few isolated distorted ovarian follicles remain.

Kidney: The cells have invaded these organs so completely as to make it difficult to find any normal tissue. The path of infiltration is between convoluted tubules. Many glomeruli are left as prominent islands in a surrounding mass of cells. (Fig. 7.) Spicules of bone are lying in the tumour mass near its pelvic attachment.

Thymus: Traces of this gland remain, but the bulk of the tissue consists of masses of tumour cells, with many isolated necrotic areas.

Spleen: This organ is involved to the extent that tumour cells, many showing mitotic figures, are scattered impartially throughout both red and white pulp. There are occasional groups of cells, but these are quite microscopic. It appears as though the spleen was only lately involved in the general condition. Masses of actively proliferating cells are attached to the surface of the splenic capsule, in the form of macroscopical tumours.

Adrenal gland: Appears quite normal microscopically.

Summary: An unusual type of malignant neoplasm is described in a two-year-old Buff Rock hen. The cell type appears to be an intermediate between a myeloblast, as witnessed by the embryonic appearance of the nucleus, and a myelocyte, judging from the granular nature of the cytoplasm. In view of these characters, the growth would appear to be a myelocytoma, and it seems probable that the primary growth originated in the sternal marrow.

Acknowledgments

I am indebted to Professor Grahame, of the Anatomy Department, for permission to have the photo-micrographs taken, and to Mr. P. G. D. Morris,

M.A., M.R.C.V.S., of the same Department, who took such pains over their preparation.

Also I am grateful to Mr. Whittick, B.Sc., M.R.C.V.S., of the Pathology Department, for giving me a second opinion as to the probable nature of the neoplasm.

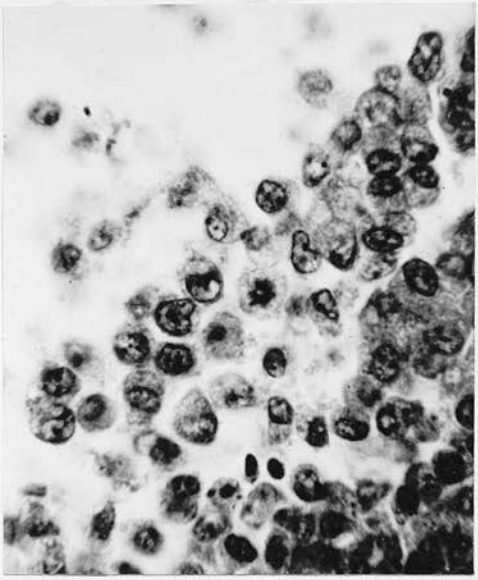

FIG. 1: Tumour cells X 1000. Mitotic figure in centre.

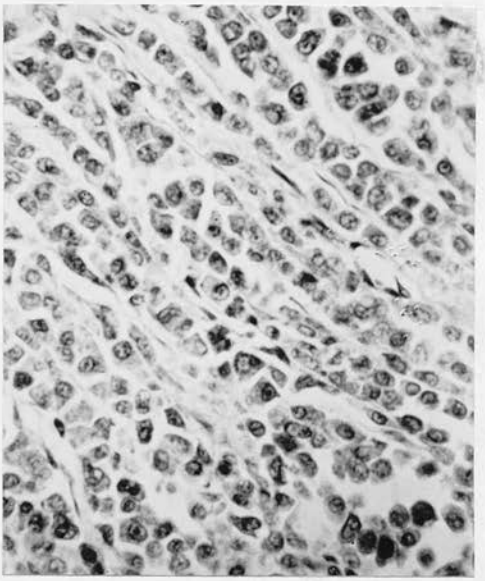

FIG. 2: Strands of cells, with delicate connective tissu fibres in Ovarian tumour.

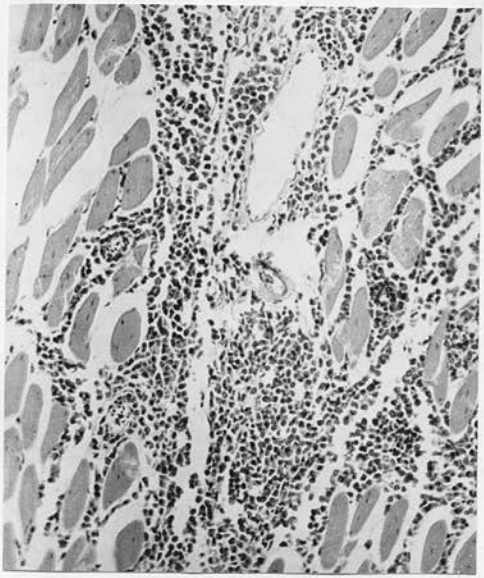

FIG. 4: Pectoral muscle fibres separated by infiltrating cells.

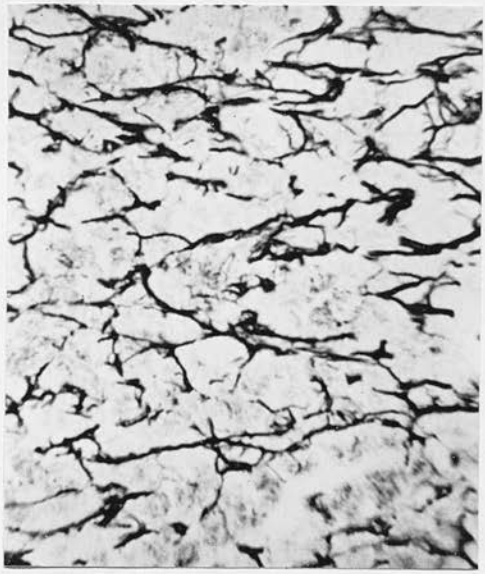

FIG. 3: Argyrophil reticulin fibrils surrounding groups of cells.

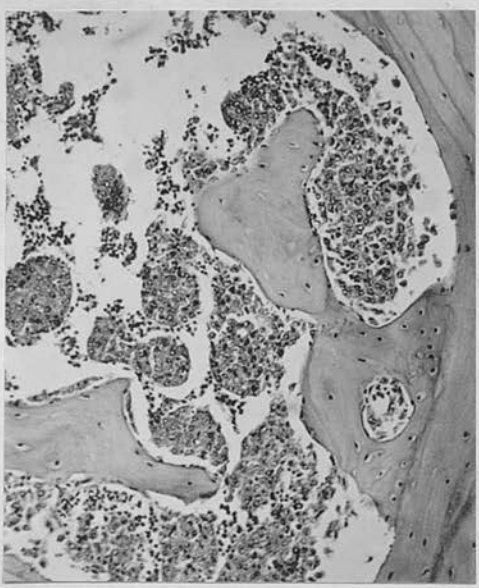

FIG. 5: T.S. Femur, showing groups of tumour cells, bone erosion, bone formation, and scattered groups of Normoblasts.

FIG. 6: Cells proliferating in inter-lobular septum invading alveolar walls.

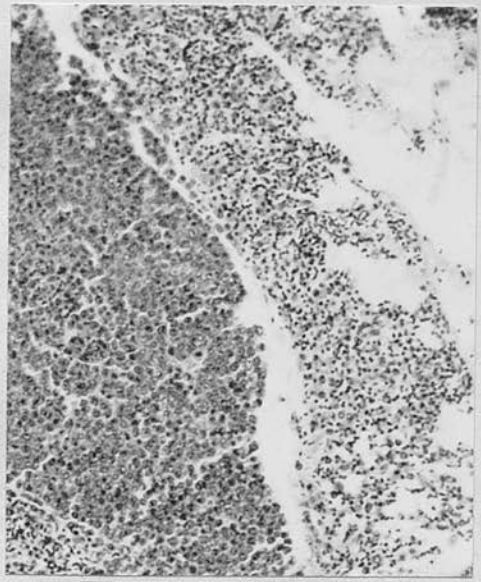

FIG. 6a: Enlargement of Fig. 6.

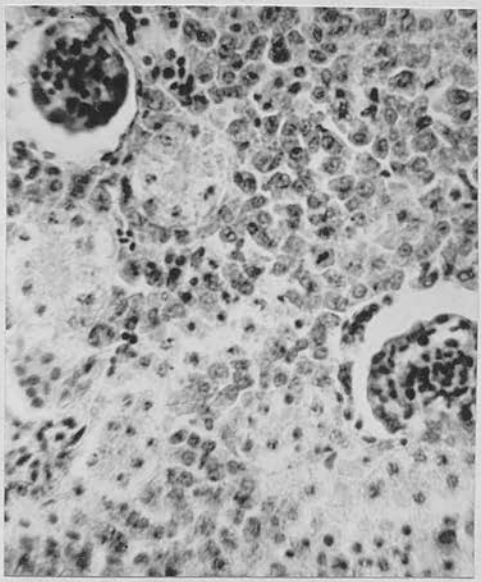

FIG. 7: Cells infiltrating the kidney. The tubles are degenerate.

EMBRYONAL NEPHROMA IN THE DOMESTIC FOWL

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APART from lymphocytomas and other closely allied conditions, neoplasms involving the kidney of the fowl are of comparatively rare occurrence. Hedren[1] describes an adenomyosarcoma with bone formation, and remarks that a tumour of similar nature occurs in human pathology. Fox[2] has reported twelve primary tumours of kidneys in birds, all of which occurred in a zoological garden. He does not mention any cases in domestic fowls. Five of his cases were papillary adenomatous tumours with cyst formation, or the production of solid nests of epithelial cells, with no acinous or duct formation. Mathews[3] has recorded twelve adenosarcomata out of fifty-five renal tumours discovered in the course of two thousand post-mortem examinations. His specimens were attached to the periosteum of the spinal column and were separated from the kidney by a fibrous capsule. In all cases the left kidney was involved. McKenny[4] had two cases of embryonal carcinoma in white leghorn hens. These grew within the kidney, were not encapsulated, and not attached to the spinal column. There were no metastases and the tumours were unilateral. Macroscopically, they showed areas of hæmorrhage and necrosis, with bands of fibrous tissue running through the mass. In one case there were large masses of undifferentiated cells; in the other, definite tubular and glomerular-like structures were seen. There were many large cysts containing colloid-like material and lined with flattened epithelial cells.

Eber and Malke[5] report sixteen cases of kidney tumours in fowls, including eleven sarcomas, one carcinoma, and two adenomas.

Feldman and Olson[6] had one case of bilateral embryonal tumour in a cross-bred hen aged 10 months, used as a subject for transmission experiments with erythroleucosis, and which died from this disease. Their specimen, which they classified as an adenosarcoma, was characterised by a variety of cellular elements.

They reported that adenomatous structures were common, cells of many of these having undergone keratinization, with formation of numerous cornified nodules.

From the foregoing it will be seen that the histological picture of this interesting tumour varies considerably, and it was therefore thought desirable to place on record a further case of this by no means common condition.

Post-Mortem 7883

The present case occurred in a white leghorn hen aged 18 months. This bird appeared normal until it was frightened and jumped from a high shed roof, making an awkward landing and appearing to hurt its left leg. When examined, the limb showed a partial spastic paralysis, and the bird could walk only with difficulty. A focus of pain was thought to be detected at the tibio-tarsal joint, and severe sprain was diagnosed. The joint was mildly stimulated with Ung. Iodi. and temporarily immobilised with a light Cellona P.O.P. dressing.

The bird, however, showed a progressive deterioration in condition, and

as at the end of nine days it was completely unable to use either leg, it was destroyed and autopsied.

Subject was in moderate condition, weighing 4 lb. The left sciatic nerve was found to be enlarged for about 3 cm. of its length in its course down the thigh. This was the only abnormality in the peripheral nervous system which could be detected macroscopically.

On removing the abdominal viscera, a whitish firm growth was seen involving the mesial aspect of the anterior lobe of the right kidney, which was enlarged. Cranial to this, i.e., immediately lateral to the ovarian attachment, was a subcapsular hæmorrhage. (See Fig. 1). There was no attachment to the spinal periosteum.

A longitudinal section of the growth and anterior pole of the kidney showed it to have a well marked capsule separating it from the renal tissue, which was compressed. The presence of this capsule negated the idea of lymphocytoma of the kidney.

The dimensions of the growth were 23 by 20 by 18 mm. It was firm, and yellowish-white on the cut surface. There were areas of compact tissue, areas of hæmorrhage and necrosis, and areas of small cysts.

Microscopical examination of frozen sections of the left sciatic nerve failed to show any infiltration with round cells. Instead, there were areas of hæmorrhage and degeneration in the swollen part, suggesting traumatism.

Histological Features of Renal Tumour

A description of the microscopical picture of the tumour can conveniently be divided into three parts corresponding roughly with the three areas already mentioned, i.e., cystic, hæmorrhagic and solid.

The first area consists of irregular vesicles or cysts of various sizes, lined with epithelium ranging from flattened cubical to columnar cells, and containing homogeneous colloid-like substance staining pink with eosin, but not with toluidin blue. This substance is therefore not epithelial hyalin or mucin. The epithelium frequently projects into the lumina of the vesicles in a papilliform manner. (Fig. 2.)

Separating the walls of the vesicles is a celullar stroma in which lie many acinar groups of cells having the appearance of sections of straight renal tubules, together with small blood vessels, etc. Some of these tubules contain the same colloid-like material, whilst others have a definite lumen. It seems probable that the large vesicles are simply cystic tubules. With higher magnification the cells composing both tubules and walls of cysts are seen to have vesicular nuclei, and the cytoplasm to have a dark "brush" border next to the lumen. Small clear vacuoles at the edge of the colloid mass appear to be analogous to the secretion vacuoles in the thyroid gland.

The stroma is composed of a preponderance of cells resembling young connective tissue cells, together with a few mature fibroblasts with their elongated deeply staining nuclei. Many delicate capillaries lie amongst these cellular intercystic groups.

The solid yellowish-white areas exhibit a more undifferentiated appearance.

There is a great abundance of cellular tissue, for the main part structureless, but here and there forming tubule-like structures, some of which are enlarging to form cysts. (Fig. 3.)

The component cells are exactly as already described in the intercystic stroma, and remind one of sarcomatous tissue, but judging from the scarcity of mitotic figures and the other findings, the neoplasm appears to be of a benign character.

The whole tumour is surrounded by a comparatively thick (1 mm.) fibrous tissue capsule, beyond which the renal tissue shows signs of compression from the growing mass.

The left kidney was also sectioned, and examination of several slides taken from the upper lobe shows isolated masses of cells characterised by darkly staining irregular nuclei, with the chromatin arranged in clumps, surrounded by a moderately extensive zone of cytoplasm. Interspersed with these, but in smaller numbers, are cells with lighter staining vesicular nuclei, with the chromatin tending to form a narrow ring within the nuclear membrane. These cells are provided with a moderate cytoplasm. Some of them appear to be arranged in the form of acini. The possibility that here we have a group of embryonic epithelial and mesenchymal cells, just commencing to develop into a similar type of tumour to that already described, makes these sections particularly interesting. (See Fig. 4.)

The above mentioned post-mortem and histological findings suggest that the condition is one of embryonal nephroma.

Other Cases

One other case of embryonal nephroma has so far been observed in the fowl. This was definitely sarcomatous in character, as compared with the adenomatous type just described. The following renal tumours have also been diagnosed in other species of birds :—

Mixed hypernephroma and adeno-carcinoma, with a certain amount of spicular bone formation in a sulphur-crested cockatoo (cf. Hedren's case).

Two embryonal adeno-sarcomas from budgerigars and a doubtful spindle cell sarcoma from another bird of the same species. (It seems possible that the latter case developed as the result of renal irritation from some sort of parasite, unidentifiable portions of which were found in the neoplasm, and therefore the condition may have been a connective tissue hyperplasia.)

Etiology of the Tumour

Embyronal nephromata, when found in human beings (children) are called Wilms's tumours. They have also been reported in rabbits and pigs.

Wilms's view is that they develop from a fœtal rest or displacement, that they develop from the same type of embryonic cell, and, according to the stage of development of this cell, exhibit various histological features, e.g., if they develop from a comparatively undifferentiated cell, the resulting tumour shows a fairly complicated structure, because of the multipotency of undifferentiated mesodermal cells. The final tumour, on the other hand, may be of a simple nature having developed from a more differentiated embryonic cell.

Summary

An embryonal nephroma occurring as a unilateral tumour in the kidney of a white leghorn hen aged 18 months is described, together with a possible stage of development of the same type of tumour in the other kidney. Other reported cases are noted, and the probable etiology mentioned.

Acknowledgments

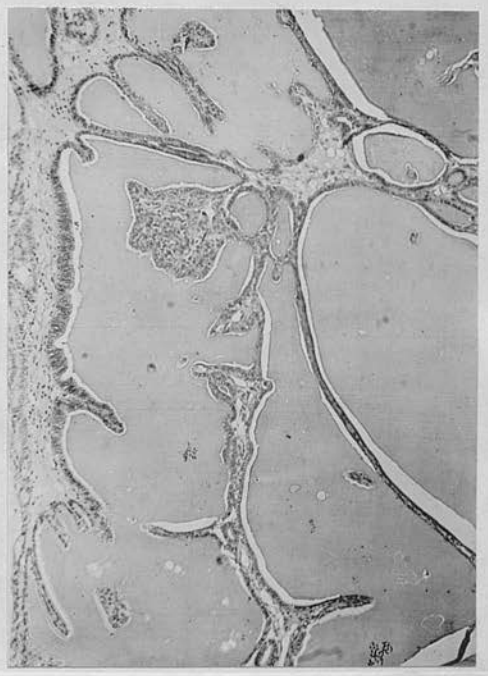
I am indebted to Professor D. C. Matheson, F.R.C.V.S., of the Pathology Department, for his constructive criticism in the preparation of this paper; also to Mr. David MacFarlane, B.Sc., M.R.C.V.S., of the same Department, who examined the sections, and to Mr. P. G. D. Morris, M.A., M.R.C.V.S., of the Histology Department, who took the photographs.

REFERENCES

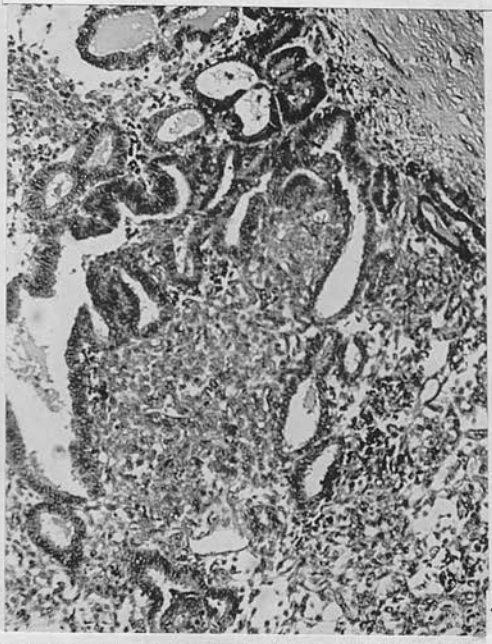
(1) Hedren (1907): "Embryonal Adenomyosarcoma of the Kidney of the Fowl." *Ziegler's Beiträge Z. Path. Anat., 40,* 1.
(2) Herbert Fox (1923): "Diseases in Wild Mammals and Birds." *Lippincott.*
(3) Mathews (1929): "Adenosarcoma. Kidney of Fowl." *J.A.V.M.A., 74,* 238.
(4) McKenney (1931): "Embryonal Carcinoma." *Amer. Journ. Canc., 15,* 122.
(5) Eber and Malke (1932): "Geschwulstbildung beim Hausgeflügel Zusammenfassender Bericht über 392 bei 16,460 Geflügelsektionen ermittelte Neubildungen." *Ztschr. f. Krebsf, 36,* 178-192.
(6) Feldman and Olson (1933): "Embryonal Nephroma. Kidney of Fowl." *Amer. Journ. Canc. 19,* 47.

these forms of cellular elements; and in those that have been

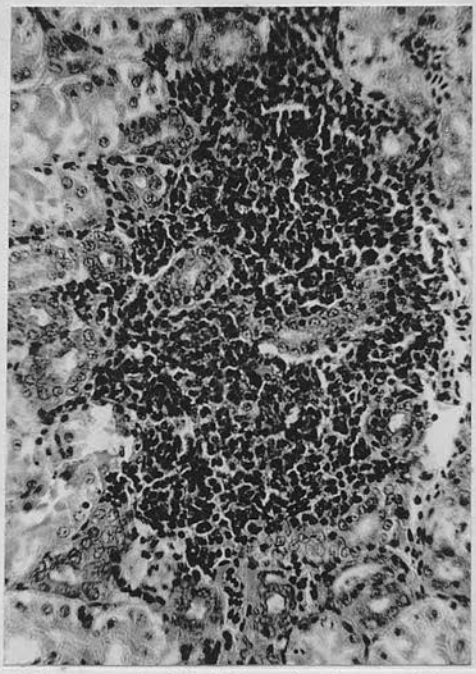
1. Embryonal Nephroma. Right kidney x ½



2. Section of cystic area. Renal tumour x 100.



3. Cellular part, showing sarcomatous appearance tubule formation and a portion of the fibrous capsule x 160.



4. Cell group in left kidney showing possible tubu formation x 390.

these forms of cellular elements; and in those that have been sub-

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HISTIOCYTIC AND FIBROPLASTIC SARCOMA (MIXED-CELL SARCOMA) IN THE DOMESTIC FOWL

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INTRODUCTION

A NUMBER of spontaneous connective tissue tumours occur, particularly in birds, the assessment of which offer considerable difficulty, owing to the apparent lack of a type cell, and the extraordinary pleomorphism of the cellular elements composing them. Such tumours are well known in human pathology, but there is very little discussion of them in standard textbooks, and most authorities avoid assigning any cell type to them.

MacCallum (1937) describes tumours which are characterised by cells showing great variation in shape and size, some of them containing very large cells (tumour giant cells) showing much diversity of form, and bizarre modifications of their nuclei. Such tumours he calls "mixed-cell sarcomas."

Sarcomata are common in the fowl, though whether the inclusion under this heading of such conditions as the leucoses is desirable is open to question. This is at any rate the practice of Malke (1930) who, in his paper on "Neoplasms in the domestic fowl," classifies 66 per cent. of his sarcomata as "sarcoma mixtocellulare"—including lympho-sarcomata or aleukaemic lymphoid leucosis.

Jackson (1936) has suggested a cytological classification of these mixed-cell tumours in conformity with the growing tendency to avoid a purely morphological nomenclature. Thus he arranges them under three general headings: (1) Fibroblastic (or better, fibroplastic, since it indicates the morphological multipotentiality of the cell); (2) mixed cell (*i.e.*, histiocytic and fibroplastic) sarcoma; and (3) histiocytic sarcoma. He points out, however, that difficulty may arise over intergrades and this fact demonstrates that all types are but variants of one basic neoplastic cell type.

Carrell and Ebeling (1926) and Haddow (1933) have published work suggesting that the macrophage of the fowl can be changed into a neoplastic histiocyte or a Rous sarcoma cell. This view, however, is not in accordance with the findings of Ludford (1936) or Halberstaedter *et al* (1941), whose results appear to show that it is the fibroblast and not the macrophage which fixes and is cancerised by the virus of Rous or Fujinami tumours.

In certain strains of the Rous sarcoma, mixed-cell growths have appeared: conversely McGowan (1928) refers to naturally occurring mixed-cell tumours as being sarcomata of the Rous type.

Maximow and Bloom (1939) say that in inflammation, the fixed histiocyte or macrophage may be regarded as a modified fibroblast, differing from the fibroblast *per se* in that it tends to be stellate and to form reticulin instead of collagen fibrils. Besides fibroblasts, it can also produce free phagocytic cells such as monocytes and giant cells. Some workers claim it can produce the free multipotential precursors of the circulating blood elements (the haemocytoblasts of the Monophyletists), although Maximow denies this.

It will be seen then that mixed-cell tumours may well show all these forms of cellular elements; and in those that have been sub-

jected to critical cytological examination, especially by Jackson, the collagen producing fibroblast, the reticulin producing fixed histiocyte, and the phagocytic free histiocyte or macrophage, together with groups of primitive blood cells have all been recognised.

A study of these new growths, therefore, is likely to throw fresh light on a number of debatable histogenetical problems, and the case described in detail below appears to confirm several debatable points. The tumours occurred in a Buff-rock hen, weight 4½ lb., which had been killed by dislocation of the neck.

MACROSCOPICAL EXAMINATION

Post-mortem examination showed both lungs to contain many firm, white tumours growing in the form of rounded masses. These protruded somewhat from the surface, giving each lung a nodular appearance. On section, these masses were seen to be present throughout the lung, and in places they were merged to form diffuse white areas. The average diameter of the isolated growths was 3 to 4 mm.

There was a firm whitish tumour growing in the anterior lobe of the right kidney (25 × 22 × 17 mm.). This was firmly attached to the 6th-7th ribs and their costo-vertebral junctions by connective tissue. There was no erosion of the ribs. The left kidney appeared normal. The right adrenal gland was somewhat larger than normal, measuring 12 mm. in cross-section as against the normal 7 to 8 mm. Its cut surface had a greyish appearance. The left gland was normal in size and colour.

The oviduct was impacted with inspissated yolk, and had several small (0·2 mm.) shining nodules growing in the wall of the magnum. The ovary, alimentary tract, liver, spleen, thyroids, blood and bone marrow all appeared normal.

Tissue taken from lung, kidney, adrenal and oviduct, as representing the abnormal organs, and from the above-mentioned macroscopically normal organs, was fixed in 10 per cent. formalin in physiological saline. Paraffin and frozen sections were cut. The following stains were used on the paraffin sections: Harris's haematoxylin and eosin, phosphotungstic acid haematoxylin, thionin blue, a modified Mallory stain and the Gros-Schultze method for nerve-fibres. Frozen sections were impregnated by both Cajal's silver and gold chloride-sublimate methods. Further formol fixed material was impregnated by Levaditi's method, embedded and paraffin sections cut at 15μ.

MICROSCOPICAL EXAMINATION

Lung.—A general low power survey of the lung shows the tumours to be growing in the walls of the alveoli. All stages of involvement are seen from a minute growth in one wall to complete obliteration of the normal lung structure over an area corresponding to 40 to 50 alveoli. The pulmonary vessels are all congested.

Examination under higher power shows a great diversity of cellular elements in different parts. These may be classified as follows: (1) fixed histiocytes, (2) free macrophages, (3) fibroblasts,

(4) tumour giant cells, and (5) lymphoid and myeloid elements. All degrees of transition exist between the first four types.

Owing to the inferior cytology of the lung tumours compared with that in other organs, a detailed description of the cells will be given later, and only a general account will be given of the lung. The histiocytic type of cell predominates in the lung tumours. These are characterised by great pleomorphism, but in the main they are elongated cells, with many fine protoplasmic prolongations which frequently merge with those of neighbouring cells. The histiocytes lie in loose interweaving strands and fasciculi (Fig. 1). In many areas the fixed histiocytes take on a more rounded shape, and judging from their amoeboid forms and the fact that they often contain cellular debris, they are actively motile and phagocytic.

Differentiation to the fibroblast type has taken place in other areas, and these cells are always associated with abundant collagen fibres. Groups of giant cells occur. They are frequently multinucleated and exhibit active phagocytosis. They may be lying in a reticular mesh, or occurring free in the lumina of the alveoli, together with a pink-staining homogeneous material.

The bronchial submucosa contains large numbers of plasma cells and a few lymphocytes. In certain parts a myeloid type of cell with eosinophilic granules in the cytoplasm predominates. Portions of bronchial epithelium in the form of a few palisaded columnar cells are distinguishable in parts of the tumour adjacent to the bronchi.

Reticulin fibrils are intimately associated with the fixed histiocytic moiety. They run mainly parallel to the long axis of the cell and branch frequently. The giant tumour cells usually lie enclosed in a reticulin mesh.

Kidney.—The growth in this organ has a similar structure to those in the lungs, with the exception that although the strands and bundles of fixed histiocytes still occur, they are not so much in evidence, the main part of the tumour being composed of neoplastic fibroblasts and groups of large roughly spherical cells lying in a loose framework of fixed histiocytes. The growth is not encapsulated, and is actively invading the kidney substance. A few delicate blood vessels occur. Some isolated sections of renal tubules remain in the invading mass.

High power shows the histiocytic element to be similar to that in the lung. The cells, which are very faintly staining, measure about $5 \times 17{\cdot}5\mu$, although it is difficult to arrive at an accurate measurement of the length owing to the tapering of the cytoplasm into fibrillar prolongations. The nuclei are pale, vesicular and oval and measure about $6{\cdot}25 \times 3{\cdot}75\mu$.

The free macrophages measure 7 to 14μ in diameter. They have a foamy, somewhat basophilic cytoplasm, a prominent vesicular nucleus with well-marked nuclear membrane, and a large acidophilic nucleolus. The giant phagocytic cells measure anything from

20 to 50μ in diameter. Generally speaking, the larger the cell the more frequently it is multinucleated. Cells showing amitosis are not uncommon. They frequently contain eosinophilic leucoctyes lying in vacuoles in their cytoplasm. These large cells lie in the meshes of a fibrillar plexus. With their basophilic granular cytoplasm, large vesicular nucleus and prominent nucleolus they bear a strong resemblance to unipolar ganglion cells (Fig. 2). Thionin blue demonstrates a certain amount of granular substance in the cytoplasm. The nuclei of some of these tumour giant cells are often very large and bizarre. They may be indented, twisted, bent into semi-circles and be anything up to 17μ in diameter. The nucleolus also varies greatly in size, one measuring 15μ across. There may be two nucleoli present. Numbers of these giant phagocytic cells lie free in the lumina of the large proximal collecting tubules.

The fibroblasts occur as fairly dense bundles of elongated cells measuring about 30 to 50μ $\times$ 3 to 4μ. Here again it is difficult to arrive at an accurate figure. They have a faintly basophilic cytoplasm which tapers to a fine thread at each pole and a large oval or round vesicular nucleus with a well-defined nuclear membrane and a few coarse chromatin granules connected by linin threads. There is a prominent acidophilic nucleolus ; sometimes there are two such nucleoli. The prolongations of these cells stain as for collagen with Mallory's connective tissue stain, and Anderson's modification of Jakob's stain for nervous tissue gives an excellent demonstration of the intimate association between cytoplasm and collagen production, the former staining red and the latter blue. (Fig. 3.)

In view of the resemblance to ganglion cells, several impregnation methods were used, but neither Cajal's silver method nor Levaditi's method demonstrated neurofibrils. Similarly an attempt was made to demonstrate neuroglia by Cajal's gold chloride-sublimate method, with negative results. A study of the recticulum shows a rich plexus of reticulin fibrils enmeshing the giant cells, and an abundance of delicate branching fibrils in intimate relation to the more undifferentiated (histiocytic) part of the tumour. Very few argyrophil fibres occur amongst the fibroblasts.

Adrenal Gland.—The capsule of the gland is intact. There is a group of normal ganglion cells attached to the outside of the capsule. The medulla has completely disappeared, its place being taken by small groups of round or polygonal cells, and strands and bundles of interweaving elongated fibroblasts. A few giant phagocytic cells are scattered throughout this tissue. Islands of cortical cells remain intact.* (Fig. 4.)

* In his discussion of secondary tumours in the adrenals, Willis (1934) points out that : " Despite the intimate anatomical relationship of the cortex and medulla, the cortex long resists neoplastic invasion from medullary deposits. No mechanical or vascular conditions suffice to explain this, and serious consideration must be given to the view that the metabolic qualities of the adrenal cortical tissue are such as to inhibit tumour invasion."

Transition forms between the macrophages and the giant phagocytic cells can be seen. Some of these intermediate types of cell show an indistinct or no nuclear membrane, although the nucleolus is still prominent. The cytoplasm is coarsely granular or hyaline. These are probably degeneration forms. Occasionally large " dumb-bell " shaped forms occur. They have a rather coarse granular cytoplasm with a small basophilic nucleus at each pole. Such cells appear to be undergoing amitotic division.

The rest of the cellular elements are as already described in the lung and kidney. The intercellular substance is composed of fibres which stain very faintly with eosin, but show up better when Mallory's connective tissue stain or phosphotungstic acid haematoxylin is used. The fibres can then frequently be traced to cytoplasmic prolongations of the fibroblasts. A few delicate blood vessels traverse the tumour. Wandering cells of the myeloid series occur in the intercellular stroma.

Laidlaw's silver impregnation shows wavy reticulin fibrils branching throughout the tissue replacing the medulla. The fibrils enmesh small groups of cells and even individual cells of the large type. The cortical cell islands appear normal and are not pervaded by reticulum.

Other Organs.—The liver, spleen, ovary, adjacent sympathetic ganglia, and longitudinal sections of marrow in rib and femur show no abnormality.

The nodules growing in the wall of the magnum of the oviduct are fibro-adeno-leiomyomata.

DISCUSSION

The case is an unusual one as regards the primary site (kidney) and the distribution of metastases. In Jackson's (1936) ten recorded cases, the commonest primary site was the ovary (5), other sites mentioned being the wattle (1), oesophagus (1) and unrecorded (3). The commonest sites for metastatic growths were bowel serosa and mesentery (strictly speaking, peritoneal implantation) from the primary ovarian tumour. True haematogenous metastases were observed in liver, spleen, kidney, heart and muscle, each in one case respectively.

Another interesting point is the very close resemblance of some of the giant phagocytic cells to ganglion cells, and the coincidence of neoplasia in the adrenal medulla—a common site of origin for neuroblastomata and ganglion-neuromata. However, the large cells frequently show cyto-phagocytosis ; no neuroblasts are present, no neuro-fibrils can be demonstrated, the presence of reticulin fibrils (*vide* Foot and Day, 1925) and the normal appearance of the ganglion cells adjacent to the adrenal and ovary, all point to the growth not being a neurogenic one.

The direct transformation of cytoplasm to collagen—a process which has been the subject of much controversy—is well shown in

this tumour. Two schools of opinion exist regarding the formation of collagen, one holding that it is laid down between the cells through a condensation of liquid intercellular substance secreted by the cells, and the other that it originates by direct transformation of cytoplasmic prolongations.

The multipotentiality of the histiocyte is well illustrated in this case. All transition forms from this cell, whether fixed or mobile, through fibroblasts to multinucleated phagocytic giant cells exist. The resemblance of those parts of the tumour which consist of loosely arranged neoplastic histiocytes to the Rous Sarcoma No. 1, is very striking.

SUMMARY

A histiocytic and fibroplastic sarcoma (" mixed-cell sarcoma "), the primary growth probably being in the right kidney, with metastases to the right adrenal and to both lungs.

ACKNOWLEDGMENT

I wish to express my thanks to my colleague, Mr. P. G. D. Morris, of the Anatomy Department, Royal (Dick) Veterinary College, for the photomicrographs.

REFERENCES

Carrell, A., and Ebeling, A. H. (1926). *J. exp. Med.*, **43,** 461.
Foot, N. C., and Day, H. A. (1925). *Amer. J. Path.*, **1,** 431.
Haddow, A. (1933). *J. Path. Bact.*, **37,** 149.
Halberstaedter, L., Doljanski, L., and Tenenbaum, E. (1941). *Brit. J. exp. Path.*, **22,** 179.
Jackson, C. (1936). *Onderstepoort J.*, **6,** 249.
Ludford, R. J. (1937). *Amer. J. Cancer.*, **31,** 414.
MacCallum, W. G. (1937). *Textbook of Pathology*, p. 1066. W. B. Saunders & Co.: 6th ed.
McGowan, J. (1928). *On Rous, Leucotic and Allied Tumours in the Fowl.* London: Lewis & Co.
Malke, E. (1930). *Zeit. Krebsf.*, **31,** 47.
Maximow, A. A., and Bloom, W. (1939). *Textbook of Histology*, chap. 5. W. B. Saunders & Co.: 3rd ed.
Willis, R. A. (1934). *The Spread of Tumours in the Human Body.* p. 277. Churchill.

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LEGENDS TO PLATE I

FIG. 1. Portion of lung tumour, showing neoplastic tissue composed of fixed histiocytes. Mallory's connective tissue stain. × 200.

FIG. 2. Giant phagocytes (macrophages) in the kidney tumour. Note resemblance to ganglion cells. Thionin blue and eosin. × 350.

FIG. 3. Fibroblastic cells, also from the kidney tumour. The long collagen fibres arising from the cells are well shown. The intra-cytoplasmic formation of collagen can be seen in the two macrophages to the right of the photograph. Anderson's stain. × 500

FIG. 4. Neoplastic fibroblasts in the adrenal medulla. The cortex is not affected. Thionin blue and eosin. × 500.

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PLATE I.

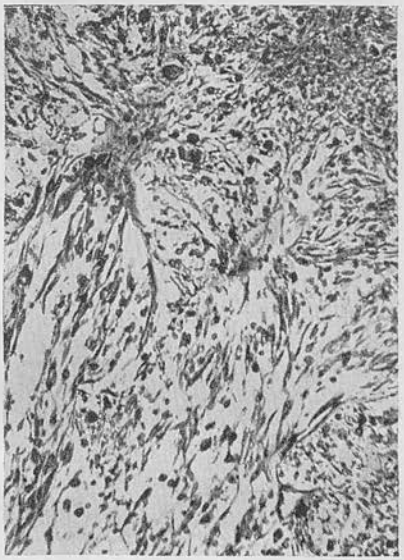

FIG. 1

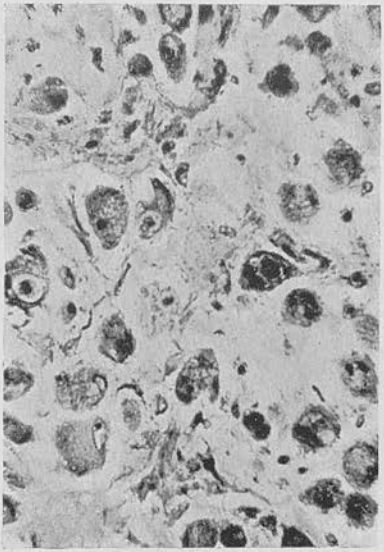

FIG. 2

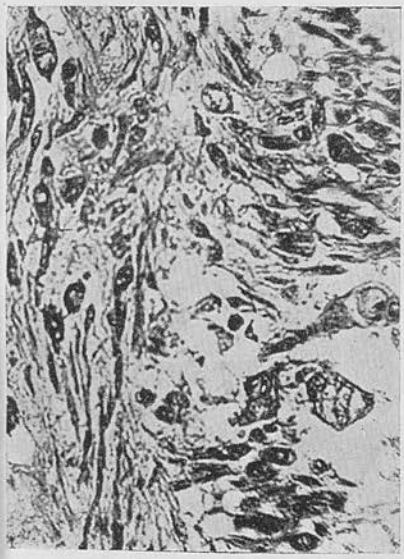

FIG. 3

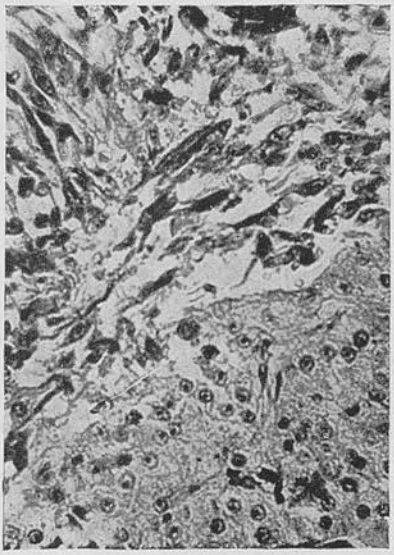

FIG. 4

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NEOPLASTIC DISEASE OF THE FOWL WITH SPECIAL REFERENCE TO ITS HISTORY, INCIDENCE AND SEASONAL VARIATION*

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INTRODUCTION

THAT the incidence of tumorous conditions is high in fowls compared with other animals is well known to comparative pathologists, who, recognising the peculiar liability of avian tissues to undergo neoplastic changes, make use of this in the experimental induction of tumours by chemical means. The transmissibility of some spontaneous tumours and leukaemias, either by tissue inoculation or cell-free filtrate, is also well established since Peyton Rous's (1910) discovery, and a large amount of experimental work has subsequently been accomplished in this field, yielding information of first-class importance and giving an impetus to cancer research, in which branch particular mention must be made of Fould's work (1934, 1940). In contrast, little has been published on the incidence of spontaneous tumours in fowls generally. Only two articles (Goss, 1940, and Bullis and Olson, 1943) give figures for the comparative incidence in the more common breeds, and there is a scarcity of information recorded on the incidence in fowls generally of the various specific neoplastic conditions and on organ distribution and seasonal incidence.

To estimate the incidence of tumours in fowls generally is obviously very difficult for it entails the maintenance of large flocks of healthy birds under observation, all of which would have to be subjected to a very thorough examination when they died or were destroyed. For this reason, the somewhat meagre information on the subject relates almost entirely to the proportion in which it is observed in disease in general in the *post-mortem* examination records of poultry laboratories engaged in routine diagnosis. The value of the few attempts to discover the absolute incidence of the tumours has usually been limited by failure to submit the material to histological examination. Some attempts at classification have been made but these are not of much significance because they have been based on macroscopic observations, faulty histological diagnosis or, very frequently, obsolete classification. It is certain that many undifferentiated carcinomata primary in the ovary have been classed as "fowl paralysis tumours," lymphocytoma and so on; that many cases of erythroleucosis have been called lymphatic leukaemia, and that granulomata have been variously diagnosed as endotheliomata,

* Based on a portion of a thesis presented for the Fellowship Diploma of the Royal College of Veterinary Surgeons.

peritheliomata or round-cell sarcomata, depending on the observed abundance of proliferating capillaries or the degree of infiltration with round cells. Critical examination of the gross and histological characters, special staining techniques and a study of the distribution of the tumour should suffice in most cases to establish its identity. Routine bacteriological examination of all suspicious growths, together with the employment of bacterial stains, would eliminate most of the granulomata.

Revision of nomenclature has invalidated much of the early work on the relative incidence of specific tumours. The term " sarcoma " is now entirely confined to use as a suffix to a definite word, *e.g.*, fibro-sarcoma, osteo-sarcoma, and so on; and there is now a strong tendency to discard the term completely and to speak of a fibroblastoma, osteoblastoma, etc. In the light of present knowledge on the histogenesis of tumours the term " round-cell sarcoma," so commonly used in the past, is obviously no longer desirable. Formerly, however, " round cell sarcoma " was frequently the diagnosis for tumours which are now classified as lymphoblastoma and were therefore included among the connective tissue tumours.

One cannot be certain, moreover, from examination of the figures for the incidence of leucotic tumours, whether those growths which are manifestations of the visceral form of fowl paralysis have been included or not. Histologically such growths, even when they occur in birds, it should be emphasised, with evident neurolymphomatosis, are indistinguishable from multiple discrete lymphocytomata and have a similar distribution, although, as will be mentioned later, it is possible that the organ incidence differs. Therefore, it seems reasonable to include such tumours among the lymphoblastomata, although in subsequent tables they have been placed in a separate column for the sake of comparison and clarity.

Finally, neglect to examine the brain as a routine at autopsy no doubt accounts for the rarity of nerve tissue tumours reported in the literature. Without a careful examination of the brain, nervous symptoms cannot be dismissed as attributable to fowl paralysis or dietetic deficiency.

The earliest descriptions of neoplastic disease in the domesticated fowl appear to have been by Roloff (1868) and Caparini (1896). There was then a lapse of several years, during which no publications are traceable, until an article by Butterfield (1905). This was followed by three articles in 1907 by Kon, Ehrenreich and Warthin respectively, and by Ellermann and Bang's articles in 1908–09. Other early contributors were Tyzzer and Ordway (1909), Hirschfeld and Jacoby (1909 and 1912), and Schmeisser (1916). Of these writers, Ehrenreich (1907) attempted to ascertain the incidence of neoplastic conditions in the fowl, examining 4,000 carcases destined for human consumption and finding seven malignant tumours. All the other papers dealt exclusively with leucotic conditions. Bürger (1914) was the only other of the earlier workers to attempt to find the incidence of tumours in fowls, recording among the 852 birds examined that 12 (1·4 per cent.) had tumours and that seven of these affected the ovary. To Curtis (1915), however, must belong the credit of having been the first

to make an accurate assessment of the frequency of tumours in the domestic fowl. A flock of 880 birds was kept under observation and all that died were examined *post-mortem*. The incidence of neoplastic disease in the whole flock was 8·98 per cent. and a distinct rise was noticed among the older birds. Figures are given for organ distribution but unfortunately the tumours were only classified as solid or cystic, without histological examination.

Other workers who have investigated the incidence of neoplastic disease in fowls or who give figures from which the incidence can be computed, are :—

Worker	*Number of Fowls Examined*	*Percentage affected*
Klee (1917)	611	14·5
Lentz (1922)	297	2·6
N.J. State—		
(1924)	142	11·9
(1925)	106	6·6
	861 (pullets)	7·5
Matheson (1927)—		
Flock A	424	3·3
Flock B	402	13·3
Flock C	516	7·1
Kerr (1928)	460	9·8
N.Y. State Vet. Coll. (1929–30) ...	1,436 (60% adults)	14·6
	1,350 (40% adults)	4·2
Malke (1930)	858	3·8
Babic (1931)	647	6·4
Bullis and Olsen (1943)	2,304	12·9

Matheson and Wilson (1933) give an incidence of 12·1 per cent., the estimation being based on total death figures over a period of 19 years. Goss (1940) had six flocks under observation. In flock A, the incidence of tumours during a period of three years was 16·48, 14·16 and 16·69 per cent. respectively. Flocks B, C, D and E were under observation for one year only and showed 10·62, 20·39, 23·18 and 24·61 per cent. respectively. Flock F showed 24·7 per cent. in White Leghorns, 23 per cent. in Rhode Island Reds and 52·81 per cent. in Barred Plymouth Rocks. The totals show that neoplastic disease occurred in 1,446 fowls (19·51 per cent.) out of 7,408 examined. Dudley, Dobson and Gordon (1941), analysing their figures for the years 1929 to 1937, show that 8 to 10 per cent. of the total mortality was due to tumours of the reproductive tract. The incidence of tumours in other organs varied from 3 to 12·7 per cent. while the leucotic complex accounted for 4·6 to 8·6 per cent. of the total mortality.

It will thus be seen that the incidence of neoplastic disease as reported by various workers during the last four decades varies between 0·17 per cent. (Ehrenreich, 1907) and 19·5 per cent. (Goss, 1940).

In view of this wide variation, it was thought that an examination of the figures of the Poultry Diseases Department of the Royal (Dick) Veterinary College might yield interesting results, especially as particular attention has been paid to neoplastic diseases in fowls during the past five years. During this time, every bird having tumours or leucosis was submitted to a detailed autopsy and all the

main organs, whether grossly abnormal or not, were submitted to histo-pathological examination.

MATERIALS AND METHODS

The material used in this investigation was obtained from fowls sent to the laboratory for autopsy during the five-year period 1939–1944. The birds were generally dead when received but a number of living fowls were examined in which neoplastic disease was diagnosed and in these cases it was possible to submit material to cytological examination, a procedure which is not possible with the ordinary *post-mortem* material of one or two days' duration. At the same time the latter material was generally sufficiently fresh for all routine histo-pathological study, and the few fowls with tumours which were in such a state of decomposition when received as to make histological examination worthless were less than 1 per cent.

Complete records were kept, including breed, sex, age (when known), whether destroyed or died, organ distribution and appearance of tumours, histological details and photographs. Tissues were fixed in 10 per cent. formol-saline and embedded in paraffin wax. Sections were usually cut at 5 to 8 μ and stained with Harris' alum haematoxylin and eosin, unless they were to be photographed, when Erlich's acid haematoxylin was substituted, as it was found to be a more intense stain and therefore more suitable for photography. If considered necessary, special fixatives and special stains were used. In a large number of cases, the distribution of the reticulum was studied, using Laidlaw's silver-carbonate technique. In all leucotic conditions, blood films and marrow or organ impressions were examined after staining with May-Grünwald-Giemsa. Marrow and organ impressions stained better if they were dipped in absolute alcohol for a few minutes, then dried and stained. This was attributed to the dissolving out of intracellular lipoids by the alcohol, thus allowing better penetration by the stain. In cases of suspected granulomata, bacteriological methods frequently gave useful information. Bacterial stains were also used on sections from such cases. In this way, many lesions liable to be confused with true neoplasms were eliminated.

CLASSIFICATION

No entirely satisfactory scheme for the classification of neoplasms is yet available, and none will be available until the exact histogenesis of all tumours is known. In avian pathology difficulties of classification are increased by the occurrence, as already mentioned, of lymphoid tumours in association with a specific disease, *viz.*, fowl paralysis, and thus the evidence for and against the inclusion of fowl paralysis tumours with other lymphoid neoplasms was carefully examined. It was decided to include them and designate the group "lymphoblastomata." Similar difficulties exist with erythroleucosis. Some evidence, based chiefly on Furths' (1931) observations that the virus isolated from erythroleucotic fowls sometimes produced erythroleucosis and sometimes myelogenous leucosis, suggests that this condition should be included in the myeloblastomata. Certainly a mixed myeloid and erythroleucosis is not infrequently seen in the same bird. On the other hand, Jordan

(1936) claims to have shown that avian red blood cells develop from small lymphocytes, which he therefore prefers to call lymphoid haemoblasts. If this is true, erythroleucosis should be grouped with the lymphoblastomata, but with such conflicting evidence it is safest at present to place erythroleucosis in a provisional group by itself termed erythroblastomata.

That all three leucotic conditions in the fowl are related is suggested by the fact that in almost all reptiles, birds and mammals, the erythrocytopoietic and granulocytopoietic tissues are intimately associated. In many species of sub-reptilian vertebrates, these tissues are located in different organs : the Salamander, for example, has erythrocytopoietic tissue in the spleen and granulocytopoietic tissue in the liver. But in each of these organs, the haemopoietic tissue is associated with identical lymphoid tissue, so that lymphoid cells may be ancestral to both erythrocytes and granulocytes (Jordan, 1936).

The classification adopted (Table I) is essentially that of Mallory (1914), modified to conform with avian neoplasia. Mallory's classification is based on the fact that tumours can originate from any of the sixteen or so different types of cell constituting the healthy animal body with a separate histological identity, which normally arise during embryogenesis.

RESULTS AND DISCUSSION

The following is a list of the various types of neoplastic disease found during the investigation. (It will be noted that owing to uncertainty as to their classification, thymoma, adrenal-cortical tumour, osteoclastoma and thecoma have been omitted from the classification, Table I.)

Adenocarcinoma fibromatosum.	Erythroleucosis	Fibroma
Adenocarcinoma leiomyomatosum	Myeloid leucosis	Fibro-sarcoma.
Hepatocellular carcinoma.	Lymphoid leucosis	Histiocytic sarcoma
Cholangiocellular carcinoma	Myelocytoma	Reticulum-cell sarcoma
Hepatoma (adenoma)	Lymphocytoma	Fibro-leiomyoma
Benign cystadenoma of lung	Thymoma	Thecoma (theca cell tumour)
Anaplastic carcinoma	Adrenal-cortical tumour of ovary	Astrocytoma
Haemangio - endothelioma	Embryonal nephroma	Spongioblastoma
Lymphangio - endothelioma		Neuroma
		Osteoclastoma
		Osteogenic sarcoma
		Chondroma

TABLE I

CLASSIFICATION OF AVIAN NEOPLASTIC CONDITIONS
(MODIFIED AFTER MALLORY)

Type Cell	*Normal Product*	*Tumour*
Fibroblast	Connective tissue	Fibroblastoma, including fibroma, fibro-sarcoma, reticulo-sarcoma and mixed-cell tumours (histiocytic and fibroplastic sarcoma).
Leiomyoblast	Smooth muscle	Leiomyoblastoma, including leiomyoma (fibro-leiomyoma of broad ligament) and leiomyosarcoma.
Endothelioblast	Blood and lymph vessel endothelium	Endothelioblastoma, including haemangio-endothelioma and lymphangio-endothelioma.
Lymphoblast	Lymphocyte	Lymphoblastoma, including lymphocytoma or aleukaemic lymphoid leucosis, lymphatic leukaemia and fowl paralysis tumour.
Erythroblast	Red blood cell	Erythroblastoma, aleukaemic or leukaemic (erythroleucosis).
Myeloblast	Myelocyte	Myeloblastoma, including myelocytoma and myeloid leucosis, leukaemic and aleukaemic.
Glioblast	Neuroglia cells	Glioblastoma, including glioma and gliosarcoma.
Osteoblast	Bone tissue	Osteoblastoma, including osteoma and osteogenic sarcoma.
Chondroblast	Cartilage tissue	Chondroblastoma, including chondroma and chondro-sarcoma.
Epithelioblast	Epithelium	Epithelioblastoma, including papilloma, adenoma and carcinoma.
Neuroblast	Nerve cell	Neuroblastoma, including neuroma.
Foetal anlage	Normal organ, *e.g.*, kidney	Misplaced and disordered growth producing teratoma, *e.g.*, embryonal nephroma.
	Not Observed	
Mesothelioblast	Mesothelium of serous surfaces	Mesothelioblastoma, including mesothelioma.
Myxoblast	Mucous connective tissue	Myxoblastoma, including myxoma and myxosarcoma.
Lipoblast	Fat tissue	Lipoblastoma, including lipoma and liposarcoma.
Melanoblast	Pigment cell	Melanoblastoma, including melanoma and malignant melanoma.
Rhabdomyoblast	Striated muscle	Rhabdomyoblastoma, including rhabdomyoma and rhabdomyosarcoma.

The tables and diagrams are self-explanatory but a few elucidatory notes are appended.

NEOPLASTIC DISEASE 1940–44 INCLUSIVE

TABLE II (A)

BREED INCIDENCE

Breed	*R.I.R.*	*W.L.*	*Br.L.*	*Bl.L.*	*W.W.*	*B.R.*	*L.S.*	*X.*	*Others*	*Total*
Number examined ...	813	398	108	91	126	98	110	60	259	2,063
Number with Neoplastic disease ...	144	78	26	16	13	38	16	12	43	386

From the above, the value of $\chi^2 = 36{\cdot}2$ and with n. = 8 then p. is less than 0·001.

The incidence of Neoplastic disease in the Buff Rock breed is therefore significantly high.

Table II (A) has been arranged so that the Chi-squared test as outlined by Mather (1943) can be employed as a test for significance. It will be seen that out of a total of 2,063 fowls examined, neoplastic disease in some form or other occurred in 386 (18·7 per cent.). This incidence agrees closely with the observations of the New Jersey State Agricultural Experimental Station (1924). Of the nine breeds examined, the Buff Rock and White Wyandotte are prominent in that the former shows a high and the latter a low incidence as compared with the average for all breeds (18·7 per cent.). A statistical check on this apparent feature is therefore desirable.

In the case of the Buff Rock breed, the individual χ^2 (obtained by dividing the difference between the observed number of cases of neoplastic disease and the expected number, squared, by the expected number) is sufficiently high to warrant further investigation. The sum of these values for all breeds is 29·49 which gives a χ^2 of 36·2. To check these results, the contribution to χ^2 by the numbers in each breed without neoplastic disease is computed, and the sum of these contributions, added to the sum of the contributions already obtained, gives a result of 36·06, which agrees closely with the first result.

If Fisher's tables are consulted, it is found that with " n " (the number of degrees of freedom, *i.e.*, in this case the number of different breeds minus one) equal to 8, then the probability that the incidence figures in the Buff Rock breed are high simply by chance is about 1 : 1,000. The conclusion is that they are highly significant.

The next highest contribution to χ^2 is given by White Wyandottes, and in order to test the significance of the apparently low incidence of neoplastic disease in this breed, a 2 × 2 contingency table has been constructed (Table II (B)) comparing this breed with the remaining breeds but excluding Buff Rocks. This gives a χ^2 of 3·762, and with n = 1, p. lies between 0·1 and 0·05.

To summarise the findings: the greatest deviations from the average incidence is shown by the Buff Rock and White Wyandotte

TABLE II (B)

SIGNIFICANCE TEST FOR WHITE WYANDOTTES

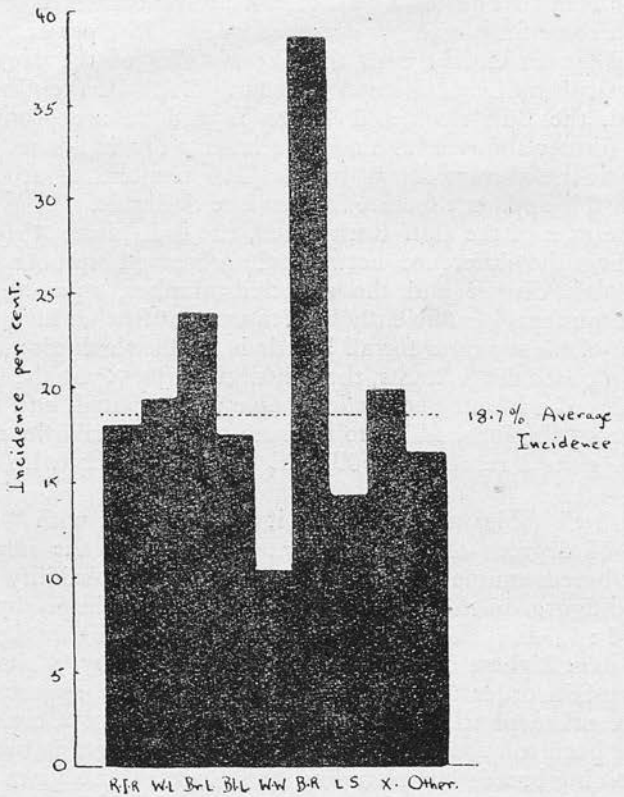
	(a)	(b)	
W.W.	Number examined 126	Number with N.D. 13	$n_1 = 139$
	(c)	(d)	
Not W.W. *Less B.R.*	Number examined 1,839	Number with N.D. 335	$n_2 = 2,174$
	n_3 1,965	n_4 348	$N = 2,313$

The contingency $\chi^2 = \dfrac{(ad - bc)^2 N}{n_1 x\ n_2 x\ n_3 x\ n_4}$

Applying this formula to the above table we get: $\chi^2 = 3{\cdot}762$. With n. = 1, p. lies between 0·1 and 0·05 and the figures for W.W. are therefore significant.

FIGURE I.

SHOWING INCIDENCE OF TUMOURS IN VARIOUS BREEDS OF THE DOMESTIC FOWL.



(The horizontal interrupted line represents the average relative incidence for all the above breeds. The figures for White Wyandotte and Buff Rock are statistically significant.)

breeds. Of the former, nearly 39 per cent. were affected, as compared with a little over 10 per cent. of the latter. None of the other breeds showed statistically significant deviations from the average incidence of 18·7 per cent. Fig. 1 shows in diagrammatic form the incidence in the respective breeds of fowl examined. Table III (A)

TABLE III (A)

SEASONAL INCIDENCE 1940–44 INCLUSIVE

	Jan.	*Feb.*	*Mar.*	*Apr.*	*May*	*June*	*July*	*Aug.*	*Sept.*	*Oct.*	*Nov.*	*Dec.*	*Total*
No. examined	126	97	152	164	176	235	240	206	218	164	192	93	2,063
No. with Neoplastic disease	32	16	47	35	31	43	27	31	33	37	30	24	386

The value of χ^2 as calculated from the above = 37·6 and with n. = 11, *p. is less than* 0·001.

gives the result of the χ^2 test on the varying incidence of neoplastic disease from month to month over the five-year period. A cursory examination of the figures reveals a high incidence in March and a low one in July, but the annual figures indicate this variation to be constant each year. The calculated result for χ^2 is 37·6, and with n = 11, p. (in Fisher's tables) is less than 0·001. As an additional check, a 2×2 contingency table (Table III (B)) was constructed, comparing summer with winter months, and again p. is found to lie between 0·01 and 0·001.

TABLE III (B)

SIGNIFICANCE TEST FOR INCIDENCE IN SUMMER AND WINTER MONTHS

(*a*) Number examined 1,239	(*b*) Number with N.D. 200	$n_1 = 1,439$
(*c*) Number examined 824	(*d*) Number with N.D. 186	$n_2 = 1,010$
$n_3 = 2,063$	$n_4 = 386$	$N = 2,449$

$\chi^2 = 9·087$

With n. = 1, *p. lies between* 0·01 *and* 0·001.

The difference in incidence between summer and winter is therefore significant and as Table III (A) shows, March and July are the critical months.

It is probable, therefore, that there is a significant seasonal variation of neoplastic disease in the fowl which may be due to increased endocrine activity in the spring causing a flare-up of latent pre-neoplastic cells. The possibility that the figures from month to month might be misleading has not been overlooked. If, for example,

there is a dearth of other diseases in the spring months, the figures for neoplasms, assuming an even distribution throughout the year, would show an apparent increase. Similarly in summer there might be more general disease, making neoplastic disease appear to have a low incidence. This argument could be strengthened by examination of the monthly figures, for it will be seen that the total number of autopsies in the five successive spring months (March) is 153, while the figure for the five summer months (July) is 240. According to this argument, however, and postulating that neoplastic disease is fairly evenly distributed throughout the year, it might be assumed that the highest figure for that disease would appear in December, during which month only 93 autopsies were performed over the five years, whereas in fact it occurs in March. Thus it seems reasonable to conclude there is a seasonal variation.

In further support of these observations, the findings of other workers may be cited. Schaaf (1936) found that over a period of three years the incidence of spontaneous leucosis in fowls had its maximum in March and its minimum from June to October. His explanation was that spring-hatched birds reached the critical age for leukaemia in the following November to May (*i.e.*, 9 to 14 months). While this may be correct in his particular observations, other workers such as Engelbreth-Holm (1942) and Peacock (1935) have reported a similar seasonal variation in the percentage successes in transmission passages of leukaemia and experimentally induced sarcomata. Furthermore, Lambin and Gérard (1934) have observed a seasonal variation in the incidence of leukaemia in man, maximum figures tending to occur in the winter and spring months.

Table V shows the distribution of the various main types of neoplastic condition in the breeds of fowl examined. The figures are based on a four-year survey only but it will be seen by comparison with Fig. 1 that the total incidence is not significantly different. Carcinomata and lymphoblastomata were most common, each accounting for 25·5 per cent. of cases ; " fowl paralysis tumour," or visceral lymphomatosis came next at 18·6 per cent., followed by true leukaemic conditions (mainly erythro- and myeloid leukaemia). The incidence of sarcomata was 6·3 per cent. and plain muscle tumours nearly 4 per cent. The remaining types varied from 3 to 0·3 per cent. The only significant breed difference was the absence of leukaemia from the White Wyandottes and Light Sussex. The failure to find fowl paralysis tumours and aleukaemic lymphocytomata in " other breeds " obviously has no significance.

If lymphoblastoma *per se* and visceral lymphomatosis are grouped together, tumours with lymphoid cells as cell type account for just over 44 per cent. of all types. This finding agrees closely with that of Jackson (1936), who recorded 93 lymphoid tumours in a collection of 203 : incidentally, the incidence of lymphoid tumours in fowl paralysis was 25·6 per cent. Pappenheimer, Dunn and Cone (1926) found 10 per cent. of their cases to have tumours.

TABLE IV (A)

Visceral lymphomatosis	(*a*) Intestines involved 8	(*b*) Intestines not involved 48	$n_1 = 56$
Lymphocytoma	(*c*) Intestines involved 39	(*d*) Intestines not involved 38	$n_2 = 77$
	$n_3 = 47$	$n_4 = 86$	$N = 133$

The contingency $\chi^2 = 14{\cdot}77$. With n. = 1 p. = $<0{\cdot}001$

TABLE IV (B)

Visceral lymphomatosis	(*a*) Liver involved 11	(*b*) Liver not involved 45	$n_1 = 56$
Lymphocytoma	(*c*) Liver involved 43	(*d*) Liver not involved 34	$n_2 = 77$
	$n_3 = 354$	$n_4 = 79$	$N = 133$

$\chi^2 = 8{\cdot}219$

With n = 1 p. is between 0·01 and 0·001

In view of the present uncertainty regarding the relationship between lymphocytoma and fowl paralysis tumour (visceral lymphomatosis), it was thought that a comparison of the organ distribution of the two conditions might throw some light on the problem. In 56 cases of lymphoid tumours associated with fowl paralysis, the intestines were involved in eight and the liver in 11. Conversely, in 77 cases of lymphocytoma with no detectable nerve lesions, the intestines were involved in 39 and the liver in 43. Applying the 2×2 contingency test to these figures it is found that in Table IV (A) the contingency χ^2 is 14·77, and with n = 1, p. is less than 0·001, while in IV (B) $\chi^2 = 8{\cdot}219$, and with n = 1, p. lies between 0·01 and 0·001. Thus it appears that in lymphocytoma the intestines and liver are involved in a significantly greater proportion of cases than in visceral lymphomatosis (fowl paralysis). This suggests that there may be an aetiological difference between the two conditions.

TABLE V

A.—PERCENTAGE INCIDENCE OF DEATHS DUE TO NEOPLASIA
B.—SHOWS THE RELATIVE INCIDENCE OF NEOPLASTIC CONDITIONS IN INDIVIDUAL BREEDS OF THE DOMESTIC FOWL, AND IN FOWLS GENERALLY

	A			B																									
				Fowl Paralysis		*Aleukaemic Lymphocytoma*		*Leukaemia*		*Aleukaemic Myelocytoma*		*Carcinoma*		*Sarcoma*		*Leiomyoma*		*Endothelioma*		*Glioma*		*Osteogenic Sarcoma*		*Embryonal Nephroma*	
Breed	*Examined*	*Tumours*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*	*No.*	*Incidence*
Rhode Island Red	653	108	16·5	23	21·2	22	20·3	17	15·7	3	2·7	27	25·0	5	4·6	3	2·7	5	4·6	2	1·8	0	0·0	1	0·9
White Leghorn... ...	326	61	19·0	10	16·4	21	34·4	4	6·5	2	3·2	15	24·5	3	4·9	2	3·2	0	0·0	1	1·6	0	0·0	3	4·9
Brown Leghorn... ...	90	22	24·4	3	13·6	6	27·2	4	18·1	1	4·5	6	27·2	2	9·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
Black Leghorn	54	11	20·3	2	18·1	3	27·2	4	36·3	0	0·0	2	18·1	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
White Wyandotte ...	108	11	10·1	3	27·2	2	18·1	0	0·0	0	0·0	3	27·2	1	9·0	2	18·1	0	0·0	0	0·0	0	0·0	0	0·0
Buff Rock ...	87	31	36·7	3	9·6	10	32·2	1	3·2	1	3·2	7	22·5	4	12·9	3	9·6	0	0·0	0	0·0	1	3·2	1	3·2
Light Sussex ...	96	13	12·5	3	23·0	6	46·1	0	0·0	0	0·0	3	23·0	0	0·0	0	0·0	0	0·0	1	7·6	0	0·0	0	0·0
Cross Bred ...	246	39	15·8	9	23·0	7	17·9	3	7·6	2	5·1	11	28·2	3	7·6	2	5·1	2	7·6	0	0·0	0	0·0	0	0·0
Other breeds ...	32	5	15·6	0	0·0	0	0·0	1	20·0	0	0·0	3	60·0	1	20·0	0	0·0	0	0·0	0	0·0	0	0·0	0	0·0
Total	1,692	301	17·7	56	18·6	77	25·5	34	11·2	9	3·0	77	25·5	19	6·3	12	3·9	7	2·6	4	1·3	1	0·3	5	1·6

SUMMARY

During a five-year investigation of spontaneous neoplastic disease in the domestic fowl, 2,063 birds were submitted to detailed examination. In 386 cases of neoplastic disease encountered, with an incidence of 18·7 per cent., 28 distinct types of neoplasia were observed.

Neoplasms were most common in the Buff Rock breed (38·8 per cent.), and least in White Wyandottes (10·3 per cent.). The breed incidence for the various types of neoplastic disease was also investigated, and the only significant difference was the absence of leukaemia in the White Wyandotte and Light Sussex breeds. The seasonal variation showed a maximum incidence in the spring months, particularly March, which was tentatively attributed to the stimulation of latent pre-neoplastic cells by sex hormones.

A comparison of tumour distribution in visceral lymphomatosis (fowl paralysis) and lymphocytoma showed that in the latter the liver and intestines were involved more frequently than in fowl paralysis.

A brief summary of the literature dealing with the incidence of neoplastic disease is given and the problems associated with the classification of avian neoplastic disease are discussed.

ACKNOWLEDGMENT

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REFERENCES

Babic, I. (1931). *Veterinarski Archiv.*, **1**, 158. (Cited by Goss.)
Bullis, K. L., and Olson, C. (1943). *Amer. J. vet. Res.*, **4**, 382.
Bürger, M. (1914). *Z. f. Krebsforsch.*; **14**, 526.
Butterfield, E. E. (1905). *Folia Haematol.*, **2**, 649.
Caparini, U. (1896). *Clin. Vet.*, **19**, 433. (Cited by Engelbreth-Holm.)
Curtis, M. R. (1915). *J. agric. Res.*, **5**, 397.
Dudley, F. J., Dobson, N., and Gordon, R. F. (1941). *Vet. Rec.*, **53**, 323.
Ehrenreich, M. (1907). *Med. klin. Jahrg.*, **3**, 614.
Ellermann, V. E., and Bang, O. (1908). *Z. Bakt.*, **44**, 595.
Engelbreth-Holm. (1942). *Leukaemia in Animals.* Edinburgh: Oliver & Boyd.
Fölger, A. E. (1917). *Ergebn. allg. Path. path. Anat.*, **18**, 372.
Foulds, L. (1934). *Imp. Canc. Res. Fund. 11th Scientific Rept.* (*Supplement*); (1940). *Amer. J. Canc.*, **39**, 1.
Fürth, J. (1931). *Arch. Path.*, **12**, 1.
Goss, L. J. (1940). *Cornell Vet.*, **30**, 75.
Hirschfeld, H., and Jacoby, M. (1909). *Berlin klin. Wschr.*, **4**; (1912). *Zeitschr. klin. Med.*, **75**.
Jackson, C. (1936). *Onderstepoort J.*, **6**.
Jordan, H. E. (1936). *Amer. J. Anat.*, **59**, 249.
Kerr, W. R. (1928). *Vet. J.*, **84**, 584.
Klee. (1917). (Cited by Folger, A. F.)
Kon, J. (1907). *Virchow's Archiv. path. Anat.*, **190**, 338.

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THE INTRACELLULAR LOCALIZATION OF β-GLUCURONIDASE.

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It has been suggested that the activity of the enzyme β-glucuronidase reflects the degree of cellular proliferation taking place in a tissue or organ (Levvy, Kerr and Campbell, 1948 ; Kerr, Campbell and Levvy, 1949). Evidence has been presented showing that the enzyme activity of tissue extracts is increased in damaged organs undergoing repair, during liver regeneration subsequent to partial hepatectomy, and in the organs of young mice as compared with adults.

Biochemical assay was necessary in order to obtain a quantitative expression of β-glucuronidase activity, but this gives no indication of the actual site of the enzyme in the cell. The recent publication of two histochemical methods (Friedenwald and Becker 1948), now enables the location of the enzyme to be studied.

EXPERIMENTAL.

The methods used for the histochemical detection and localization of β-glucuronidase were essentially those devised by Friedenwald and Becker (1948). Their technique depends upon the hydrolysis at 37° C. of a conjugated glucuronide by the enzyme in fresh frozen sections of tissues, with either the direct liberation of an insoluble azo dye, or the formation of a precipitate of ferric hydroxyquinoline which is converted into Prussian blue. In both methods microscopically visible substances are deposited at the presumptive site of enzyme activity in the tissue.

(1) " 1-*ortho-hydroxyphenylazo*-2-*naphthol glucuronide* " (Friedenwald and Becker, 1948). The substrate solution was made up by dissolving 50 mg. of the glucuronide in 1 litre 0·1 N acetate buffer at pH 5 with warming. When cool, the solution was filtered. Unfixed frozen sections, between 20–40μ in thickness, were incubated in a water bath at 37° C. in 100 ml. substrate solution, in stoppered vessels of such capacity that the air space above the solution was small.

After incubation, the sections were washed in distilled water, fixed in 10 per cent formalin and 1 per cent acetic acid, and either mounted directly in glycerine jelly, or counterstained with 0·5 per cent methyl green in 0·5 per cent acetic acid and then mounted.

Controls for the specificity of the method consisted of incubating the substrate alone, incubating with inert foreign matter, e.g. paper pulp, and incubating with frozen sections of boiled mouse kidney. In no case did hydrolysis occur, even after 18 hours.

(2) 8-*hydroxyquinoline glucuronide* (Brahm, 1899). The substrate solution was prepared as described by Friedenwald and Becker (1948). After the addition

of 0·03 N ferric chloride, followed by incubation at 37° C. for 2 hours, it was found necessary to carry out a double filtration through Whatman No. 40 paper to remove the very fine suspension of ferric hydroxyquinoline which formed. Filtration was carried out in an incubator saturated with water vapour.

It was found advantageous to place the frozen sections in chilled 0·1 N acetate buffer pH 5, as soon as they were cut and to remove as much buffer as possible by touching the sections against a Whatman No. 3 paper before transferring them to the substrate. Small weighing bottles capable of holding about 6 ml. were used as incubation vessels. These had the advantage that there was only a small air space between the liquid surface and the stopper.

The substrate was used on the day it was prepared, and sections were placed in it within 2 to 3 minutes of cutting. One of the main difficulties associated with this method was to obtain a complete conversion of the enzymatically liberated ferric hydroxyquinoline to Prussian blue. This was largely overcome by increasing the concentration of potassium ferrocyanide from 1 per cent to 2 per cent in the ferrocyanide-hydrochloric acid mixture. Sections were fixed as in method (1) and counter-stained with basic fuchsin 1 : 5000 in 1 per cent acetic acid.

RESULTS.

Location of the enzyme.

Fig. 1 shows that the enzyme is localized to the cortical region of the male mouse kidney, and Fig. 2 is an adjacent section incubated in a control solution in which 8-hydroxyquinoline glucuronide was replaced by an equal volume of buffer. A higher magnification (Fig. 3) shows the enzyme to be mainly localized to the proximal convoluted tubules. In suitably thin sections at a still higher power, the enzyme appears to be localized in the basal parts of the cells constituting the proximal convoluted tubules, coincident with the parallel rods frequently to be seen in ordinary histological preparations. These rods are thought to be mitochondria (Maximow and Bloom, 1948). The distal convoluted tubules do not show such an intense reaction, and there is only a very slight staining of Bowman's capsule and the glomerulus of the male kidney (Fig. 5 and 6). Similar localization to the mitochondria was seen in frozen sections of liver (Fig. 7).

Paraffin sections of tissue fixed according to the Gomori technique for phosphatase, showed only a very faint staining with " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide," and none at all with 8-hydroxyquinoline glucuronide.

Influence of pH.

In a series of trials with both glucuronides in which the pH of the substrate was respectively 4·5, 5, 5·2 and 5·5, the best results were obtained within the range 5–5·2 using the normal male mouse kidney.

Inhibition by saccharic acid.

Saccharic acid is known to be an effective inhibitor for β-glucuronidase *in vitro* (Karunairatnam and Levvy, 1949). Its action was utilized to test the specificity of the histochemical methods. Potassium hydrogen saccharate was dissolved in water to give a concentration of 0·1 M. Before final adjustment

of the volume the pH was adjusted to 5 (glass electrode). This solution was added during preparation of either substrate solution in place of part of the acetate buffer.

Using 8-hydroxyquinoline glucuronide as substrate, 0·001 M saccharate caused almost complete inhibition of hydrolysis by a section of male mouse kidney (Fig. 4). Fig. 4 should be compared with Fig. 3, which shows another section from the same kidney, incubated with substrate in absence of saccharate.

To see whether saccharic acid penetrates intact cells, a bisected mouse kidney was incubated overnight in 8-hydroxyquinoline glucuronide solution containing 0·001 M saccharate. As control, the other kidney, also bisected, was incubated in substrate alone. Serial frozen sections from the control kidney showed penetration of the glucuronide to a depth of 20 to 30 cells. Inhibition by saccharate was almost complete at all depths.

Using " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide " as substrate, inhibition by saccharate was not pronounced, although the inhibitor appeared to delay the reaction. A more pronounced inhibition resulted when sections were incubated with 0·001 M saccharate before placing in the substrate and inhibitor. With this glucuronide it appears that the insolubility of the reaction product is so great that the reaction is governed more by the rate of diffusion of the substrate than by the amount of enzyme available. After 2 or 3 hours incubation the fullest possible intensity of staining is seen, independently of the activity of the enzyme present. At shorter periods, some discrimination in intensity is possible between different preparations, but the reaction is not very suitable for comparative work.

Influence of sex on results.

It was incidentally observed that male mouse kidneys gave a more intense staining reaction to both methods than female organs. A suitably short period

EXPLANATION OF PLATES.

(All photographs prepared from frozen sections.)

PLATE I.

FIG. 1.—Longitudinal section of male mouse kidney showing localization of β-glucuronidase to the cortex. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 12 hours. × 12.

FIG. 2.—Control section incubated for the same time in the absence of the glucuronide. × 12.

FIG. 3.—Localization of the enzyme mainly to the proximal convoluted tubules of the kidney. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 12 hours. × 36.

FIG. 4.—Succeeding section to that shown in Fig. 3. Hydrolysis has been inhibited by 0·001 M saccharate. × 36.

PLATE II.

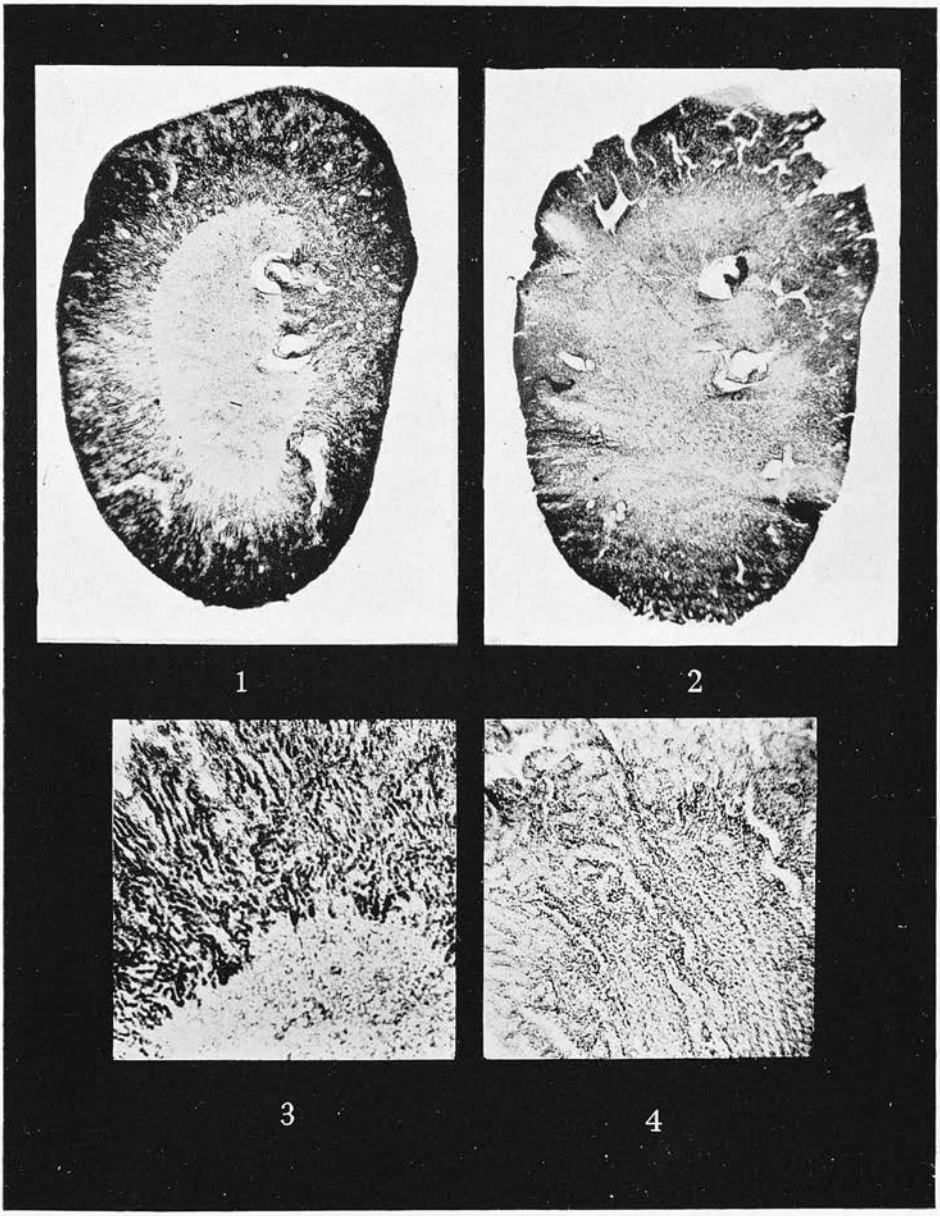
FIG. 5.—Proximal convoluted tubules of the kidney, showing localization of staining to the mitochondria. Substrate: " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide." Incubation time 80 minutes. × 300.

FIG. 6.—As for Fig. 5. The glomerulus is unstained. Incubation time 160 minutes. × 300.

FIG. 7.—Specific staining of the mitochondria of mouse liver cells. Substrate: " 1-ortho-hydroxyphenylazo-2-naphthol glucuronide." Incubation time 80 minutes. × 300.

FIG. 8.—3:4 benzpyrene induced squamous cell carcinoma of skin of mouse, showing intense staining of the malignant cells growing down into the corium. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 15 hours. × 36.

FIG. 9.—Granular and filamentous mitochondria staining specifically in the G.R.C.H./15 chicken fibro-sarcoma. Substrate: 8-hydroxyquinoline glucuronide. Incubation time 10 hours. × 300.



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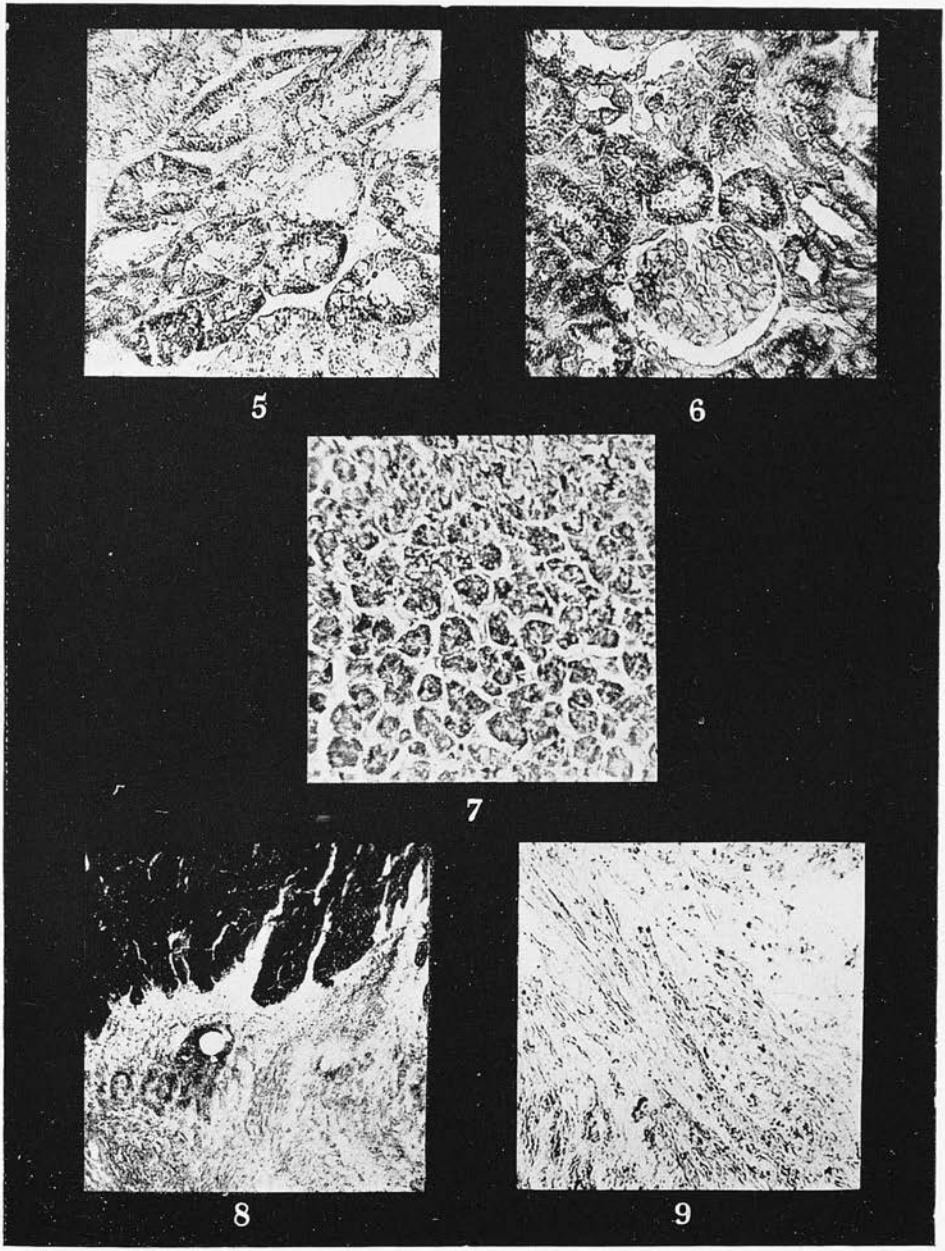

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of incubation was used in Method 1. The hydrolytic reaction was reduced in castrate male mouse kidneys, and increased in kidneys from ovariectomized mice, in both cases 8 weeks subsequent to operation. Localization of the enzyme was identical in male or female kidneys.

Results with tumour tissues.

Great difficulty was experienced in obtaining satisfactory histochemical pictures using rapidly-growing malignant tumours, because of frequent widespread necrotic changes, while in certain growths the presence of large amounts of mucoid material interfered with the reaction. However, although results were often unsatisfactory from the photographic aspect, a fair picture of the distribution of the enzyme was obtained in a preliminary study. As a whole, tumours seemed high in enzyme activity compared with normal liver and kidney. Table I summarizes the findings in those tumours examined.

TABLE I.

Tumour.	Enzyme distribution.
Rous sarcoma, fowl	Scattered discretely in cytoplasm.
G.R.C.H./15, fowl	,, ,,
Ovarian adenocarcinoma, fowl . .	Traces of enzyme confined to glandular epithelium. None in schirrous areas.
Mouse mammary carcinoma . . .	Rich in anaplastic areas, none in blood cysts, degenerated parts or connective tissue.
Squamous cell carcinoma, skin of mouse .	Rich in poorly differentiated down-growing epithelium. Traces in dermis.
Papilloma, skin of mouse . . .	None.

Assessing these preliminary observations, it may be concluded that β-glucuronidase is most rich in those parts of tumours where cell division is a prominent feature. This is especially well shown in Fig. 8 where a very pronounced staining is practically confined to the down-growing malignant epithelial cells of a mouse skin epithelioma induced by an injection of 3 : 4 benzpyrene. As in normal tissues, suitable preparations show the enzyme to be confined to the cytoplasm of tumour cells (Fig. 9).

DISCUSSION.

Fishman (1940) showed that the prolonged administration of menthol to mice caused a rise in the activity of β-glucuronidase in the liver, kidney and spleen. He postulated that this enzyme acts synthetically, but this has not yet been established and other workers take the view that the function of the enzyme in the animal body is purely hydrolytic (Levvy, 1947 ; Karunairatnam, Kerr and Levvy, 1949.)

In a later report, Fishman and Anlyan (1947) demonstrated a high β-glucuronidase content in some human tumours. It is interesting to note that

in Table I of their communication, the activity of the enzyme was closely correlated with the clinical malignancy of the tumour. For example, benign tumours showed a low activity, tumours such as schirrous carcinoma also low, whilst adenocarcinoma and medullary carcinoma had high enzyme activities. It is difficult in cancer studies to obtain the corresponding normal tissue unless an organ like the liver is involved, and a serious criticism may be made of the normal tissue controls used by Fishman and Anlyan (1947). Whereas an organ may be a complex of tissues, only one of these is usually involved in neoplasia. The fact remains, however, that striking elevations of the enzyme figures occurred in malignant tumours compared with normal tissues or organs from which those tumours arose. This work has been confirmed and extended by Odell and Burt (1949).

Kerr and Levvy (1947) at the same time as Fishman's work on tumours, showed that a rise in enzyme activity occurred in mouse liver or kidney following administration of toxic agents not known to form glucuronides, dependent on the site of damage. The action of menthol, which was found to be a liver and kidney poison could be explained on the same terms. Kerr, Levvy and Campbell (1947) found that the rise in the enzyme activity was not detectable in the early stage of poisoning, as, for example, with mercuric nitrate or chloroform in the kidneys of the male mouse, but occurred as soon as repair was histologically demonstrable. Furthermore, sub-total hepatectomy of mice, either by ligation or cautery, caused rapid hypertrophy of the remainder of the organ, a great deal of mitotic activity and an elevation of β-glucuronidase activity. It was also found that the liver, spleen and kidney of baby mice showed much higher enzyme levels than in adults. This was also true of uterus (Kerr, Campbell and Levvy, 1949).

It has been shown by Crabtree (1941) that there is a morphological difference in the cells of Bowman's capsule in male and female adult mice. Eschenbrenner and Miller (1945) demonstrated the specific toxicity of chloroform for the convoluted tubules of the male mouse kidney. Furthermore they showed that the epithelium of Bowman's capsule in castrated male mice tended to change to the female type, and that the kidneys of such mice were no longer susceptible to chloroform poisoning. These findings were confirmed (Kerr, Campbell and Levvy, 1949) and it was also shown that a rise in β-glucuronidase occurred in male kidneys subsequent to the administration of chloroform, but not in female kidneys or kidneys of castrated male mice, and the rise, when demonstrable, was coincident with repair processes in the damaged organ.

Further evidence for a relationship between cell proliferation and β-glucuronidase activity appears to be provided by Fishman and Fishman's (1944) observation that the administration of oestrogens to ovariectomized mice causes a rise in uterine glucuronidase, their explanation being that glucuronidase is primarily concerned with the conjugation of oestrogen. A more likely explanation would be on the basis of proliferating endometrium. The effect can be prevented by the concurrent administration of androgen (Kerr, Campbell and Levvy, 1949). Odell and Burt (1949) consider that the association between enzyme activity and proliferative activity provides the best explanation of their results for human cervical cancer, and those conditions characterized by an increased rate of cell division such as pregnancy and the proliferating corpus luteum.

The first part of the present paper deals with preliminary findings using histochemical methods for the localization of β-glucuronidase. The association with mitochondria is perhaps not unexpected, as it does not seem unreasonable to assume that many intracellular enzymes must be associated with these organoids, which in the living cell are in constant movement, exhibit changes of form, and frequently show a well marked tendency to polarity, all indicative of intracellular activity.

It is significant that the enzyme is confined to that part of the kidney parenchyma, the cortex, which is damaged in chloroform poisoning.

The experiments with saccharate suggested that the test with 8-hydroxyquinoline glucuronide at least, is specific for glucuronidase. It is interesting to note that the experiments give unequivocal evidence of penetration of saccharic acid into the intact cell. The failure of saccharic acid to influence glucuronide synthesis by surviving liver slices (Karunairatnam and Levvy, 1949) thus provides excellent grounds for believing that β-glucuronidase is not involved in the synthesis of glucuronides *in vivo*. In the few histochemical observations made to date and described above, β-glucuronidase appears to be particularly rich in actively growing tumours or parts of tumours.

SUMMARY.

Using techniques devised by Friedenwald and Becker (1948), the localization of β-glucuronidase has been studied in normal mouse kidney and liver, and in several chicken and mouse tumours. In the kidney, enzyme activity is practically confined to the proximal convoluted tubules in the cortex.

The intracellular site of the enzyme in both liver and kidney appears to be coincident with the mitochondria.

The hydrolysis of 8-hydroxyquinoline glucuronide, by fresh frozen mouse kidney sections is inhibited by 0·001 M saccharic acid. Serial frozen sections of a bisected kidney treated in the same manner, show penetration of the cells by saccharic acid, as indicated by inhibition of hydrolysis.

In unit time, the male mouse kidney shows a stronger staining reaction in these histochemical methods, than the corresponding female organ. Castration of males diminishes this effect, whereas ovariectomy augments it, when compared with the enzymatic activity of kidneys from intact animals of the like sex.

In a preliminary study the enzyme activity is found to be high in malignant tumours, and is mainly localized in anaplastic areas where cell division is prominent.

The results obtained by a number of workers can be interpreted in terms of a relationship between β-glucuronidase activity and proliferative activity.

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REFERENCES.

Brahm, C.—(1899) *Hoppe-Seyl. Z.*, **28**, 439.
Crabtree, C.—(1941) *Anat. Rec.*, **79**, 395.
Eschenbrenner, A. B., and Miller, E.—(1945) *Science*, **102**, 302.

FISHMAN, W. H.—(1940) *J. biol. Chem.*, **136**, 229.
Idem AND ANLYAN, A. J.—(1947) *Cancer Res.*, **7**, 808.
Idem AND FISHMAN, L. W.—(1944) *J. biol. Chem.*, **152**, 487.
FRIEDENWALD, J. S., AND BECKER, B.—(1948) *J. cell. comp. Physiol.*, **31**, 303.
KARUNAIRATNAM, M. C., AND LEVVY, G. A.—(1949) *Biochem. J.*, **44**, 599.
Idem, KERR, L. M. H., AND LEVVY, G. A.—(1949) *Ibid.*, in press.
KERR, L. M. H., CAMPBELL, J. G., AND LEVVY, G. A.—(1949) *Ibid.*, **44**, 487.
Idem AND LEVVY, G. A.—(1947) *Nature*, **160**, 463.
Iidem AND CAMPBELL, J. G.—(1947) *Ibid.*, **160**, 572.
LEVVY, G. A.—(1947) *Ibid.*, **160**, 54.
Idem, KERR, L. M. H., AND CAMPBELL, J. G.—(1948) *Biochem. J.*, **42**, 462.
MAXIMOW, A. A., AND BLOOM, W.—(1948) 'A Textbook of Histology.' London (W. B. Saunders Co.), p. 10.
ODELL, L. D., AND BURT, J. C.—(1949) *Cancer Res.*, **9**, 362.

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SOME UNUSUAL GONADAL TUMOURS OF THE FOWL.

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NEOPLASMS involving the ovary are common in the fowl (Olson and Bullis, 1942 ; Campbell, 1945). The great majority fall into one or other of two categories, namely adenocarcinomatous growths in various stages of differentiation from the frankly anaplastic type to the sclerosing tumour, or, secondly, lymphomatous growths associated with the leucotic complex, e.g., lymphocytoma, fowl paralysis tumour, aleukaemic lymphoid leucosis, etc. Most of these lymphoid growths should not be classified as ovarian tumours, since in view of the generalized nature of the leucotic process it is usually impossible to be certain whether they are primary in that organ or not. They are mentioned simply because of the frequent reference in avian pathological literature to " lymphoid ovarian tumours."

In human pathology, besides the common adenocarcinomatous types of ovarian tumour, several other rarer forms are recognized as follows : Brenner tumour (oöphoroma folliculare) : granulosa-cell, thecal, and luteal tumours ; arrhenoblastomas ; dysgerminomas.

A survey of the literature dealing with neoplastic disease of the fowl shows the apparent extreme rarity of such tumours. Seifried (1923) recorded a Brenner tumour of the fowl and compared it with its human counterpart, and Friedgood and Uotila (1941) detailed 5 cases of ovarian " tumours " associated with virilism in the fowl. In two of these latter cases, tuberculosis of the ovary complicated the picture, and the remainder were cystic or contained small growths which appeared to be tumours in various stages of degeneration. Tentative diagnoses of arrhenoblastoma were made, but it was also suggested that they might be luteal-cell tumours or " hypernephromata." The only record of an ovarian teratoma in the fowl is by Jackson (1936).

With regard to the male bird, there appear to be no reports of spontaneous seminoma or interstitial-cell tumour, but several cases of spontaneous teratoma testis have been recorded (Sheather, 1911 ; Jackson, 1936 ; Olson and Bullis, 1942).

It seemed reasonable to believe that the absence of reports of the rarer forms of ovarian and testicular tumours was simply an indication of a lack of intensive study in this branch of comparative pathology. It was therefore determined to examine as many tumours arising in these sites as possible. To date approximately 2000 cases have been submitted to a thorough post-mortem and histological scrutiny, and, as was anticipated, several hitherto unrecorded types of tumour of the fowl gonads have been encountered.

It is desirable that an account of these should be put on record, not only because of their interest from the viewpoint of comparative oncology, but also

because the study of such tumours may throw a light on the origin of their human counterparts, and may help to settle the much debated questions of inter-relationship and correlation between histological structure and endocrinological function.

METHODS.

All the tumours described in this paper arose spontaneously. Some of the birds came to autopsy without any history. Others were obtained alive with a history of unusual behaviour such as the development of male traits in the hen, cessation of laying, enlarging abdomen, etc. At post-mortem a thorough search was made for metastases, and the possibility that the gonadal tumour was not the primary growth was ruled out as far as possible. Blocks of tissue were taken from many organs for histological examination, whether they showed visible abnormality or not. They were fixed in formal-saline, Susa or Helly, embedded in paraffin wax and cut at 8μ, and were stained by a variety of methods. Frozen sections were studied when the occasion permitted. Photographs of gross specimens were taken in many cases.

PATHOLOGY.

Tumours of the Female Gonad.

Tumours of the female gonads will be dealt with first.

(1) This tumour was encountered in a Rhode Island Red hen in its third year. Upon cessation of laying the bird was killed for table purposes, and the carcase was brought to the laboratory upon the same day.

The ovary was inactive, and a large part of it was replaced by a firm, faintly yellow and smoothly bosselated tumour 4·0 × 3·5 × 3·8 cm. in dimension. A few small cysts were visible on the surface, and a larger cyst filled with a brownish mucoid fluid occupied the caudal pole of the mass. The growth offered some resistance to the knife, and its cut surface showed pale yellow fibrous areas containing numerous small cysts and islands of pinkish tissue. There were no implantations in the abdominal cavity and no metastases were found.

Histologically a large part of the tumour consists of a stroma of interlacing bands and whorls of fibrous tissue, mostly dense, but having a looser texture in some places. There is a certain amount of plain muscle (characteristic of the avian ovary especially at the hilus) in one part of a section. Embedded in the dense fibrous tissue are numerous epithelial-lined cysts of varying size, some exhibiting a single layer of flattened cells, others lined by several layers, the innermost of which are typical columnar mucous secreting cells (Fig. 1). No cilia are detectable. The lumen of many cysts contains a granular eosinophilic material. Scattered between these structures are a few solid nests of undifferentiated epithelial cells with large pale nuclei. These show a tendency to develop a lumen (Fig. 2).

In view of its undoubted ovarian origin, its benign character and its strikingly similar structure to the Brenner tumour of the human subject, this case—the only one of its kind in the whole series of ovarian tumours so far examined—was classified as such. According to Willis (1948) it is probable that Brenner tumours arise in the human from nests of cells first described by Walthard in 1903. These may be found on the surface of the ovary, Fallopian tube or broad ligament. At the same time he points out that no extra-ovarian Brenner tumour has ever been

reported, which, in view of the distribution of Walthard nests, raises the question of the validity of this etiological theory.

In an effort to establish whether comparable " nests " are present in the fowl, a considerable number of ovaries, oviducts and dorsal ligaments have been examined both by naked eye and histologically, with negative results. Such a finding of course does not rule out their existence in the fowl, but it does indicate that should other workers fail to find them, an alternative explanation will have to be considered. It may be relevant to indicate here that in the hilus of the fowl's ovary and in the oviduct ligament, a vestigial structure is to be found representing a rudiment of the genital part of the mesonephros and corresponding to the epididymis of the male. A comparison of this structure (Fig. 3) known as the parovarium, with the tumour described above shows a great similarity between the two. A comparable structure occurs in the human subject, and is well illustrated by Nicholson (1950).

(2) The next ovarian tumour to be considered is the granulosa-cell type, of which 4 cases have been studied in this series. They all occurred in adult hens, namely 2 Buff Rocks and 2 crossbreds. Unfortunately none of these was seen alive. In 2 cases there were extensive implantations from multiple ovarian tumours (Fig. 4) to the serous surface of the gut, oviduct, liver and kidney, and in one instance a metastatic growth was found in the lung. In the other 2 cases, the tumours were found at the site of the rudimentary right gonad, and one was associated with a small persistent cystic right oviduct. In both cases the left functional ovary appeared normal.

The gross appearance of these tumours varied somewhat. In general they were fairly firm, white, or with yellowish areas, sometimes cystic and occasionally haemorrhagic. It was interesting to note that in the first 2 cases mentioned above, although the ovary was not functional, the oviduct had the appearance and was nearly the size of an active organ.

Histologically, these tumours are characterized by a quite variable structure in different regions or even in different areas of the same section. One case, for example, shows mainly a " cylindroid " type of growth, with cords of polygonal epithelial cells with large clear nuclei and one or two prominent acidophilic nucleoli, lying in a fine connective tissue stroma (Fig. 5). A folliculoid formation is common in some areas, and the stroma between these groups of cells shows hyalinization (Fig. 6). Other cases show a more undifferentiated structure, but still a tendency to form " follicles," and in one case a very marked luteinization of the granulosa cells was present (Fig. 7). So noticeable is this feature in certain areas that a diagnosis of luteoma would be justifiable. " Typical " rosettes as seen in human tumours, characterized by a stellate arrangement of cells around a cystic space containing fluid or a degenerating cell, apparently do not occur in avian granulosa-cell tumours.

(3) One example of a theca-cell tumour was found at autopsy in a Buff Rock hen in her second year. It consisted of a yellowish irregular nodular mass measuring $5 \times 6 \times 4{\cdot}5$ cm. (Fig. 8). It was of a soft consistency and the exterior tended to be friable. On section, it was cream to yellowish in colour and displayed bands of tissue which had a tendency to radiate from the centre of the growth. The vascularity appeared to be very poor. The rest of the ovary seemed normal, with a few atretic follicles, and the oviduct was that of a bird in full lay. Histologically, this tumour is mainly composed of round oval or fusiform cells, with a

fine chromatin network in the nucleus and a rather foamy cytoplasm (Fig. 9). Mitosis is extremely rare. In some regions, particularly near the periphery, these cells become polyhedral, with round nuclei and vacuolated cytoplasm. Frozen sections stained with Sudan IV show abundant lipoid material in such cells. The periphery of the growth is largely necrotic. Radiating bands of smooth muscle occur in the interior of the section.

Willis (1948) states that ovarian fibromata are probably the end result of a theca-cell tumour. In that case, the rarity of the latter in fowls may account for the equally rare occurrence of fibrous tissue tumours associated with this organ. As an instance of this the only case of ovarian fibroma encountered in this series is worth mentioning. It was found in a Rhode Island Red hen in her third year, and weighed 570 g. including the small inactive ovary (Fig. 10).

(4) Glynn (1921) states that there is no evidence (in the human subject) that heterotopic adrenal cortical tumours arise in the ovary. Willis (1948) agrees, and adds that no acceptable report exists showing the presence of accessory adrenal tissue in the ovary, and he concludes with Glynn that " these growths are all luteinized ovarian tumours." Later, however (p. 510). Willis admits the possibility of very rare adrenal cortical tumours primary in the ovary. Maximow and Bloom (1948) state that what are usually considered chromaffin cells (sympathicotrophic cells) are to be found at the hilus of the human ovary. These apparently have much in common with the medullary cells of the adrenal, although they cautiously conclude that until these cells have been shown to elaborate epinephrin, their relationship to adrenal medulla must remain unproven. The chromaffin reaction of such ovarian cells is unreliable but this also applies to the adrenal medullary cells, where the staining by chrome salts may vary from practically none to a very deep brown.

As far as can be ascertained there is no record of chromaffin cells or heterotopic adrenal cortical tissue in the avian ovary. That such cells may in fact be occasionally present seems to be implied by the following cases, and a thorough cytological examination of the region of the hilus should clear up this point.

The first of these tumours to be described occurred in a Brown Leghorn hen which was brought for examination after developing signs of masculinization. The bird was said to have ceased laying, commenced crowing and to have endeavoured to copulate with other hens in the run. The comb was decidedly male in character, and plumage changes were evident especially in the neck and tail.

Upon post-mortem, a rounded soft yellow-brown tumour measuring 3·8 × 3·5 × 2·8 cm. was found in the ovary, which was inactive. The oviduct was atrophied. Both adrenals were intact and were removed with other organs for examination.

Histologically the tumour has a distinct capsule. Numerous irregular trabeculae containing vessels arise from this and pass into the interior of the growth. Between these are solid is ands of large polyhedral epithelial cells which are in turn divided into small irregular groups by fine connective tissue staining as for reticulum. The cytoplasm is eosinophilic and faintly granular. In places it is vacuolated. The nuclei are spherical and fairly constant in size, being situated usually in the centre of the cell. They have a finely scattered chromatin content and one or two inconspicuous nucleoli. There are no mitotic figures (Fig. 11).

This case is an excellent example of the pitfalls of diagnosis based upon purely

morphological grounds, especially when applied to gonadal neoplasms. Superficially the tumour may be thought to resemble a luteoma, but the secretion of androgens weighs against this. It is of interest however to compare it with a small group of thecal luteal cells in the fowl's ovary (Fig. 12). A " corpus luteum " derived from the granulosa cells presents an entirely different picture (Fig. 13).

Other possible explanations are two in number. Firstly the growth may represent a heterotopic adrenal cortical tumour, which Ewing (1942) states to be highly masculinizing but otherwise difficult to differentiate from luteomas. The views of Willis (1948) on the subject have already been stated. Or secondly it may be a case of arrhenoma with such advanced luteinization (Fig. 16) that it bears no resemblance to " text-book " illustrations of that tumour.

It may be stated here that Meyer (1931) concludes that there is hardly any ovarian tumour pattern which cannot be reproduced in the arrhenomata. This view is substantially supported by Burrows (1943). If this is the case, then obviously ovarian tumours can no longer be classified according to their histology but only by their endocrine effects, if any, on the host.

What are we to conclude ? A comparison of the histology of this tumour with normal adrenal cortical tissue of the fowl shows a striking resemblance (Fig. 14). Silver preparations for reticulum also resemble the pattern found in the adrenal cortex. Measurements of thecal luteal cells in the ovary, adrenal cortical cells, and those in the tumour under discussion show that the last two have a similar range in size, whereas the thecal cells are smaller (thecal luteal cells 11·2μ, nucleus 3·2μ, adrenal cortical cells 16·0μ, nucleus 4·8μ, and tumour cells 19·2μ, nucleus 4·8μ, all measurements averages from fixed cells.)

It is now generally agreed that morphological resemblance does not necessarily imply physiological identity of function. Indeed Willis (1948) goes so far as to say " It would not be surprising if further research should show that the two classes of tumour (œstrogenic and androgenic) are really one, and both derived from the ovarian parenchyma." If this should prove to be the case, the naming of such tumours on morphological grounds cannot be justified, and they should be classified according to their endocrine activity.

This tumour was associated with virilism, and is therefore presumably a heterotopic adrenal cortical cell growth or an arrhenoma. The first view is favoured as being the more probable.

(5) The next case, though not a masculinizing growth, is worth recording for its unusual nature, and because its interpretation could mean that besides adrenal cortical cells, chromaffin cells resembling those of the adrenal medulla may occur in the fowl's ovary and may undergo neoplasia in common with other heterotopic tissues.

During the examination of a crossbred fowl which was found dead, a large intra-abdominal blood clot was found proceeding from a rupture in the substance of a smoothly lobulated brownish-yellow tumour of soft consistency, measuring 6·5 × 5 × 4·3 cm. and occupying the site of the left (functional) ovary. It was freely movable, being only attached at the hilus, and was therefore easily dissected out. Numerous small cream-coloured tumours were scattered in the substance of the liver, spleen and kidneys. The adrenals seemed normal and this was subsequently confirmed by histological study.

Section of the gross specimen showed it to have a richly vascular outer zone

and an irregular inner zone, of a spongy brown character. The periphery of the growth tended to strip off in layers of what appeared to be necrotic tissue.

It is fortunate that the several blocks of tissue taken from various regions were fixed in Helly's fluid, as well as other fixatives, since it is possible that the unusual nature of the tumour would have been largely missed, had it not been chromed.

Histological examination of the outer zone shows it to be necrotic in some parts. The healthy remainder is composed of connective tissue cells with spherical to spindle-shaped nuclei, and eosinophilic granular cytoplasm, surrounding aggregations of large polyhedral cells with abundant finely granular eosinophilic cytoplasm, large nuclei and very prominent nucleoli (nuclear-nucleolar ratio 1:3 to 1:5).

An abundant infiltration with eosinophils is present in many parts of the small epithelial-cell moiety.

A well-marked but thin layer of connective tissue separates this outer zone from the inner, which is composed of small lobules of cells separated from each other by fine trabeculae of connective tissue. The cells composing these groups are large, with giant nucleoli similar to those mentioned above, and have a brownish-yellow cytoplasm in bichromate fixed haematoxylin and eosin preparations. They are frequently arranged in palisade formation at the periphery of the lobule, and their disposition and appearance in the interior of the groups is strikingly similar (save in size of nucleoli) to the cells composing the avian adrenal medulla (Fig. 15 and compare with previous figure). Areas of necrosis and spaces filled with fibrinous material are common in the inner zone, which is also very vascular.

Other regions of the tumour, and the metastatic lesions in the liver, spleen, and kidneys, consist mainly of polyhedral cells with large vacuolated nuclei and prominent nucleoli, tending to palisade small acini whose basement membrane is composed of reticulum fibrils. The cytoplasm of these cells is finely granular and heterophilic, and mitotic figures are common. At the edge of one series of sections, the only indication of ovarian origin is the presence of a few follicles. Bands of smooth muscle are scattered throughout the section.

The presence of such large numbers of cells showing a distinct chromaffin reaction justifies a diagnosis of chromaffinoma or paraganglioma. One hesitates to speak of a phaeochromocytoma of the ovary since no record of such tumour is in existence, but the term is really synonymous. The nature of the remaining cells of this tumour is debatable. Some are similar to the chromaffin cells, except there is no brown staining of the cytoplasm, and could conceivably be young forms. Others are mesenchymal in nature and are strongly reminiscent of thecal cells, a view which is supported by the fact that isolated groups of such cells are seen to be undergoing luteinization; and finally the polyhedral cells palisading the small acini which make up the rest of the tumour and metastatic growths, resemble granulosa-cells. This tumour, then, is a complex in which chromaffin, theca and granulosa-cells all occur, the latter metastasizing to the liver, spleen and kidneys.

The ovarian tumour just described apparently had no noticeable effect on the behaviour of the bird. In the human subject phaeochromacytomas are associated with raised blood pressure. It may not be entirely coincidence that this bird died as a result of an internal haemorrhage.

(6) The following case is another example of a masculinizing tumour. It occurred in a Brown Leghorn " hen " at this Centre, and was originally studied by Drs. Blyth and Carr, who supplied the following details.

The bird appeared to be a male when killed, although originally it had a normal female appearance. It was used to propagate the Duran-Reynals filterable sarcoma. *Post-mortem* examination showed a small tumour at the site of the left ovary, normal in shape for that organ, but with no macroscopically-visible ova, measuring 1·2 × 0·7 cm. It was closely adherent to the left adrenal gland, and was sectioned with it. This tumour is composed of well-developed testiculoid elements lying in a stroma of undifferentiated spindle-shaped cells (Fig. 16). Many solid cords of epithelioid cells lie just below the germinal epithelium investing the surface. The growth appears to have arisen within the adrenal capsule.

Another small (0·5 × 0·2 cm.) tumour was found in the site of the rudimentary right gonad and closely adherent to the dorsal wall. This shows not only testiculoid elements, but also a considerable area composed of whorls and strands of rapidly dividing mesenchymatous cells, presumably a metastasis from the experimentally transplanted sarcoma.

The histology of both gonadal growths resembles Ewing's (1942) Fig. 316, illustrating a " testicular adenoma of the ovary," a well-differentiated tumour belonging to the arrhenoma group. The nests of cells below the germinal epithelium resemble the primitive sex cords of the indifferent embryonic gonad. This tumour illustrates well the bisexuality of the embryonic fowl's ovary. The *rete ovarii* formed from the primitive sex cords is a homologue of the testis and as is well known can develop into one following left ovariectomy of the hen, where the right gonad hypertrophies and differentiates even to the extent of producing spermatozoa (Benoit, 1923).

Another tumour of the ovary associated with masculinization in a Light Sussex hen is shown in Fig. 17. Folliculoid formation and luteinization is apparent, and the structure is comparable to a granulosa-cell tumour. In fact, this diagnosis would be justifiable on histological grounds alone, but has to be modified in view of the androgenic effects on the host.

It would appear that the rudimentary right ovary is not the only place where in theory at any rate, testiculoid structures could arise in the intact bird. Champy, Lavedan and Marquez (1939) state that the adrenals of either male or female castrates in the fowl frequently contain " regenerating gonadal cells." Birds' adrenals constantly contain Wolffian duct remnants in the capsule on the ventral internal aspect. These slowly hypertrophy following castration to form an epididymis-like structure, whilst some form epithelial tubules similar to those found in the right ovarian rudiment subsequent to castration. Spermatogenesis has not been seen in the adrenal capsule however. There does not seem to be any record of spontaneous arrhenoma arising in the adrenal capsule of the fowl, but it seems possible that the foregoing is a case in point.

(7) The last of the tumours of the female gonad to be described was originally considered to be a teratoma. It is unfortunate that no comparison can be made of this case with the only comparable ovarian tumour of the fowl on record (Jackson, 1936), since apart from stating its didermic character, no other details were given, and the single photograph is of the gross specimen.

The present case occurred in a Brown Leghorn hen aged about 3 years, at the Poultry Research Centre. The bird had ascites and ovarian tumour was diag-

nosed. Red blood cells, macrophages and epithelial cells were seen in a smear of the centrifuged deposit from the brown turbid fluid, 140 ml. of which was withdrawn from the abdomen. After death 4 days later, an ovarian tumour was found measuring 5 × 4 × 4 cm. It was mottled pink, brownish and cream, of firm consistency and mucinous on section. Other growths, pinkish and soft, were attached to the wall of the oviduct, and to the serous investment of the duodenum, pancreas and liver.

Histologically the ovarian tumour is the only one to show complexity of structure. Immediately below a thin capsule there are nests of large pale staining epithelial cells, some of which have differentiated into cysts of varying size, lined with one or several layers of columnar epithelium. In many cases these cells are actively secreting mucus. Large areas of the tumour consist of a vascular myxo-chondromatous matrix showing all stages of development of hyaline cartilage from a myxomatous tissue to irregular islands of well developed cartilage. Complicated folded, branched and invaginated epithelial tubules, and clumps of actively dividing cells often forming folliculoid structures complete the picture (Fig. 18). The implants on the viscera show a typical folliculoid type of granulosa-cell tumour.

As with many ovarian tumours, this case is not easy to classify. At first sight two embryonic layers appear to be involved, namely mesoderm and ectoderm. The latter may be represented by the cyst epithelium forming mucinous glands, and possibly as the complex epithelial tubular structures. The remainder of the tumour is mesodermal in origin. If this is the case the presence of these derivatives of two germinal layers presumably puts it in the same category as Jackson's (1936) "didermic teratoma." Willis (1948) defines a teratoma as "a true tumour or neoplasm composed of multiple tissues of kinds foreign to the part in which it arises." The presence of cartilage can be explained by the conversion of connective tissue normally present in the ovary, to cartilage by mucoid degeneration and hyalinization. At first sight the mucus-secreting epithelial cells of the cysts seem to satisfy the definition of Willis, but such structures have been described by Harvey, Dawson and Innes (1939), in Brenner-cell tumours in the human subject, and therefore presumably arise from cells indigenous to the ovary. Also it should be noted that the same authors express the view that Brenner and granulosa-cell tumours are intimately related, an opinion also held by Willis (1948).

Is a diagnosis of teratoma justifiable ? Nicholson (1950) stresses the necessity of the identification of tissues representing all three germinal layers before a diagnosis of teratoma is made. It has been shown that in this case it is doubtful whether even two layers are in fact represented, and so the view is taken that we are here dealing with a neoplasm demonstrating the close relationship between Brenner tumour, and pseudomucinous cystadenoma, in which metaplasia of myxomatous tissue has given rise to hyaline cartilage, whilst other elements have differentiated into a mature type of epithelium secreting mucus, and an embryonic moiety represented by the granulosa-cell elements has spread by implantation to organs in the abdominal cavity.

Tumours of the Male Gonad.

1. It seems extraordinary that no report exists of spontaneous seminoma in the fowl. Champy, Lavedan and Marquez (1939) describe 4 cases of "semi-

noma " in the regenerating testes of birds following partial castration, but the identity of these growths with true seminoma seems doubtful. One of their cases exhibited metastases, but no details are given of the histology of this secondary growth or its site.

Several seminomas have been studied during the past 11 years and two of these are selected for description here. The first case was found involving the left testis of a White Wyandotte cockerel. The gland was enlarged, measuring 6·0 × 3·2 × 3·3 cm., but retained its normal shape and consistency. The right testis was normal both in size and microscopic structure, and no metastases were found.

Microscopically the left testis consists of poorly delineated lobules of pale staining epithelial cells arranged in diffuse sheets and penetrated here and there by blood vessels. Mitotic figures are frequent (Fig. 19).

The cells are mainly spherical to polygonal through mutual pressure, but the faintness of cytoplasmic outline often gives a syncytial appearance. They take on a plump spindle-shape in some areas. This tumour is the counterpart of the " embryonal carcinoma " of Chevassu (1906).

2. A second testicular tumour—also involving the left organ—was seen at autopsy in a Buff Rock cockerel. It measured 4·5 × 3·8 × 3·5 cm., and on section showed an irregular yellowish area near the caudal pole, compared with the rest of the tumour which was of a cream colour. The right gonad was atrophied.

Tissue taken so as to include the two areas showed, at subsequent histological examination, two distinct cell types. The cream-coloured area, forming about two-thirds of the tumour, is composed of typical seminoma cells, showing in many places a marked acinar structure. Luteinization of these cells is proceeding in small isolated areas. The alveoli are formed by an argyrophil reticulum, which is thickened in parts to form fibrous bands. There are numerous mitoses.

Contiguous with these cells, and in parts merging with them, are very large polygonal cells with a granular acidophilic cytoplasm and relatively small peripherally situated nuclei. These resemble the interstitial or Leydig cells of the testis and occur in the form of irregular acini separated by delicate bands of connective tissue. There are no signs of cell division in this region.

In some areas there is a gradual transition between seminoma and interstitial cells (Fig. 20), whilst others show an abrupt change separated by a zone of compressed and distorted seminomatous tissue. In this case, two distinct cell types are present as contiguous growths in the one tumour. The question arises whether the interstitial-cell moiety represents a concommitant neoplasia of the stroma, as the acceptance of Firket's (1920) view on the connective tissue origin of interstitial cells would imply. In other words, is this an example of a " mixed tumour "—or alternatively is it an indication of the teratomatous nature of seminoma, as Ewing (1942) believes, but which Willis (1948), Nicholson (1950), and others dispute ?

Still another possibility exists, namely that the two cell types have arisen from a common ancestral totipotent germinal cell capable of giving rise to a variety of tissues, in accordance with the view put forward by Innes, Harvey, and Dawson (1938).

A re-examination of Fig. 20, which is representative of most of the junction between the two components, shows that the seminoma cells and the interstitial

cells merge indistinguishably, and moreover, lie in the same connective tissue-bounded cords. Another testicular tumour in the writer's collection shows a similar transition, but this time between seminiferous epithelium and seminoma (Fig. 21), a finding which is not in accord with Ewing's (1942) hypothesis.

It therefore appears that in the fowl at any rate, seminiferous epithelium can give rise to both seminoma and interstitial-cell tumours, and that it is not necessary to invoke the theory of a one-sided development of a teratoma to explain these cases.

3. The last testicular tumour of special interest occurred in a cross-bred fowl, which showed during life a marked atrophy of the comb, and cessation of crowing and of other activities associated with the adult male bird.

At post-mortem, a greatly enlarged (9 × 10 × 6 cm.) left gonad was found, a flattened oval in shape and firm in consistency. The right testis was small, fairly rich in interstitial cells and showed little spermatogenesis upon histological examination. On medial section, the tumour presented two prominent nodules and several well-defined coloured areas varying from cream through yellow mottled with haemorrhages to reddish-brown. A prominent fleshy band ran through the centre of the growth, which also contained several cysts filled with a brownish mucinous fluid. There was a well marked vascular capsule (Fig. 22). Tissue blocks were taken from five different regions for study.

The area marked " A " in the figure, consists of small cysts or irregular glands lined with either mucus-secreting cells or flattened epithelium, lying in a stroma of mesenchymal tissue and bands of plain muscle (Fig. 23). Numerous islands of interstitial cells occur in various stages of degeneration, and frequently lying within the lumen of the glands, which otherwise contain a colloid-like material.

Area " B " is much more cellular, being composed of masses of rather loosely arranged small pale staining epithelial cells, in diffuse sheets or tending to form follicles or " rosettes." A few cysts are present, similar to those described in area " A," and one of these is partly lined with a tall columnar epithelium with basally situated nuclei and a striated free border, resembling intestinal epithelium (Fig. 24). The whole region is divided into irregular trabeculae by thin cords of plain muscle and connective tissue. It is rather vascular and in parts is heavily infiltrated with granular leucocytes. Necrotic changes are plentiful, and other parts of the tumour taken for examination show a great deal of degeneration.

The teratomatous nature of this tumour seems fairly certain, although it does not contain such a variety of tissues as the experimentally induced testicular teratomata of Bagg (1936), which incidentally, as Falin (1940), points out, offers good evidence for the theory of the multipotency of the spermatocyte. Seminomatous tissue is not present. Willis (1948) says in this connection : " I know of no example of associated seminoma and teratoma in an animal." Instead, there is in parts, epithelial tissue resembling arrhenoma, and it is an interesting though admittedly highly debatable point whether these parts are of the nature of the so-called " androma," a benign testicular tumour associated with feminization in the human subject, described by Teilum (1946), and of which only two cases have been studied. While it is doubtfully justifiable to speak of feminization in this cockerel, it did at least lose certain of its male characteristics both in appearance and behaviour. Willis (1948) mentions that certain tumours in the human ovary, designated a " combination of granulosa-cell tumour and arrheno-

blastoma " have contained lipoid-laden cells regarded as Leydig cells, and also glandular structures lined by columnar mucous epithelial cells.

DISCUSSION.

In the fowl, as in man, there exists for a brief period in embryonic development, an indifferent gonad characterized by the presence of a medullary or male component, and a cortical or ovarian part. Suppression or stimulation of this bipotential gonad during development leads to the formation of testis or ovary as the case may be. For example, it is known that the injection of oestrogen into the fertile egg at the indifferent stage (from the third to the sixth day), results in the formation of an ovo-testis in genetic males.

The phenomenon of sex-reversal is based on this bisexual organization, and it seems probable that the formation of androgen-secreting tumours of the ovary with the resulting appearance of secondary male sex characters, is due to the suppression of the female component by disease, followed by neoplastic transformation, possibly due to endocrine imbalance, of the rudimentary male component. It may be that the normal presence of a rudimentary right ovary increases the probability of this occurrence in birds.

This tendency to change to a state of " maleness " is much more pronounced than the opposite case of a generic male developing secondary female characters. The reason for this is not clear unless it is assumed that in the male embryo the cortical region of the differentiating gonad is usually completely suppressed. That this may not always be the case is shown by an instance of the development of female type feathers in a Brown Leghorn male after castration (Greenwood and Blyth, 1932). At subsequent autopsy this bird had a small nodule at the site of the right gonad, whose histological structure, in the opinion of the present writer, resembles that of a thecal-cell tumour.

Teilum's (1946) case of " androma " of the testis in man may be another example of a feminizing tumour arising from a persistent remnant of the " ovarian" cortex, and the presence of arrhenomatoid tissue in a testicular teratoma of a fowl which lost certain of its male characters has already been described in the present paper.

Turning to granulosa-cell and related tumours, the development of the ovary throws some light on the inter-relationship of these oestrogen-secreting growths. In the embryonic ovary it is commonly stated that the granulosa cells arise from coelomic epithelium derived from the genital ridge, whereas the thecal cells, being stromal in nature arise from the mesenchymal elements of the mesoderm. Examination of the early ovary, however, shows primary follicles composed of a central ovocyte surrounded by a ring of indifferent epithelial cells indistinguishable from the stromal cells composing the rest of the organ. These indifferent cells appear to be the precursors of the granulosa cells, whereas the remainder of the stroma forms the theca. It seems probable that this common ancestry is reflected in the behaviour of thecal and granulosal cells in the neoplastic state, where both secrete oestrogens as judged by the muscular and glandular hypertrophy of the oviduct of the tumour-bearing bird, and both undergo the phenomenon of luteinization.

Granulosa-cell tumours in the fowl are not morphologically identical to these in man in that they do not exhibit the characteristic " rosettes " which are often

a feature of human tumours, and which are considered to be identical to the bodies of Call-Exner occurring normally in the stratum granulosum of the human ovary. Such bodies are not found in the granulosa layer of the ovarian follicle because this is usually only one, or at the most two or three cells thick. Call-Exner bodies are usually considered to be due to cystic degeneration of granulosa cells. This does not occur in avian tumours where there is ample space for such a change to take place, and it therefore seems possible that these structures may serve some specific purpose as for example a glandular function, in the ovaries of those animals in which they occur.

With regard to thecal-luteal cells and the interstitial cells of the testis, it is interesting to note that they appear to have a common ancestory. Fell (1924) states that the ovarian thecal-luteal cells of the fowl are derived from remnants of aborted medulla in the development of the ovary. Thus they have the same histogenesis as seminiferous tissue. We have already seen that the testicular interstitial cells appear to be derived from seminiferous epithelium. This common embryonic origin may account for the confusion regarding the so-called

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EXPLANATION OF PLATES.

FIG. 1.—Brenner tumour, showing cysts in various stages of development, with epithelium varying from flattened to a columnar mucus-secreting type, lying in hyperplastic ovarian stroma. H. & E. × 85.

FIG. 2.—Section from the same tumour showing nests of epithelioid cells, one of which is being transformed into a cyst. The stroma is myxomatous in this region. H. & E. × 370.

FIG. 3.—Hilar region of ovary of fowl, showing the parovarium. H. & E. × 135.

FIG. 4.—Granulosa-cell tumour of ovary. × $\frac{1}{3}$.

FIG. 5.—Granulosa-cell tumour showing cylindroid type with a trabecular-like stroma. H. & E. × 190.

FIG. 6.—Granulosa-cell tumour showing folliculoid type, developing from solid masses of cells. The stroma is hyalinized. H. & E. × 320.

FIG. 7.—Section of a liver implant from a granulosa-cell tumour, showing areas of luteinization. H. & E. × 320.

FIG. 8.—Thecal-cell tumour of ovary. × $\frac{2}{3}$.

FIG. 9.—Structure of the above, showing vacuolated cells, due to removal of lipoids, to the left (some nuclei are pyknotic) and cells resembling fibrous tissue to the right of the figure. H. & E. × 400.

FIG. 10.—Transection of an ovarian fibroma weighing 570 g. Note the small regressing ovary.

FIG. 11.—Adrenal cortical ? tumour of ovary. H. & E. × 380.

FIG. 12.—Group of thecal luteal cells from an active ovary. Masson's stain. × 515.

FIG. 13.—Corpus luteum derived from granulosa cells. Normal active ovary. H. & E. × 370.

FIG. 14.—Adrenal cortical and medullary cells. Bichromate fixed. H. & E. × 400.

FIG. 15.—" Chromaffinoma " of ovary. Note conspicuous nucleoli and attempted orientation of cells. Bichromate fixed. H. & E. × 370.

FIG. 16.—Testiculoid tubules in " ovary." A well differentiated arrhenoma which appeared to have arisen in the adrenal capsule. H. & E. × 370.

FIG. 17.—Folliculoid formation and luteinization in an arrhenoma. Note resemblance to granulosa-cell tumour. H. & E. × 330.

FIG. 18.—" Mixed ? " ovarian tumour, consisting of areas of tortuous glands, mucinous cysts, cartilage, solid nests of epithelial cells and a myxomatous stroma. H. & E. × 42.

FIG. 19.—Seminoma or " embryonal carcinoma " of the testis. H. & E. × 370.

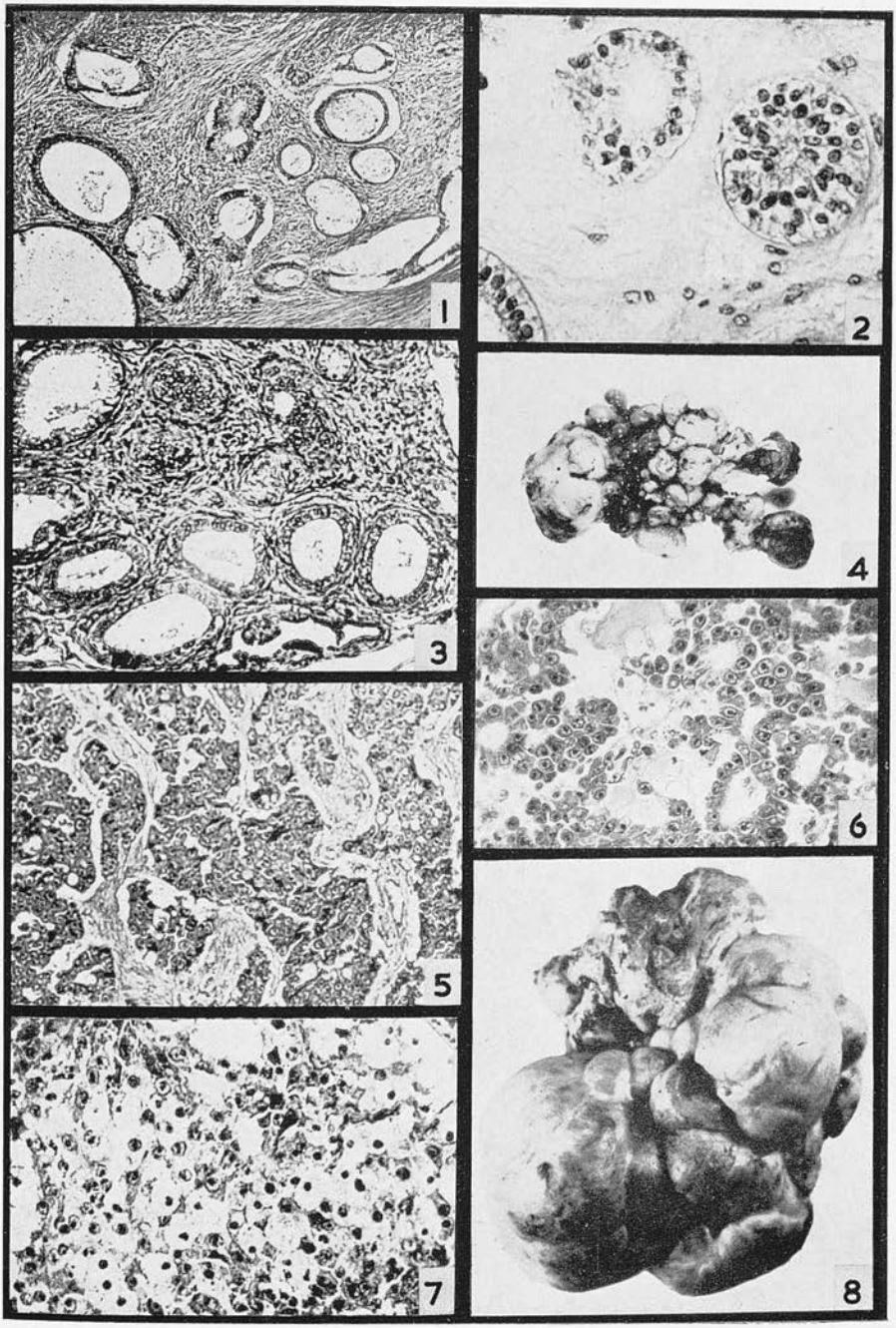
FIG. 20.—Transition zone between interstitial cell tumour (top left) and seminoma. Note that the cells lie within common connective tissue-bounded columns. H. & E. × 370.

FIG. 21.—Section showing seminoma cells differentiating into primary spermatocytes. H. & E. × 370.

FIG. 22.—Teratoma of testis, showing the two main nodules, cysts, a central muscular band, and well formed capsule. × $\frac{2}{3}$.

FIG. 23.—Section from nodule " A " (Fig. 22), showing epithelial cysts, connective tissue and plain muscle. H. & E. × 85.

FIG. 24.—Section from nodule " B " (Fig. 22) showing tendency of cells to form folliculoid structures, and part of a cyst lined with intestinal epithelium. H. & E. × 370.



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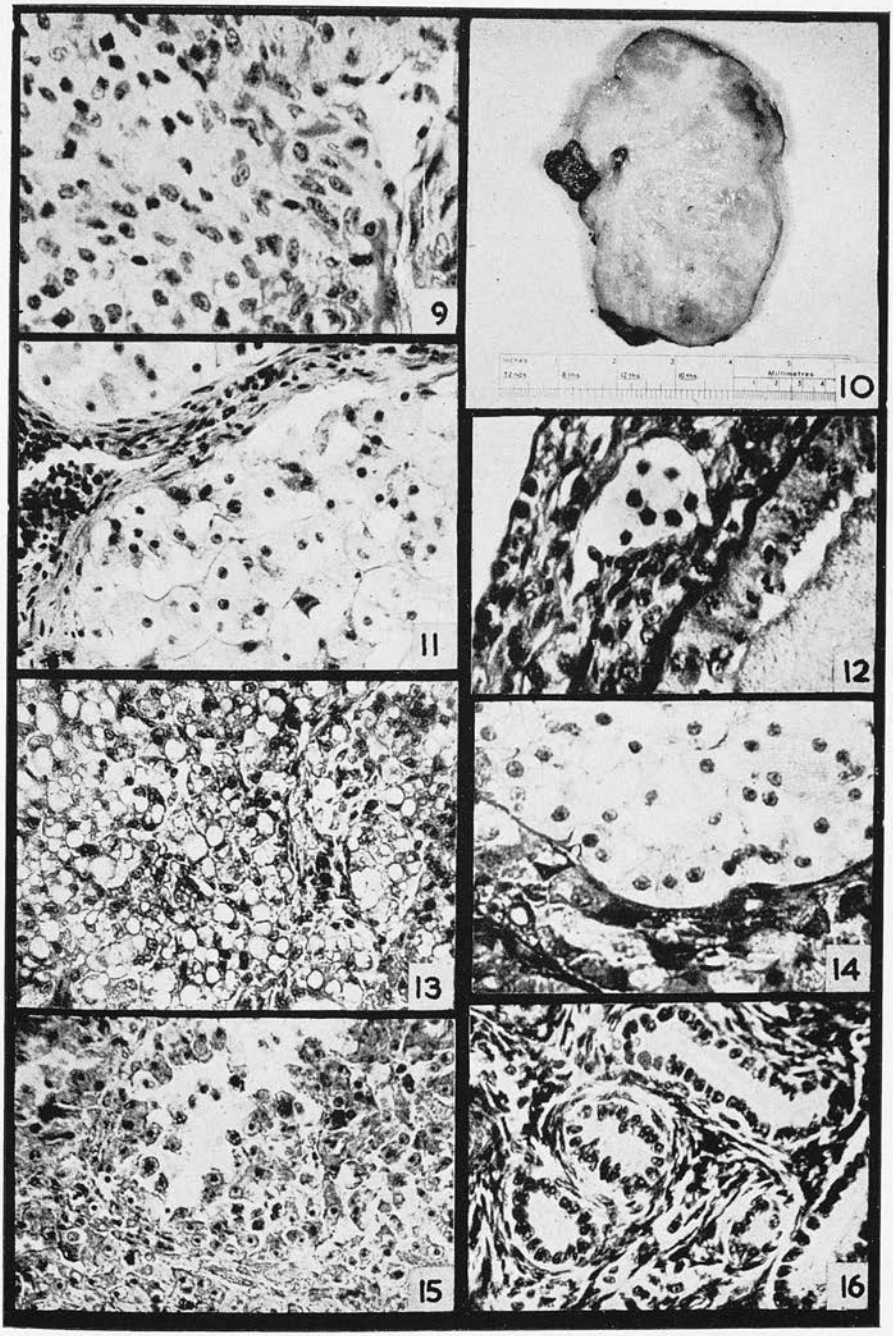

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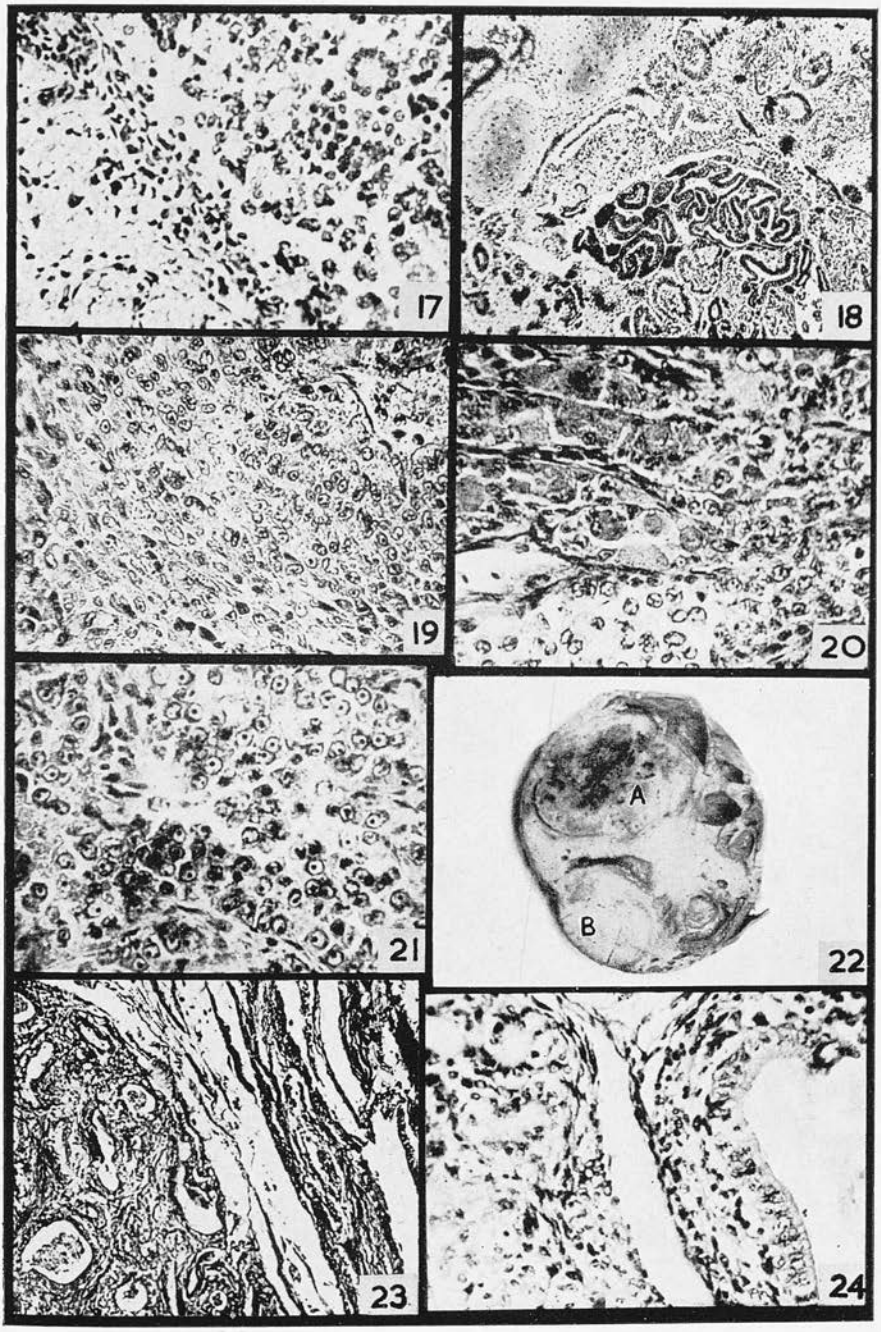

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" luteal " cells of the testis which were originally recorded by Boring and Morgan (1918), and regarding the nature of which there has been much debate.

If it is the case that cells in the testis may occasionally resemble luteal cells and exert an oestrogenic effect on the plumage of the male bird, then tumours of such cells, perhaps in a more anaplastic state, but still capable of elaborating oestrogen would account for the so-called " androma " of Teilum (1946), which is apparently indistinguishable from an arrhenoma.

Conversely, ovarian growths resembling luteal-cell tumours may secrete androgens, e.g., arrhenomas, regarding which Burrows (1943) states (in the human subject), " Some ovarian tumours which induce hirsuties and other masculine phenomena are the colour of corpora lutea, and are composed of cells which resemble those of luteal tissue. Others might be described from their cytological appearance as thecomas, or as granulosa-cell tumours, and yet others look like tumours derived from adrenal tissue."

It should not be forgotten that arrhenomas and seminomas may also undergo luteinization. In view of the origin of all these tumours in tissues with similar embryonic derivation, of their capacity for secreting either male or female sex-hormones independent of their cytology, and of the fact that luteinization is common to all, it seems reasonable to conclude that there is a very close relationship between ovarian tumours and between these and tumours of the testicle. This relationship tends to be obscured by the present confusing system of classification, which could well be revised.

In conclusion it is considered that the suggestion of Burrows (1943), should be more widely adopted, i.e., that tumours secreting androgens should be called arrhenomas, and tumours secreting oestrogens should be called theelomas, irrespective of their histological structure or site of origin in the body. Only by adopting some such simple and logical classification (with additional details where necessary regarding malignancy, etc.) will order be brought to the unwieldy and unscientific classification of gonadal tumours, adopted by most present-day pathologists.

SUMMARY.

1. Most, if not all, of the rarer ovarian and testicular tumours of the human subject have their counterparts in the fowl.

2. Brenner tumours appear to arise from the parovarium and not from Walthard nests. They show affinities to granulosa-cell tumours and to pseudo-mucinous cystadenomata.

3. Granulosa and theca-cell tumours are characterized by their oestrogenic effect as evidenced by a hypertrophied oviduct in the non-laying fowl. Both types undergo luteinization. Call-Exner bodies are not present in the thin granulosa of the fowl ovary, and typical rosettes were not found in granulosa-cell tumours.

4. The occurrence of both adrenal cortical and chromaffin tumours in the ovary is adduced as evidence in favour of the occasional presence of heterotopic adrenal tissue in that organ, presumably due to developmental displacement.

5. Arrhenomata may not only arise from the rudimentary right ovary, but also from Wolffian duct remnants within the adrenal capsules. These essentially masculinizing tumours may also undergo luteinization.

6. The phenomenon of luteinization is also to be seen in seminomas.

7. Seminomas arise from seminiferous epithelium and can give rise to cells indistinguishable from Leydig's cells.

8. A testicular teratoma showed regions indistinguishable from arrhenoma. The host had lost much of its male characteristics.

9. It is evident that there is a very close relationship between the various ovarian tumours, and between these and tumours of the testicle.

REFERENCES.

Bagg, H. J.—(1936) *Amer. J. Cancer*, **26**, 69.
Benoit, J.—(1923) *C.R. Soc. Biol. (Strasbourg)*, **89**, 1326.
Boring, A. M., and Morgan, T. H.—(1918) *J. gen. Physiol.*, **1**, 127.
Burrows, H.—(1943) *J. Obstet. Gynaec.*, **50**, 430.
Campbell, J. G.—(1945) *J. comp. Path.*, **55**, 308.
Champy, C., Lavedan, J. P., and Marquez, J.—(1939) *Arch. Zool. exp. gén.*, **80**, 389.
Chevassu, M.—(1906) 'Tumeurs de testicule.' Paris. (Monograph.)
Ewing, J.—(1942) 'Neoplastic Diseases.' London (W. B. Saunders Co.).
Falin, L. I.—(1940) *Amer. J. Cancer*, **38**, 199.
Fell, H. B.—(1924) *Brit. J. exp. Biol.*, **1**, 293.
Firket, J.—(1920) *Arch. Biol., Paris*, **30**, 393.
Friedgood, H. B., and Uotila, U. U.—(1941) *Endocrinology*, **29**, 47.
Glynn, E. E.—(1921) *J. Obstet. Gynaec.*, **28**, 23.
Greenwood, A. W., and Blyth. J. S. S.—(1932) *J. Genet.*, **26**, No. 2, 199.
Harvey, W. F., Dawson, E. K., and Innes, J. R. M.—(1939) *Edinb. med. J.*, **46**, 256.
Innes, J. R. M., Harvey, W. F., and Dawson, E. K.—(1938) *Ibid.*, **45**, 36.
Jackson, C.—(1936) *Onderstepoort. J. Vet. Sci.*, **6**, No. 1 (Monograph).
Maximow, A. A., and Bloom, W.—(1948) 'Textbook of Histology.' London (W. B. Saunders Co.).
Meyer, R.—(1931) *Amer. J. Obstet. Gynec.*, **22**, 697.
Nicholson, G. W. de P.—(1950) 'Studies on Tumour Formation.' London (Butterworth & Co.).
Olson, C., and Bullis, K. L.—(1942) *Mass. Agric. exp. Sta. Bull.*, 391.
Seifried, O.—(1923) *Z. Krebsforsch.*, **20**, 188.
Sheather, A. L.—(1911) *J. comp. Path.*, **24**, 129.
Teilum, G. (1946) *Acta path. microbiol. scand.*, **23**, 252.
Walthard, M.—(1903) *Z. Geburtsh. Gynäk*, **49**, 233.
Willis, R. A.—(1948) 'Pathology of Tumours.' London (Butterworth & Co.).

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THE OCCURRENCE OF BLOOD FILAMENTS OR "PSEUDO-SPIROCHÆTES" IN CERTAIN NEOPLASTIC CONDITIONS.

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WHEN fresh blood is examined by dark-ground illumination, a number of filamentous objects may be seen which have, in the past and even quite recently, led to errors in diagnosis through misinterpretation. It is surprising that no reference is made to these structures in any of the current textbooks of hæmatology so far consulted.

The first mention of such filaments appears to be by Nuttall and Graham-Smith (1907) in one of their classical papers on piroplasmosis. They found filaments, extruded vesicles, and beaded structures in the blood of dogs, and at first they considered them to be parasites. Later, filaments were also found in the blood of various animals including the fowl. Nuttall and Graham-Smith concluded that these objects are degeneration products of red blood corpuscles, supporting this contention by showing that the production of filaments could be enhanced by warming the blood above its physiological temperature, and they appended some good drawings of typical structures.

Kuhn and Steiner (1917) studied these filaments, claiming that they were spirochætes and were the cause of multiple sclerosis. They described them as often more delicate than Weil's organism, some showing a loop, and others a highly refractile ball at each end. They claimed to have transmitted the condition to guinea-pigs and rabbits. Collins and Noguchi (1923) also experienced a preliminary difficulty in the interpretation of these structures. They state that they are abundant in blood kept at room temperature, and that there are many free-swimming forms not attached to erythrocytes. They were not stainable by Giemsa's stain, but occasionally by Loeffler's flagella stain, and were undoubtedly derived from altered red corpuscles.

Thomson (1923) records their presence in normal human blood and describes two kinds of filaments, the first being thick, motile, and 5 to 9 μ in length, and the second longer (7 to 12 μ) and extremely delicate. They exhibit no spiral movement but are actively motile (?), wriggling and bending upon themselves. He found that they may assume a spiral shape on fixing and drying. They normally show no coils or spirals, and occasionally exhibit a beaded appearance. Similar structures in the blood are mentioned by Adams *et alia* (1925).

Baer and Allen (1944) drew attention to these filaments, which they had observed in their study of cases of leptospirosis, and warned against their confusion with true spirochætes. Kuzell (1945) speaks of artificial spirochætes in cases of infective hepatitis, and the possibility of their misinterpretation; while Simmons (1946) and Gowen (1946) also mention "pseudo-spirochætes" as a source of diagnostic error. Schmidt (1947) likewise describes spirochæte-like formations which he found in the blood of patients and in normal persons.

Similar structures to these have been encountered in the course of recent work at this Centre. They were originally found in profusion in the fresh blood of fowls infected with a virus-associated erythroleukæmia, but could not be seen in fresh normal fowl's blood. They make brilliant objects with dark-ground illumination and an oil-immersion objective (Fig. 1), are easily detectable with phase contrast, but are extremely difficult to see with ordinary transmitted light. They have been stained with Giemsa's stain, but Levaditi's method, osmic acid, Feulgen's stain, and brilliant cresyl blue all failed to show them in dried films.

Subsequently, similar structures have been observed in normal fowl's blood which has been kept for 12 to 24 hours at room temperature and has been sealed under sterile conditions in Vaseline on slides. The longer these preparations are kept, the more numerous the filaments become. Typically, they appear as tenuous filaments of varying width and from 3 or 4 μ up to 30 μ in length, usually attached to a pole of an erythrocyte, and occasionally appearing to arise from the region of the nucleus. The point of attachment is marked by a bright refractile granule (Fig. 2) and a similar granule is often to be seen at the free end. They may branch, or several may be attached to one cell, and they are often seen floating freely in the plasma, fluttering and bending but certainly not exhibiting any spontaneous motility. On several occasions a long filament has been observed to contract suddenly and then form a small vesicle with "pseudopodia" adhering to an erythrocyte (Fig. 3).

A less common structure is shown in Fig. 4. It is beaded and is always terminated by a slightly larger granule. These too may branch, and may be seen apparently within an erythrocyte where, over a period of hours, they have been observed to increase in size and change their conformation (Fig. 5). On one occasion such a structure slowly disintegrated, leaving numerous dancing granules in the cytoplasm of the cell. Small tufts of beaded filaments may occur attached to a cell.

The filaments are remarkably stable, preserving their identity for many days in a sealed sterile preparation, and even increasing in number. They also show a tendency to thicken. They occur in equal abundance in heparinised leukæmic blood, or similarly treated normal blood kept at room temperature. Saponin 1 : 1,000 has no effect on them, although the cells are laked.

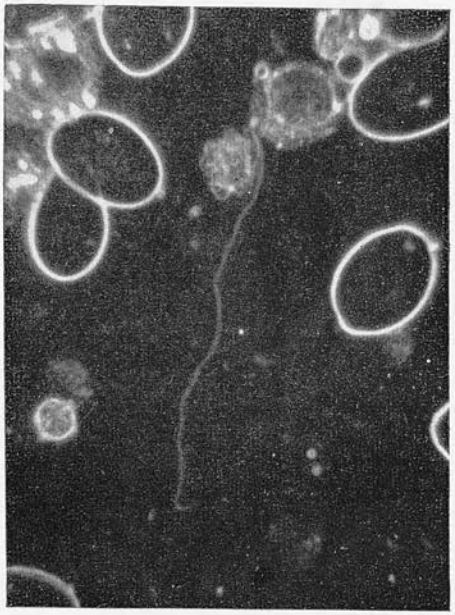

FIG. 1

An example of a long free filament. Fresh blood from a case of erythroleukæmia in a 7-week-old chicken. Dark-ground illumination × 2,800.

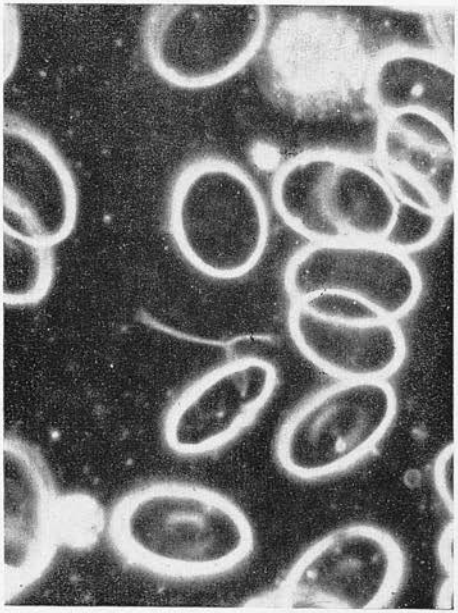

FIG. 2

A branched filament attached to an erythrocyte. The double contour and forked extremities are artefacts due to movement during exposure. Erythroleukæmia blood. D.G.I. × 2,800.

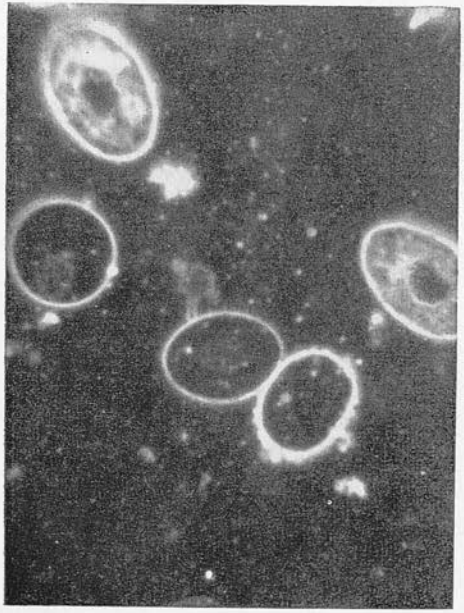

FIG. 3

A vesicle with pseudopodia projections attached to an erythrocyte. This structure was originally a long filament comparable to that shown in Fig. 1. Erythroleukæmia blood. D.G.I. × 2,800.

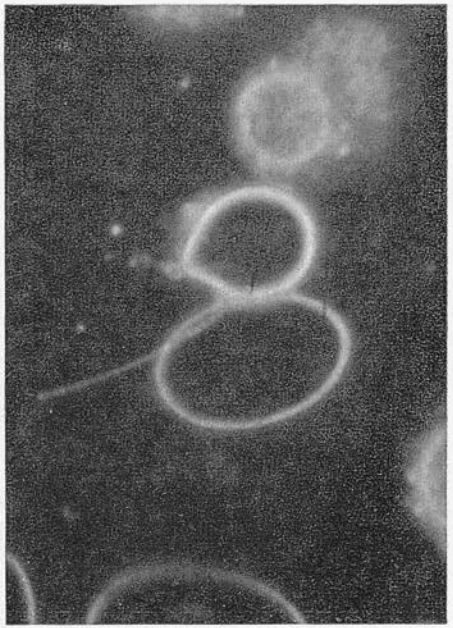

FIG. 4

A beaded filament attached to an erythrocyte. Note the larger granule at the free extremity. Erythroleukæmia blood. D.G.I. × 4,200.

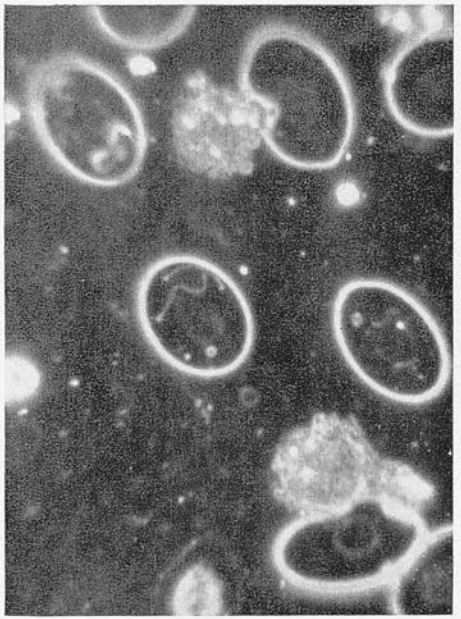

FIG. 5

A branched and beaded filament with conspicuous terminal granules within an erythrocyte. Erythroleukæmia blood. D.G.I. × 3,000.

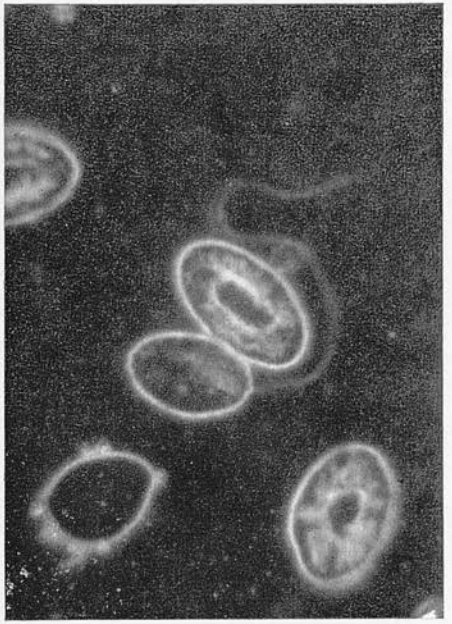

FIG. 6

Apparent formation of a filament from the nuclear region of an erythrocyte. Movement has blurred the outlines. Erythroleukæmia blood. D.G.I. × 3,000.

They are not confined to erythrocytes. Occasionally a leucocyte shows one, and some of the malignant hæmocytoblasts in leukæmic birds may carry numerous waving filaments, each with a terminal refractile granule.

The nature of these structures is not clear. In the fowl, they are frequently seen arising from the region of the nucleus. Fig. 6 shows one such apparently having its origin in a disintegrating nucleus.

Not all have this appearance, however, many being attached to quite normal-looking erythrocytes with an intact nucleus. There is no doubt that distorted or damaged cells commonly have these appendages. In successful Giemsa-stained preparations they stain a pale blue, unlike the pink reaction of the cell membrane or cytoplasm. Feulgen preparations of such tenuous filaments are probably not reliable. Osmic acid does not stain them, so they presumably do not contain much lipoidal material. Both cytoplasm and nucleoprotein can exhibit contractile phenomena, and so this criterion is not much help either.

It must be remembered that most reports of these filaments have been as the result of the examination of mammalian blood. The red blood corpuscles being non-nucleated in these animals, it is difficult to identify them with products of nuclear degeneration unless it can be shown that they arise from reticulocytes. On the other hand, it is well known that structures such as Rous sarcoma cells photographed by ultra-violet light often show a fringe of fine filaments bordering the cell, obviously of cytoplasmic origin. Blood cells in preparations of Rous tumour and in the chemically-induced G.R.C.H./15 fibro-sarcoma of the fowl also show filaments, but usually longer and thicker than these cytoplasmic threads and in every way comparable to those described in erythroleukæmic blood or in normal blood kept for several hours at room temperature.

It is quite possible that similar filamentous structures have been encountered in association with tumours in the past, and will be found also in the future. The fact that such structures are not only present abundantly wherever blood cell degeneration is going on, as in tumours, leukæmia, or jaundice, but may also be seen in normal blood or tissue cells under suitable conditions, would emphasise the need for caution in the interpretation of filaments attached to cells in association with certain virus infections such as fowl plague or vaccinia, in both of which "stalked forms of virus" have been reported (Bland and Robinow, 1939; Dawson and Elford, 1949; and Robinow, 1950).

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REFERENCES

Adams, D. K., Blacklock, J. W. S., and McCluskie, J. A. W. (1925) : *Jl. Path. Bact., 25,* 115.
Baer, L. S., and Allen, R. F. (1944) : *U.S. Nav. Med. Bull., 43,* 127.
Bland, J. O. W., and Robinow, C. F. (1939) : *Jl. Path. Bact., 48,* 381.
Collins, J., and Noguchi, H. (1923) : *Jl. Amer. Med. Assoc., 81,* 2109.
Dawson, I. M., and Elford, W. J. (1949) : *Jl. Gen. Microbiol., 3,* 298.
Gowen, G. H. (1946) : *Illin. Med. Jl., 89,* 294.
Kuhn, P., and Steiner, G. (1917) : *Med. Klin., 13,* 1007.
Kuzell, W. C. (1945) : *Bull. U.S. Army Med. Dept., 89,* 112.
Nuttall, G. H. F., and Graham-Smith, G. S. (1907) : *Jl. Hyg., 7,* 268.
Robinow, C. F. (1950) : *Jl. Gen. Microbiol., 4,* 242.
Schmidt, B. (1947) : *Klin. Wschr., 24/25,* 400.
Simmons, H. C. R. (1946) : *Schweiz. Med. Wschr., 76,* 992.
Thomson, J. G. (1923) : *Jl. Trop. Med. Hyg., 26,* No. 15.

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OBSERVATIONS ON THE " ECLIPSE PHASE " IN A VIRUS-ASSOCIATED ERYTHROLEUKAEMIA OF THE CHICKEN.

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WITH the resurgence of interest in the viral theory of the etiology of cancer, a good deal of attention has been given in recent years to the multiplication of viruses ; and in particular to that temporary phase occurring shortly after infection, during which the virus is no longer demonstrable as an agent capable of infecting and producing pathological change when passaged to a second susceptible host. This interval has been variously called the " dark period," the " eclipse phase," or the "masked stage," and during it the virus has been thought to have become "inactive," " incomplete," " toothless," or " pro-virus." In this paper the terms " eclipse " and " pro-virus " have been adopted.

Most mammalian and many avian spontaneous tumours, and all chemically induced tumours, apparently do not contain a demonstrable virus. The increasing knowledge of the non-infective stage of viruses and phage has led some workers to wonder whether virus may be present in a more or less permanent state of eclipse in all cancerous conditions, only rarely becoming demonstrable as an infectious agent after some form of stimulus whose nature is at present conjectural. With these facts in mind, it is thought that some observations on the " eclipse phase " of the virus associated with a transmissible erythroleukaemia of chickens may be of interest.

MATERIALS AND METHODS.

The strain of erythroleukaemia used in this investigation was originally provided by Professor Engelbreth-Holm of the University of Copenhagen, Denmark, and has been propagated continuously in young Brown Leghorn fowls at the Poultry Research Centre for the past four years. Routine transmission is conveniently effected by intravenous injection, using whole blood or plasma, diluted 1/20 with sterile 6 per cent dextrose solution, which preserves both cells and virus activity very satisfactorily for several days when stored at 4° C. (C. le Q. Darcel, personal communication). Heparinization is rarely necessary when leukaemia blood is added to this diluent. The dose adopted was 1 ml. of the 1/20 dilution, i.e. 0·05 ml. of whole blood or plasma, corresponding roughly to 50,000 Minimal Infective Doses. Tissue suspensions were prepared by grinding portions of the organs to be examined with 6 per cent dextrose solution in a TenBroeck tissue grinder, followed by light centrifuging to throw down the coarser fragments. The supernatant was then injected intravenously in 1 ml. doses. Difficulty was occasionally encountered when marrow suspensions were given intravenously, as the recipient birds convulsed and died suddenly probably as the result of a fat embolus in the brain. Plasma preparations were obtained from blood withdrawn into a

heparinized syringe, and spun for 30 minutes at 3000 r.p.m. in an M.S.E. Minor centrifuge. Blood or tissue preparations were only registered as non-infective if the birds injected survived at least 4 weeks after testing. With this strain of leukaemia, using the above-mentioned dose in 6-week-old chickens, the average survival time is between 10 and 18 days, according to whether whole blood or plasma is used. Each experiment consisted of 10 birds, 5 serving as controls for the infectivity of the virus and one of these providing blood or tissue for testing after varying intervals on the remaining 5.

The presence of leukaemia was confirmed by post-mortem examination of all birds dying during the experiment. No attempt at titration was made, as it was obvious that the doses given (*c.* 50,000 M.I.D.'s) were producing clear-cut results, and at this juncture it was felt that no useful purpose would be served by performing similar experiments with serial dilutions. However, a rough estimate of the actual dilutions was obtained in the following manner. The birds used in these experiments were all about 6 weeks old, and averaged 300 g. live weight. Using the Evan's blue (T. 1824) technique (Newell and Shaffner, 1950), and estimating the disappearance of the dye against time, plasma samples were examined in a Unicam spectro-photometer working at 6000Å. By plotting back to zero time the blood volume was estimated at about 20 ml. As the initial dilution of leukaemia blood was 1/20, blood removed and diluted from the control birds would represent a final dilution of 1/400 or approximately 125 M.I.D.'s would be injected into the test birds. However, as virus and cells disappear within a short time of injection, these figures are probably only significant for blood withdrawn from the controls during the first few minutes.

EXPERIMENTAL AND RESULTS.

A. Whole blood

Diluted whole leukaemia blood was injected into groups of control birds and samples withdrawn from the opposite wing vein of one of these controls at different intervals for each group. This blood was in turn diluted and injected intravenously into the test group in order to determine infectivity. The results are given in Table I. Fraction numerators indicate the number of birds dying, and denominators indicate the number of birds tested.

TABLE I.—*Determination of the Duration of the "Eclipse Phase," following Infection with Whole Blood.*

Experiment number.	Time between injection and withdrawing of blood.	Control group.	Test group.
1	1 minute	5/5	5/5
2	10 minutes	4/5	3/5
3	20 „	3/5	5/5
4	35 „	4/5	0/5
5	1 hour	3/5	0/5
6	2¾ hours	3/5	0/5
7	3 „	5/5	0/5
8	3½ „	5/5	0/5
9	6½ „	5/5	0/5
10	16¾ „	4/5	0/5
11	18 „	5/5	4/5
12	19½ „	5/5	2/5
13	52½ „	5/5	5/5
14	72 „	5/5	5/5

The results indicate that it takes about 30 minutes for infectivity to disappear after intravenous injection, that the eclipse phase lasts approximately 17 hours, and thereafter there is a return of infectivity lasting until death. In Experiment 11 the birds in the test group survived for 4 weeks, but were then killed, when their blood picture was found to be one of sub-acute leukaemia.

B. Plasma

As the above experiments were conducted with whole blood, where both virus and living malignant cells were injected, a second series of birds was tested with plasma as the infective medium, with the results shown in Table II.

TABLE II.—*The Duration of the "Eclipse Phase," using Erythroleukaemia Plasma*

Experiment number.	Time between injection and withdrawing blood (plasma).	Control group.	Test Group.
1	10 minutes	4/5	3/5
2	30 „	5/5	0/5
3	7 hours	5/5	0/5
4	17 „	4/5	0/5
5	24 „	3/5	2/5

Essentially the same results were therefore obtained when plasma alone was used for the first and second injections.

The impossibility of transmitting infection during the eclipse phase when whole blood is used seems to indicate either (*a*) that the original leukaemia cells from the donor's blood are filtered from the circulation of the recipient bird soon after injection and are retained, e.g. in the marrow or spleen, where by proliferation they give rise to homologously derived cells in addition to those autologous cells produced by the action of plasma virus on the recipients' haemopoietic tissue; or (*b*) that the introduced homologous cells are destroyed and those which appear in the circulation 2 or 3 days before death are derived solely from the host. In order to ascertain which of these possibilities is true, blood films were examined at various intervals after injection of whole leukaemic blood. It was found that apparently viable leukaemia cells, as judged by their staining properties, were detectable at 20 minutes subsequent to their introduction, then rapidly decreased in number with signs of degeneration, until there were none to be found at 3 hours. At this latter period however, dark-ground illumination showed the presence of swarms of filaments, either free in the blood or attached to cells, and at 5 hours these in turn were no longer detectable. It seems probable that these filaments are produced by the disintegration of homologous blood cells shortly after their introduction into the circulation, and that the blood stream is quickly cleared of this debris.

C. Liver, spleen and marrow.

The fate of the virus during the eclipse phase still remains undetermined, but there appear to be three possibilities : (*a*) the virus may enter the hosts' susceptible cells and the non-infective period of roughly 18 hours is occupied by a breakdown of virus into pro-virus, which is replicated within the cell before recombining to form more infective virus ; (*b*) natural immune bodies in the serum of the host may temporarily neutralise the virus; or (*c*) the virus is rapidly taken up in an unchanged state by reticulo-endothelial cells with haemopoietic properties and is

thus temporarily removed from the circulation. Experiments were therefore designed to test some of these possibilities, and are detailed below.

In order to find out whether the virus is fixed unchanged in the cells of the host, birds were killed during the eclipse period subsequent to receiving intravenous injections of infective plasma in the usual dilution. Portions of liver, spleen and marrow were then removed and ground up with 6 per cent dextrose solution in a TenBroeck grinder. The resulting suspensions, after light centrifuging, showed no intact cells, and were injected intravenously into chickens, with results shown in Table III.

TABLE III.—*The Infectivity of Tissues Rich in Reticulo-endothelium at Various Intervals Subsequent to Infection with Erythroleukaemia Plasma.*

Experiment number.	Time killed after injection. Hours.	Material.	Birds died.
1	2	Spleen	0/5
		Femoral Marrow	0/5
		Liver	0/5
2	7	Spleen	0/5
		Femoral Marrow	0/5
		Liver	0/5
3	26	Spleen	3/5
		Femoral Marrow	4/5
		Liver	1/5

Thus these experiments produced similar results to those in which whole blood or plasma was administered, and appear to rule out possibilities (*b*) and (*c*).

DISCUSSION.

Certain results of this investigation are only partly in accord with previously reported work. Crank and Furth (1931) claimed that homologous leukaemia blood cells injected intravenously into young chickens commence proliferating immediately, and if a large enough inoculum was given, the birds died from acute leukaemia in two days. This has not been the case in the present work, although probably not such large numbers of viable cells were injected as in Crank and Furth's experiments. It was found instead, that the introduced leukaemia cells, behaving like a foreign tissue, degenerate and are removed from the circulation. The same authors found that blood withdrawn from chickens 30 minutes after injection was no longer infectious, which is in excellent agreement with the commencement of the eclipse phase reported here. Rothe Meyer and Engelbreth-Holm (1933), however, found the blood of injected chickens to be infectious up to 24 hours after injection, after which infectivity disappeared, reappearing 2–15 days later. Similar results were reported by Rufilli (1938), and Storti and Brotti (1938), both cited by Engelbreth-Holm (1942). These workers also claimed that the virus could be demonstrated continuously in the marrow from shortly after injection to the time of death, although it could not be demonstrated so regularly in tissues other than marrow.

Rothe Meyer and Engelbreth-Holm (1933) found that leukaemia cells appear in the blood stream at the same time that the property of infectivity reappears. This has not been our experience, since the first indication of an abnormal blood

picture is usually observed about 5 days before death, and consists of the appearance of a few cells resembling pro-monocytes. These are rapidly followed by haemocytoblasts in increasing numbers until death of the bird.

The following sequence of events is suggested following the introduction of erythroleukaemia virus into susceptible chickens. For a brief period of about 30 minutes, infective virus in cells and plasma circulates in the blood stream. At this point, all infective virus disappears, and blood and tissues rich in reticulo-endothelium taken from the chicken during the eclipse phase, which lasts about 17 hours, are not capable of infecting further chickens. After 18 hours, such tissues are once again infective. A somewhat similar state of affairs to that outlined above has been demonstrated for the Rous I virus by Carr (1953). For the first hour or so after injection of whole leukaemic blood, apparently viable cells can be detected in the blood stream. These then degenerate and the protoplasmic filaments subsequently demonstrable may be derived from them. These filaments in turn are no longer detectable by about the fifth hour. This destruction of the homologous leukaemia cells explains why infection is not transferred simply by the injection of living malignant cells, independent of whether the virus is inactive or not. In other words, the presence of infective virus at the time of injection is essential for the propagation of this and probably other strains of leukaemia.

It has been frequently observed that spontaneous (" field ") cases of erythroleukaemia are not transmissible, even following the injection of massive doses of whole blood. The writer has found that chickens inoculated with such material are not resistant to the virus-associated leukaemia under discussion, that is, they develop no immunity as the result of previous innoculation with the spontaneous strain (unpublished data). Since a solid immunity can be easily demonstrated in chickens which have survived an infective dose of virus-associated leukaemia, and since such birds may suddenly die from acute leukaemia many months after challenge, it seems reasonable to conclude that certain birds, possessing more serum antibody than others, are able to resist infection to the extent that the virus is kept in a state of more or less indefinite eclipse, and that some, as yet unknown, factor which reduces the resistance of the bird may result in uncontrolled proliferation of the cells containing pro-virus. The presence of pro-virus might thus confer the property of potential malignancy. That neoplastic change does not depend on the transformation of pro-virus to complete or infective virus seems to be indicated by the impossibility of demonstrating infective virus in cell-free preparations obtained from either field cases or those which have suddenly flared up in resistant laboratory fowls many months after challenge.

Although leukaemia virus is formed intracellularly, it makes a rapid appearance in the plasma, possibly because of the high rate of cellular degeneration associated with the fragile undifferentiated cells in the circulation, and it does not seem improbable that the protoplasmic filaments already mentioned are one means of releasing free virus (Campbell 1952; Bather 1954).

Failure to detect infective virus in the 17-hour period of virus eclipse in marrow, spleen, and liver, all rich in reticulo-endothelial tissue with haemopoietic potentialities, eliminates the possibility of unchanged virus being held at these sites either in homologous retained cells or in those of the host. Also the hypothesis of natural serum antibody temporarily neutralizing the infective virus can probably be dismissed, since it is usually accepted that antibody cannot reach intra-

cellular virus. Therefore, although the plasma virus would be neutralized, that within the cells should be demonstrable during the eclipse phase by injection of washed disintegrated cells. A preliminary test failed to demonstrate infectivity under these conditions, and more recent experiments have confirmed this result.

SUMMARY AND CONCLUSIONS.

When blood from a case of acute virus-associated erythroleukaemia is injected into the circulation of susceptible chickens it remains demonstrable by sub-inoculation up to about 30 minutes. The virus then disappears, as do the homologous leukaemia cells which degenerate, being replaced by streaming filamentous structures visible in the blood with dark-ground illumination.

Infectivity cannot be demonstrated by sub-inoculation of blood, plasma, marrow, liver and spleen until about 18 hours later, when virus activity returns and lasts until the bird's death from leukaemia 10–18 days after injection.

It is considered that the eclipse phase, during which pro-virus is not infective in the pathological sense and therefore not demonstrable represents a form of life-cycle in which, after penetration of susceptible haemopoietic cells, virus breaks down into pro-viral units which are multiplied by replication. The pro-virus is then re-formed as complete or infective virus which again becomes demonstrable at the termination of the eclipse phase, and although this cycle continues until the death of the host, after 18 hours sufficient complete virus has been elaborated and, of course, continues to be formed, for the blood and tissues to be always thereafter infective. In addition, virus is liberated into the plasma by intravascular and intramedullary cellular degeneration. Leukaemia cells do not overflow into the circulation from haemopoietic centres such as the marrow until several days later.

Most spontaneous leukaemias, although cytologically indistinguishable from the laboratory strain, are not transferable by whole blood or tissue inocula, and birds so injected do not subsequently possess any immunity against the virus-associated leukaemia. It seems probable that these field cases are associated with pro-virus in an indefinite state of eclipse. A point of essential difference seems to be the fact that the Rous sarcoma can be propagated in the virus-eclipse phase by transplantation, but leukaemia cells are not capable of propagation in this manner, since they are destroyed upon introduction into the circulation.

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REFERENCES.

BATHER, R.—(1954) *Brit. J. Cancer*, **8**, 132

CAMPBELL, J. G.—(1952) *Brit. vet. J.*, **108**, 191

CARR, J. G.—(1953) *Proc. Roy. Soc. Edinb.*, B, **65**. Part I. 66.

CRANK, R. P. AND FURTH, J.—(1931) *Proc. Soc. exp. Biol. N.Y.* **28**, 287.

ENGELBRETH-HOLM, J.—(1942) ' Spontaneous and Experimental Leukaemia in Animals.' Edinburgh (Oliver and Boyd).

NEWELL, G. W. AND SHAFFNER, C. S.—(1950) *Poult. Sci.* **29**, 78.

RUFILLI, D.—(1938) cited by Engelbreth-Holm, J. (1942), Spontaneous and Experimental leukaemia in Animals.' Edinburgh (Oliver and Boyd).

STORTI E. AND BROTTI, V.—(1938) cited by Engelbreth-Holm. J. (1942), *Ibid.*

ROTHE MEYER, A. AND ENGELBRETH-HOLM, J.—(1933) *Acta. path. microbiol. scand.*, **10**, 261.

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Section C. Diseases and their Control

59. AVIAN LEUCOSIS: A PLEA FOR CLARIFICATION

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" Leucosis " is a term introduced by Ellerman (1920), who applied it to a group of diseases affecting the fowl, characterised by neoplasia of haematopoietic tissues. Virchow's original name for such diseases in the human subject was leukaemia (" white blood "), but as the presence of excessive numbers of primitive malignant cells in the blood stream is by no means invariable in the fowl, the term is not strictly appropriate.

The type of leucosis depends (*a*), on the particular blood cell progenitor involved, and (*b*), the mode of manifestation in the body. Thus we meet lymphoid leucosis, myeloid leucosis and erythroleucosis, the last two being often present simultaneously, suggesting that in the bird at any rate red cells and granular cells may have a common ancestral cell. Any of these may occur as a true leukaemia in Virchow's sense of the word, or as an aleukaemic form characterised either by a diffuse infiltration or discrete aggregation of malignant cells in various organs, causing hypertrophy in diffuse leucosis, or actual circumscribed tumours in the discrete type.

One cannot go far in any discussion on leucosis before mention is made of fowl paralysis, which in all its manifestations is always included in the leucotic complex, although some doubt has been expressed, particularly by Burmester *et al.* (1946), Davis *et al.* (1947) and Jungherr (1952) on the propriety of doing so. Similarly, the condition known as osteopetrosis (" marble bone ") is usually discussed under the heading " Leucoses," although, as will be seen, the evidence for so including it is slender.

A diagram of the present conception of the Leucotic complex will clarify the position.

It is the purpose of this paper to present evidence to justify the removal of fowl paralysis and osteopetrosis from the Leucoses, and to set them apart as distinct and unrelated disease conditions.

Let us consider Fowl Paralysis first. Classically, this is a disease of fowls about to attain or having recently attained sexual maturity, in which a chronic inflammatory condition affects the peripheral nerve trunks and less frequently the central nervous system, causing oedema and swelling in the nerves, and a more or less heavy infiltration with lymphocytes, monocytes and plasma cells, all usually well differentiated and histologically easily recognisable. Other characteristic pathological changes are perivascular infiltration, myelin sheath degeneration, Schwann-cell proliferation and necrosis and degeneration of ganglion cells in the peripheral nervous system.

These lesions result in a variety of nervous symptoms including spastic paralysis according to the main site of the disease. Their similarity to the pathology of Borna disease or equine encephalomyelitis, and to a lesser extent to acute anterior poliomyelitis, both due to neurotropic viruses, does not need stressing.

The type of fowl paralysis so far described is known as neurolymphomatosis, but there is a fairly general agreement that other forms of fowl paralysis occur, namely, the ocular and visceral forms. Ocular fowl paralysis or ocular lymphomatosis is associated with an infiltration of similar cells to those already mentioned into the iris and ciliary muscles, giving the well-known " pearly eye," and the ragged bordered, non-reactive pupil. Visceral fowl paralysis or visceral lymphomatosis is characterised by tumour-like aggregations of

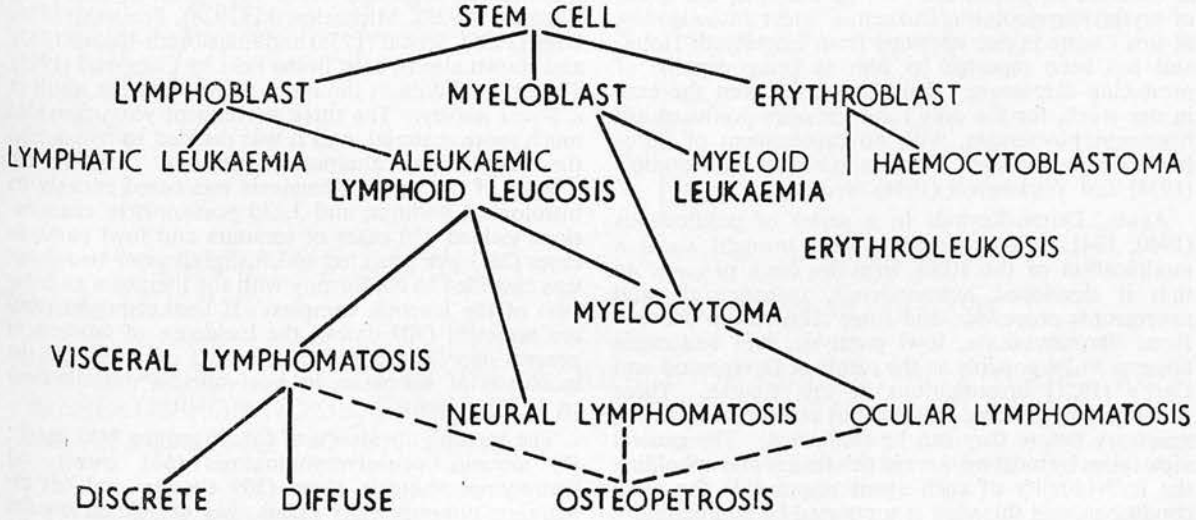


Fig. 1 Leucotic Complex as generally accepted.

more or less mature lymphocytes, etc., as above, occurring in about 10 per cent of neurolymphomatosis cases in various thoracic and abdominal organs. These are frequently confused with the true tumours unfortunately commonly classified under the heading "lymphomatosis," but which should be called lymphosarcomata or lymphoblastomata, since they are characterised by undifferentiated cells of the lymphoid series.

The condition known as osteopetrosis was originally closely identified with fowl paralysis by Jungherr and Landauer (1938), and is still described together with that disease in the leucotic complex. It is a condition in which abnormal amounts of compact bone develop on both the interior and exterior of the diaphyses of long bones, causing hypertrophy and distortion. Initially, the pathology resembles osteodystrophia fibrosa, but terminally it resembles Paget's disease or osteitis deformans. There seems little doubt that the condition is associated with a virus, though not, as will be shown presently, necessarily identical to that causing neurolymphomatosis and the ocular and visceral forms of fowl paralysis.

There is a tendency to postulate etiological unity for leucosis and the transmissible sarcomata, and by implication also for fowl paralysis and osteopetrosis. Oberling and Guerin (1933), Furth (1933) Rothe Meyer, and Engelbreth-Holm (1933) all demonstrated the production of fibrosarcomata after intramuscular or subcutaneous injection of the infectious agent associated with transmissible leukaemia, and they attempted but failed to separate a sarcoma-producing fraction from the leukaemia-producing fractions. They concluded that a single agent might be considered the cause of both fibro-sarcoma and leukaemia.

Later, however, Furth (1936), Jungherr (1937) and Cole (1941) investigated "mixed" strains and found they could isolate two agents with different pathological potencies. It is now apparent that not all strains of erythroleukaemia agent have this dual potentiality, and indeed they may behave differently in different environments. As an example, the strain of erythro-myeloblastic leukaemia under investigation at this Centre is one obtained from Engelbreth-Holm, and has been reported by him as being capable of producing sarcomata. This has never been the case in our stock, for the only local tumours produced are haemocytoplastomata, with no involvement of fibroblasts or macrophages. This is in accord with Stubb's (1938) and Wickware's (1946) results.

Again, Duran-Reynals in a series of publications (1940, 1941, 1947) claimed to have brought about a modification of the Rous virus by duck passage, so that it developed haemorrhagic, osteopetrotic and neurotropic properties, and some relationship between Rous fibrosarcomata, fowl paralysis, and leukaemia appears to be possible as the result of Greenwood and Carr's (1951) investigations at this Centre. These results are of extreme interest, but much more work is necessary before they can be confirmed. The general view taken by most workers in this field is one upholding the individuality of each agent responsible for these conditions, and this view is supported by an impressive bulk of evidence. In a paper of this size it is obviously impossible to survey the literature comprehensively, so only a few examples can be quoted, but it is considered that they represent a fair cross-section of the published work.

Durant and McDougle (1939) found no leucosis or tumour formation as the result of neurolymphomatosis transmission experiments. Osteopetrosis was claimed to have been transmitted by blood inoculation from birds with neurolymphomatosis. Affected nerves or bone itself failed to transmit the condition. Jungherr (1937) could find no evidence for a unitarian etiologic hypothesis relating fowl paralysis, lymphomatosis, erythroleucosis, myeloid leucosis or fibro-sarcoma. He pointed out that neurolymphomatosis showed nerve involvement with mainly mature cells, and a normal blood picture with no marrow involvement, whereas lymphomatosis was visceral in distribution, with no nerve involvement, but with abnormal marrow and anaemia or lymphatic leukaemia. Furth (1934, 1935) found no relationship between fowl paralysis and lymphomatosis, and the latter condition was shown by Gibbs and Johnson (1936) to be characterised by involvement of all lymphoid tissues, whereas neurolymphomatosis was not.

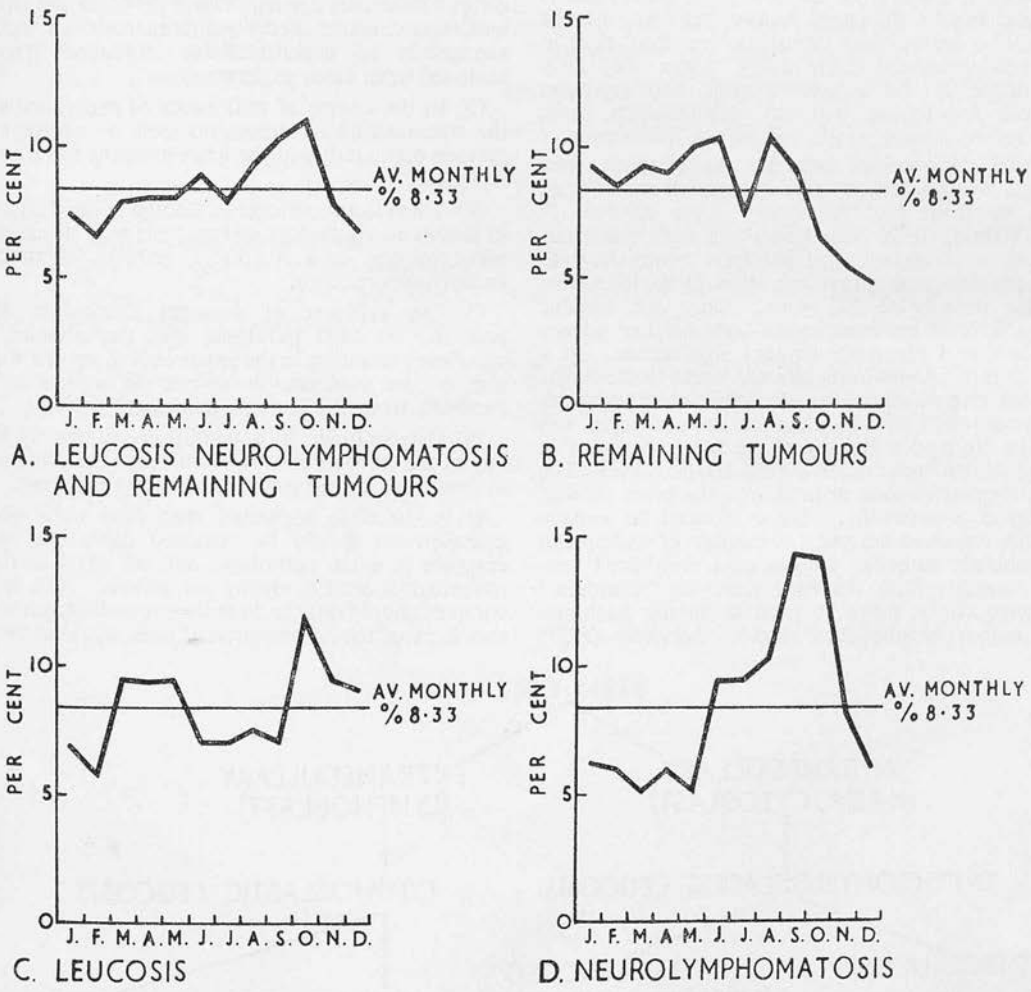
In a review, Davis *et al.* (1947) summarised the diverse reports on the transmissibility of fowl paralysis and lymphocytoma and reached the conclusion that visceral lymphomatosis is a distinct entity unrelated to neurolymphomatosis. Finally, the U.S. Bureau of Animal Industry, in their report for 1951, concludes that fowl paralysis is distinct from visceral lymphomatosis. This conclusion was reached by the writer (Campbell, 1945) on the evidence of a difference in the distribution of organ lesions between visceral lymphomatosis with no detectable nerve lesions, and lymphoid growths occurring concurrently with neurolymphomatosis.

Further evidence in favour of separating fowl paralysis from the leucoses has been obtained during a long term investigation into the phenomenon of seasonal variation of neoplastic disease in fowls, well-known, especially from experimental studies by Ellerman (1922), Michaelowski (1928), Peacock (1936), Bagg (1936), Schaaf (1936) and Engelbreth-Holm (1942), and shown also to exist in the field by Campbell (1945). The original data in the latter paper were the result of a 5-year survey. The three subsequent years provided much more material, and it was decided to re-examine the material by alternative methods. During the 8 years of the survey, diagnosis was based entirely on histological findings, and 3,285 post-mortem examinations yielded 920 cases of tumours and fowl paralysis cases (28.3 per cent), of which slightly over two-thirds was classified in conformity with the literature as being part of the leucotic complex. If fowl paralysis cases are removed (309 cases), the incidence of leucosis in general neoplasia is reduced to 38.2 per cent and the incidence of neoplasia in post-mortem examinations to 18.1 per cent.

The monthly incidence of (*a*) all groups (920 cases); (*b*) leucosis+neurolymphomatosis (661 cases); (*c*) neurolymphomatosis alone (309 cases); and (*d*) remaining tumours (259 cases) was calculated on the basis of the total number of cases in each group for the

year (=100 per cent). An arbitrary standard base line was obtained by dividing the annual total (=100 per cent) by the number of months, giving 8.33 per cent.

Fig. 2A shows the monthly fluctuations of all neoplastic disease plus fowl paralysis, averaged out over a period of 8 years, and demonstrates a fairly steady rise to a peak in October. Fig. 2B shows the result of removing the leucoses and fowl paralysis and Fig. 2C shows the leucoses plotted alone. Fig. 2D, with its spectacular rise commencing in August and remaining until the middle of October, represents fowl paralysis alone. It is obvious, apart from statistical tests, that Fig. 2A obtains its characteristic curve mainly because of the inclusion of fowl paralysis (i.e., neurolymphomatosis alone or associated with visceral lesions). Statistical tests show Figs. 2A and 2D to be significant, the latter highly so, whereas Figs. 2B and 2C are without significance. In passing, it is worth while noting that an autumn peak incidence of fowl paralysis is to be anticipated in the northern hemisphere, if the egg transmission theory of the disease is accepted; since fowl paralysis in its classical form is essentially confined to 6-9 month old birds, and the main hatching period is in the spring. Indeed, this result provides indirect evidence of the correctness of the theory.



SEASONAL FLUCTUATION IN THE INCIDENCE OF NEOPLASTIC DISEASES AND NEUROLYMPHOMATOSIS, EXPRESSED AS MONTHLY TOTAL/YEARS TOTAL, FOR THE YEARS 1940-47 INCLUSIVE.

Fig 2

For Graph A slope of regression line is 0.364, significant at 0.1 per cent level; Graph D 0.946, significant at 0.1 per cent level; Graph B slope is zero (to the limits of accuracy used); Graph C slope is 0.283, not significant.

CONCLUSIONS

We are now in a better position to answer the question—have fowl paralysis and the leucoses a common etiology? In an attempt to answer this question, we may summarise the following points:

(1) Neurolymphomatosis, in contra-distinction to the classical leucoses, cannot be classified as a neoplastic disease. The cells infiltrating the various nerve trunks and central nervous system are in the main fully mature. The impression received is that they accumulate at these sites in response to some irritant, almost certainly viral. In other words, the typical fowl paralysis nerve lesion is one of chronic inflammation and oedema. There is no marrow involvement, nor is there a change in the blood picture, but there may be tumour-like aggregations of mature lymphoid cells in peripheral nerves and in the ovary, where they have their origin in the adjacent nerves, and represent lymphoid hyperplasia with an inflammatory basis. These lesions do not invade and do not metastasise.

(2) The majority of attempts to transmit fowl paralysis by inoculation with material from affected nerves or from the " tumours " have resulted in failure (Olson, 1937). The writer has made numerous attempts to transplant fowl paralysis " tumours " to young chickens, without success, although the birds were kept for periods of 2-3 years. Only one tumour developed in these experiments—an ovarian adenocarcinoma and obviously without significance. In a series of experiments using affected nerve tissue as the inoculum given intraperitoneally into young chickens, the development in 8-14 days of haemorrhages was noted in the myocardial fat of the left atrium and in the adductor muscles overlying the sciatic nerves. The nerves themselves were normal, and the birds showed no clinical abnormality. Those allowed to survive 1-2 years remained normal. A number of widespread lymphoblastic tumours unassociated with fowl paralysis, together with the fowl paralysis " tumours " mentioned above, failed to produce similar haemorrhagic lesions (unpublished work). Jungherr (1937) reported the infectivity of blood, but stated that no other type of the leucotic complex occurred in their experimental birds; that is, he only succeeded in transmitting the nerve form. One strain of lymphomatosis which occasionally produced a Rous sarcoma-like tumour, later dissociated into the corresponding morphologically and etiologically distinct entities demonstrating the presence in the original tumour of two different infectious agents. It appears from later work (Burmester and Prickett, 1945; Burmester, Prickett and Belding, 1946; and Burmester, 1947) that his claim to have shown a common etiological agent for neurolymphomatosis and osteopetrosis cannot be accepted, since these workers demonstrated two distinct infectious agents. Olson (1947) found chickens with spontaneous neurolymphomatosis to be fully susceptible to experimentally implanted lymphoid tumours from cases of leucosis.

(3) In the course of multitudes of experiments with the transmissible leucoses, no case of neurolymphomatosis attributable to the leucosis agent has ever been reported.

(4) There is a significant difference in the distribution of lesions in visceral lymphomatosis with no detectable nerve lesions, and lymphoid growths occurring in neurolymphomatosis.

(5) The evidence of seasonal fluctuation in the incidence of fowl paralysis, and the absence of a significant variation in the leucoses is in accord with the rest of the evidence in favour of separating fowl paralysis from the leucotic complex.

(6) Osteopetrosis is a condition completely unlike any of the leucoses *per se* and evidence has accumulated in favour of its separation from fowl paralysis.

It is therefore suggested that fowl paralysis and osteopetrosis should be removed from the leucotic complex in avian pathology, and set apart as distinct pathological entities arising *sui generis*. The leucotic complex should only include those conditions involving neoplasia of the haematopoietic cells, and thus conform

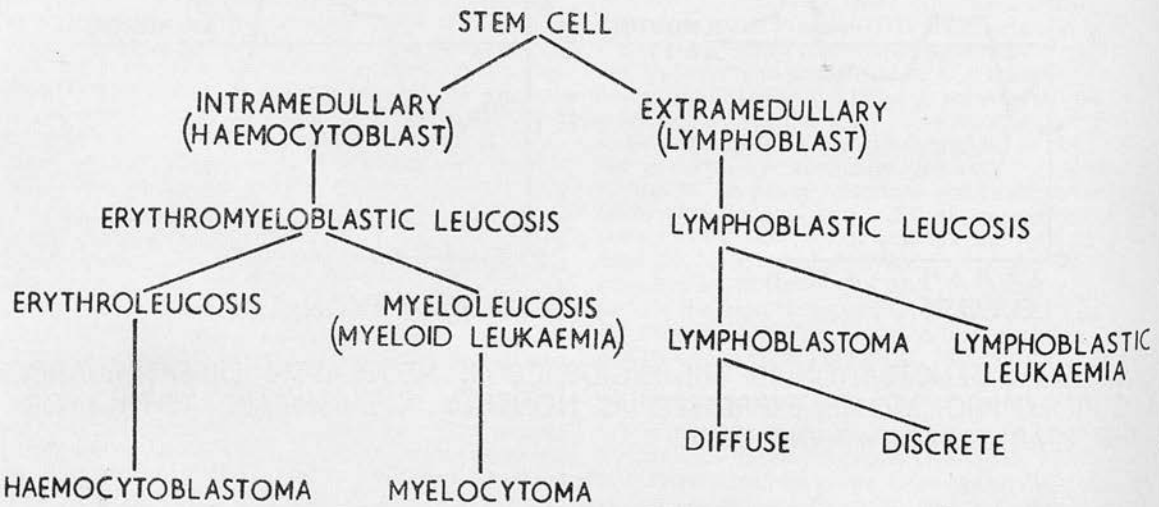


Fig. 3 The Modified Conception of Avian Leucosis.

with the basic conception of leucosis. Fig. 3 illustrates a revised classification of the leucoses founded upon the material discussed and presented in this paper.

REFERENCES

BAGG, H. J. (1936). Amer. J. Cancer. **26** : 69.

BURMESTER, B. R., and PRICKETT, C. O. (1945). Cancer Res. **5** : 652.

BURMESTER, B. R., PRICKETT, C. O., and BELDING, T. C. (1946). Poult. Sci. **25** : 616.

BURMESTER, B. R. (1947). Ibid. **26** : 215.

CAMPBELL, J. G. (1945). J. Comp. Path. **55** : 308.

COLE, R. K. (1941). Cancer Res. **1** : 714.

DAVIS, O. S., DOYLE, L. P., WALKEY, F. L., and CENKER, L. K. (1947). Poult. Sci. **26** : 499.

DURAN-REYNALS, F. (1940). Yale J. Biol. and Med. **13** : 77.

—— (1941). Science **93** : 501.

—— (1947). Cancer Res. **7** : 99.

DURANT, A. J., and MCDOUGLE, H. C. (1939). Mo. Agr. Exper. Sta. Res. Bull. 304.

ELLERMANN, V. (1920). Folia Haematol **26** : 135.

—— (1922). Cited by Engelbreth-Holm. *Leukaemia in Animals*. Oliver & Boyd. Edinburgh.

ENGELBRETH-HOLM, J. (1942). *Leukaemia in Animals*. Ibid.

FURTH, J. (1933). J. Exp. Med. **58** : 253.

—— (1934). Proc. Soc. Exp. Biol. and Med. **31** : 921.

—— (1935). Arch. Path. **20** : 379.

—— (1936). J. Exp. Med. **63** : 145.

GIBBS, C. S., and JOHNSON, C. G. (1936). Mass. Agr. Exp. Sta. Bull. 327.

GREENWOOD, A. W., and CARR, J. G. (1951). Official Rep. 9th World's Poult. Congr., Paris.

JUNGHERR, E. (1937). Storrs Agr. Exp. Sta. Bull. 218.

—— (1952). *Diseases of Poultry*. 3rd edit. Ames. Iowa.

JUNGHERR, E., and LANDAUER, W. (1938). Storrs Agr. Exp. Sta. Bull. 222.

MICHAELOWSKI, I. (1928). Virchow's Arch. **267** : 27.

OBERLING, CH., and GUERIN, M. (1933). Bull. Assoc. franc pour Etude Cancer **22** : 326.

OLSON, C. (1937). Jour. Infect. Dis. **61** : 325.

—— (1947). Cornell Vet. **37** : 231.

PEACOCK, P. R. (1935). Amer. J. Cancer **25** : 37.

ROTHE MEYER, A., and ENGELBRETH-HOLM, J. (1933). Cited by Engelbreth-Holm. *Leukaemia in Animals*. Oliver & Boyd. Edinburgh.

SCHAAF, J. (1936). Cited by Engelbreth-Holm. Ibid.

STUBBS, E. L. (1938). J. Amer. Vet. Med. Ass. **92** : 73.

U.S. BUREAU OF ANIMAL INDUSTRY (1951). 12th Ann. Rep. of the Regional Poult. Res. Lab., East Lansing, Michigan.

WICKWARE, A. B. (1946). Canad. J. Comp. Med. **10** : 74.

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INDUCTION OF MULTIPLE PRIMARY TUMOURS IN FOWLS WITH 2-ACETAMIDOFLUORENE.

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From the British Empire Cancer Campaign Unit at the Poultry Research Centre, Edinburgh.

Received for publication, December 10, 1954.

THE carcinogenic property of 2-acetamidofluorene (N-acetyl-2-aminofluorene, acetylaminofluorene, 2-AAF) for fowls has been previously demonstrated by Bielschowsky and Green (1945), who reported the induction of renal tumours, and by Peacock and Peacock (1949, 1954). The latter recorded a squamous cell carcinoma of the crop mucosa which developed along the needle track following injection of 2-AAF in arachis oil into the lumen of that viscus ; and in their later paper described 3 cases of multiple primary tumours affecting various organs out of a total of 16 fowls which had received repeated injections of aqueous suspensions of 2-AAF in 25 mg. doses into the crop. In 4 other birds, single epithelial tumours developed.

Although spontaneous carcinomata are relatively common in older fowls (Olson and Bullis, 1942 ; Campbell, 1945), they have always been regarded as difficult to produce experimentally, compared, for example, with the ease with which they can be induced in the small rodents by various carcinogenic hydrocarbons. In particular, the carcinogenicity for rats of 2-AAF has been utilized experimentally by many workers since the first report by Wilson, De Eds and Cox (1941).

In contradistinction to experimentally produced mammalian carcinoma, those few which have been produced in fowls have not been transplantable, and indeed, no one has yet succeeded in propagating any epithelial tumour of the fowl by homologous transplantation, unless the limited success reported by Campbell (1949) with liver cell carcinoma in ducks is taken as an indication that the propagation of carcinomata in birds is not impossible. It follows, then, that experimental epithelial tumours of the fowl are of some interest, not only because of their rarity, but also because of the possibility of obtaining a carcinoma which might be propagated and which could then be subjected to similar extensive studies as the transplantable connective tissue tumours. The experiments now reported were begun in 1949, and, while on a small scale, have given very encouraging results, so that further work using different techniques is already going forward.

METHODS.

Eight Brown Leghorn pullets of an inbred strain and aged about 6 months were used. Five received daily doses of 25 mg. 2-AAF in 0·6 ml. sesame oil in gelatine capsules (Size No. 1, Parke Davis) orally for 3 months, Sundays excepted. The remaining 3 birds received sesame oil in capsules and served as controls. Treatment was commenced in February, 1949. The birds were kept in battery cages and fed the normal balanced ration used at the Centre.

Tissues taken at the end of the experiment were fixed in Susa, and prepared in the usual way for embedding in wax. Sections were cut at 5–7μ and stained with haematoxylin and eosin, or by Mallory's trichrome method. Where possible all fresh specimens were photographed immediately after post mortem examination.

Transplantation experiments consisted of intra-muscular, intra-peritoneal and intra-hepatic injections of finely-minced tumour tissues in normal saline. The tumours were either prepared for implantation direct from the post mortem material, or from fragments cultured on the chorioallantoic membrane of 10-day embryonated eggs, using the method of Beveridge and Burnett (1946).

RESULTS.

None of the birds showed any ill-effects as the result of the treatment, eating and drinking normally and coming into lay between 7 and 8 months of age. Normal moults were carried out, and the plumage pigmentation was unaffected. In May, 1953, one bird was found dead, having been laying intermittently for some time previously. Examination of the remainder showed another bird to be thin but in apparent good health, and the experiment was terminated by killing the remainder in the course of the next few days. Multiple tumours of the viscera were found in 3 birds out of the 5 treated with 2-AAF, and the other two showed cirrhosis of the liver and cystic kidneys. The controls were entirely normal. Table I summarizes the details.

TABLE I.—*Results of Treating Fowls with 2-AAF.*
All experiments commenced 1/2/49.

Fowl No.	Number of doses.	Total dosage 2-AAF (g.).	Result.
1	77	1·925	Died 7.v.53. Tumours in liver, intestine, oviduct, cysts and a tumour in one kidney.
2	77	1·925	Killed 10.v.53. Tumours in liver, ovary, oviduct and kidneys. Lesion in left lung.
3	77	1·925	Emaciated. Killed 10.v.53. Tumours in liver and ovary. Contracted granular kidneys.
4	77	1·925	Killed 10.v.53. Cirrhosis of liver. Cystic kidneys.
5	77	1·925	Killed 10.v.53. Cirrhosis of liver. Cystic kidneys.
6, 7, 8	77 (oil only)	—	Killed 12.v.53. No evident abnormality.

Post mortem findings and histopathology.

1. The liver was irregularly contracted, of a brownish colour and with prominent vessels visible beneath a thickened capsule. There were numerous bile-stained tumours, some discrete, others coalescent (Fig. 1). The gall bladder was

small and fibrosed, and contained a clear watery fluid. Small cream-coloured tumours were scattered on the serosa of the gizzard, duodenum and terminal part of the ileum. The left kidney was enlarged and cystic (Fig. 2), and one cyst contained an organizing blood clot. The right kidney was normal in size, but also cystic. The oviduct contained an isolated tumour which projected into the lumen of the magnum (Fig. 3). The ovary was that of an actively laying fowl, and all other viscera appeared normal.

Histological examination showed a fibrosis of the liver capsule with irregular bands of connective tissue entering the parenchyma, causing contracture and distortion. The capsular thickening was very pronounced in the region of the portal fissure and fossa for the gall bladder. There were numerous apparently benign hepatomata consisting of well differentiated but irregularly arranged cords of liver cells. Small zones of lymphocytes were fairly frequent in these tumours (Fig. 4).

The oviduct tumour was an adenocarcinoma arising from the mucosa of the magnum (Fig. 5, 6). Although this tumour was fairly well differentiated, with but little mitotic activity, it had spread by implantation to the serosa of the alimentary tract, as demonstrated by identical structure of those tumours.

Both kidneys showed multiple retention cysts, while the left organ contained a small encapsulated and trabeculated tumour composed of anaplastic cells bearing some resemblance to those of the oviduct tumour. The metastasis was apparently within a renal vein (Fig. 7). There was a pronounced concentric intimal fibrosis of the arterioles and the glomerular tufts were very cellular and hypertrophied (Fig. 8).

2. This bird ceased to lay early in 1953, and was killed 3 days after bird No. 1 died. It was found to have multiple liver tumours showing faint bile staining, and tumours in the ovary, oviduct and kidneys. A caseated abscess was present in the left lung.

Histologically, the liver tumours were typical hepatocellular carcinomata exhibiting patches of fatty change (Fig. 9). Metastases were present in both kidneys. The ovarian and oviductal tumours were adenocarcinomata of essentially similar structure, and it was difficult to ascertain which was primary. It seemed most likely, however, that the ovarian tumour (Fig. 10) represented a secondary deposit, since the structure resembled uterine glands more closely than it did a tumour derived from ovarian tissue.

Sections of lungs showed no tumours, but there was an old encapsulated and caseated abscess, and anthracosis was a prominent feature.

3. As this hen was becoming progressively more emaciated and had ceased to lay for several months, it was killed at the same time as bird No. 2. Post mortem examination showed multiple cream-coloured liver tumours, a small cystic ovary with a solitary tumour, and contracted granular kidneys. A large white nodule embedded in the left thyroid gland proved to be a hypertrophied parathyroid. The corresponding right gland was only slightly enlarged.

The liver tumours were hepatomata formed by relatively small undifferentiated cells arranged in irregular branching cords forming blood sinusoids (Fig. 11). The ovarian tumour was of an unusual nature and offered more difficulty in diagnosis. It consisted of a fairly abundant fibrous stroma, often forming the core of papillae which were invested with a single layer of cuboidal to columnar pale-staining epithelial cells with conspicuous vesicular nuclei. Loose accumula-

tions of similar cells were present in the pockets between papillae (Fig. 12). These frequently showed degenerative changes. Numerous cysts, lined with epithelial cells were present. A diagnosis of cystic papilliform adenocarcinoma of the ovary was made, but the origin of the epithelial cells was undetermined. Neither of these tumours metastasized. The kidneys showed a proliferative glomerulitis with interstitial infiltrations of granulocytes.

4, 5. These two birds were also killed at the same time. They had previously shown no symptoms, but No. 4 had ceased laying some months earlier, whereas No. 5 still laid sporadically. At examination, bird No. 4 was found to have a firm, somewhat smaller than normal liver and cystic kidneys. Histologically a mild cirrhosis was demonstrated, with regions of marked bile duct hyperplasia. The liver of bird No. 5 was also slightly cirrhotic, and the kidneys were cystic. All other organs appeared normal. No tumours were detected in either of these birds.

Controls.—The 3 controls, dosed with sesame oil only, were all in good condition and exhibited no evident abnormality upon post mortem examination.

Transmission Experiments.

Finely minced fragments of the liver tumour from fowl No. 1 were seeded on to the chorioallantois of 4 ten-day embryonated eggs. Tumours grew in all the eggs, and at 20 days they were opened, the tumours removed and finely divided with scissors and transferred to further eggs. These also grew, but did not reach such a large size at 20 days as in the first egg passage. One membrane tumour was taken for histology, and showed a well differentiated hepatoma,

EXPLANATION OF PLATES.

FIG. 1.—Fowl No. 1. Liver, visceral surface, showing multiple hepatomata. The gall bladder has been removed. Natural size.

FIG. 2.—Fowl No. 1. Left kidney, showing several cysts and marked fibrotic changes. Natural size.

Fig. 3.—Fowl No. 1. Oviduct with isolated tumour projecting from the mucosa. Natural size.

FIG. 4.—Fowl No. 1. Histology of hepatoma. A mass of lymphocytes is at the bottom left. H. & E. × 230.

FIG. 5.—Fowl No. 1. Adenocarcinoma of oviduct. Trichrome. × 75.

FIG. 6.—Fowl No. 1. Detail of oviduct tumour. Trichrome. × 285.

FIG. 7.—Fowl No. 1. Metastasis of oviduct tumour within renal vein. Trichrome. × 285.

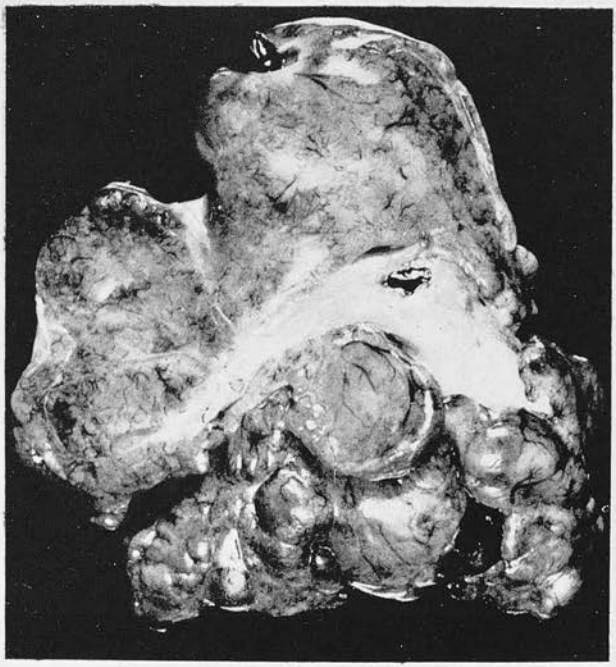
FIG. 8.—Fowl No. 1. Arterial fibrosis (sclerosis) and glomerular hypertrophy. Kidney. Trichrome. × 285.

FIG. 9.—Fowl No. 2. Hepatocellular carcinoma, with fatty change. H. & E. × 240.

FIG. 10.—Fowl No. 2. Adenocarcinoma in ovary, probably representing a secondary from the oviduct tumour, which was similar in structure. Trichrome. × 240.

FIG. 11.—Fowl No. 3. Hepatoma, showing smallness of cells, which form anastomosing cords and sinusoids. H. & E. × 240.

FIG. 12.—Fowl No. 3. Cystic papilliform adenocarcinoma of ovary. Trichrome. × 240.



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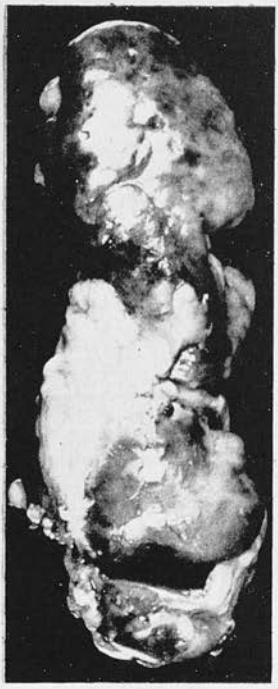

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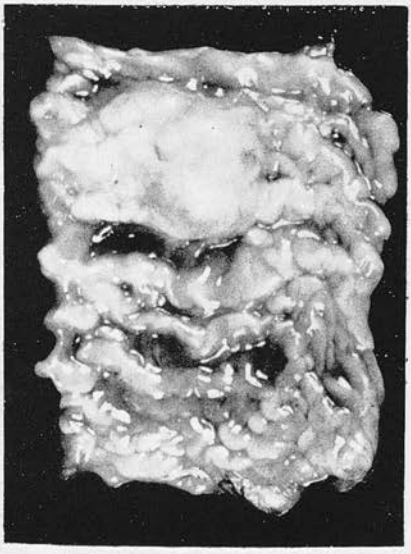

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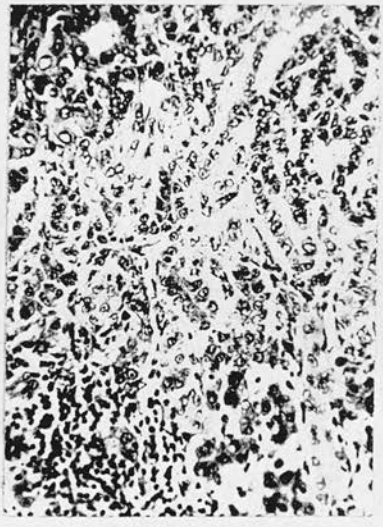

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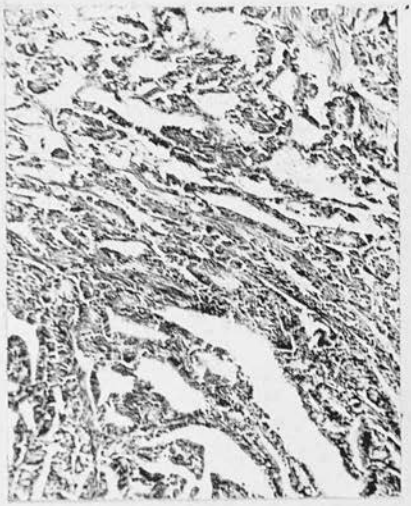

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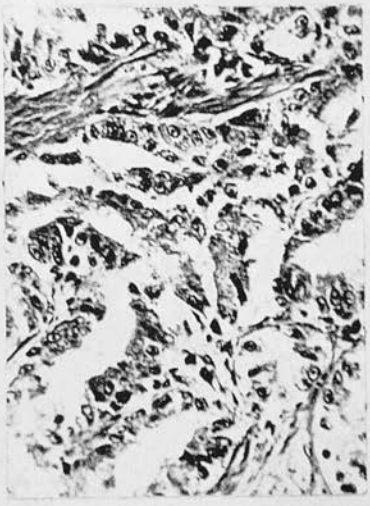

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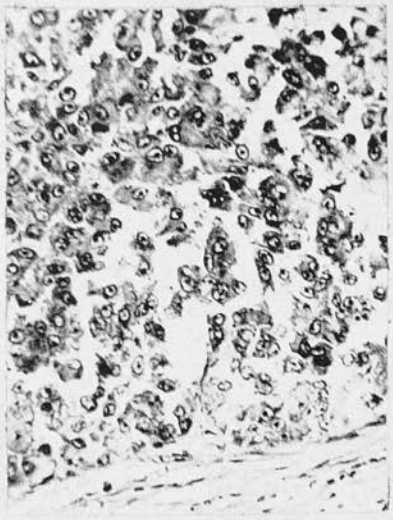

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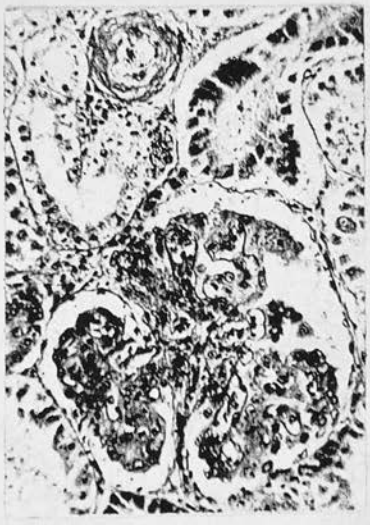

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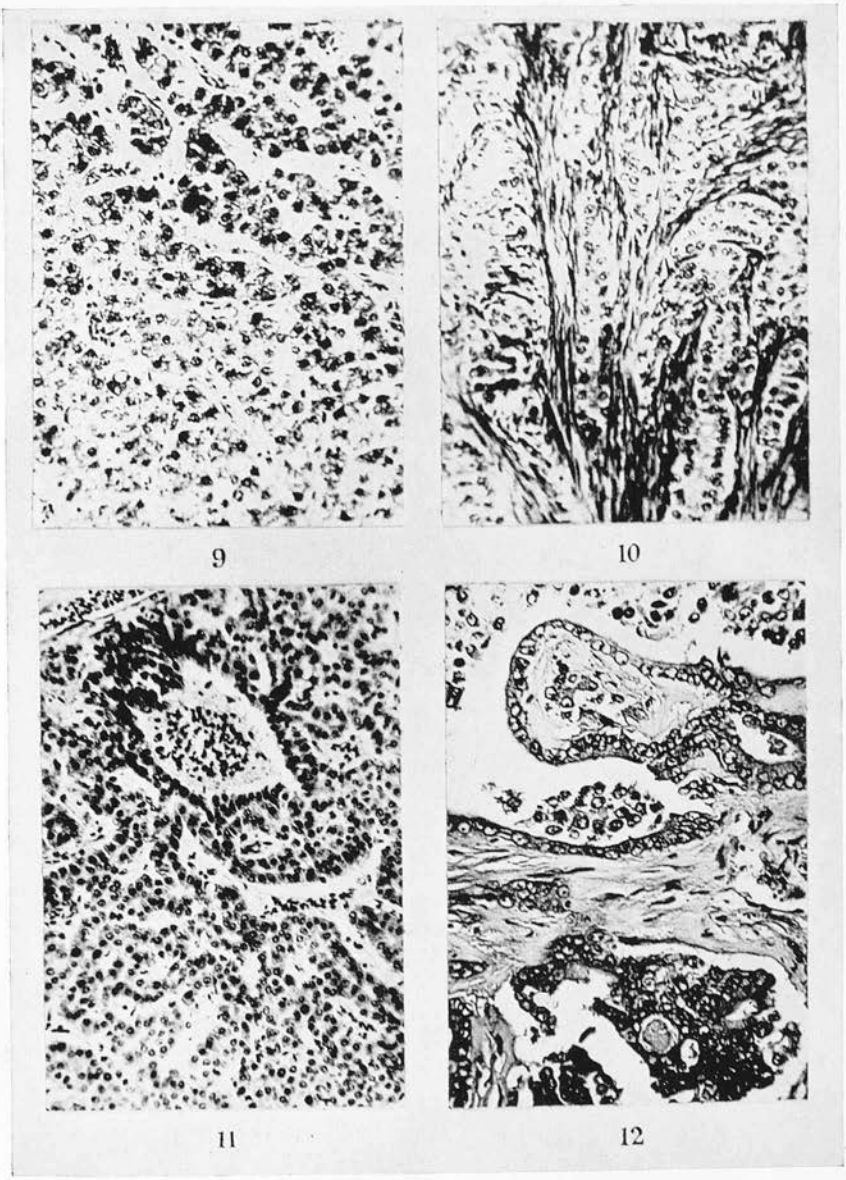

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while the remainder were bulked, minced, suspended in normal saline, and injected into the breast muscles, peritoneal cavity, and livers of 6 six-week-old chicks. In all cases the tumour failed to take. Similar negative results were obtained in 6 young birds receiving fragments of the hepatoma obtained directly from the liver at autopsy.

Attempts were made to propagate both liver and ovarian tumours from fowl No. 3, with no greater success. As before, the direct homologous implants failed to grow, whilst tumour fragments grew satisfactorily on the C-A membrane for two passages. Subsequent efforts to implant the egg-grown tumours into six-week-old chickens were unsuccessful.

DISCUSSION.

Since the incidence of spontaneous tumours in the flock at the Poultry Research Centre is very low (1·6 per cent of all birds dying annually : Greenwood, Blyth and Carr, 1948) there is no doubt that dosing with 2-acetamidofluorene induced multiple primary epithelial tumours in 3 out of 5 birds treated. It is possible that tumours would have developed in the remaining 2 birds had they been allowed to live, for they both showed abnormalities of the liver and kidneys. When multiple tumours or concomitant neoplasia occurs in the human subject it is considered sufficiently unusual to merit reporting. A considerable number of papers are scattered throughout the pathological, surgical and obstetric journals, and it would be pointless to give details here, though it may be worthy of mention that the great majority of such reports do not discuss the implications of multiple primary tumours, except to speculate in a general way on " cancer-prone " individuals. Spontaneous multiple primary tumours also occur in the lower animals, but are rarely reported. In the experimental field, however, numerous cases have been put on record.

Concomitant primary tumours in poultry are quite rare (Olson and Bullis, 1942). The present paper, together with those of Bielschowsky and Green (1945), and Peacock and Peacock (1949, 1954) demonstrates that, as in mammals, it is possible to induce multiple primary tumours in various organs of the fowl by means of a carcinogen. Some of these tumours were adenomatous, others frankly malignant, as proved by remote metastases and implantations. Indeed in fowl No. 2 there were present metastases of two distinct histological types. The propagation by transplants from certain of these tumours was unsuccessful, a not unexpected result, since past attempts at the transplantation of epithelial tumours of the fowl were not any more encouraging. However, two liver tumours and one ovarian tumour were grown on the chorioallantoic membrane of chick embryos for two passages, but failed to take in fowls when subsequently implanted. This suggests that future attempts at propagating epithelial tumours in the fowl should be made in very young chicks, even one day old.

In the present state of knowledge, speculations as to the ultimate cause of carcinogenesis is merely an exercise in unprovable hypotheses, and indeed, as Nicholson (1950) points out, we can know nothing of first principles. But it is tempting to look a little more closely into the implications of the development of multiple discrete tumours in an organ or tissue following the application of a carcinogen. Why, for example, should not the whole liver become diffusely cancerous when the animal is exposed to 2-acetamidofluorene, since all the hepatic

cells are equally exposed over the period of administration? Instead, we see initial damage to the parenchyma, followed by regeneration, fibrous tissue proliferation, duct hyperplasia, and so on. This is succeeded by a gradual change in certain foci, where the cells pass insensibly from hyperplasia to neoplasia, forming the familiar nodules of a hepatoma, interspersed with apparently normal tissue (with the exception of a variable degree of cirrhosis). It seems reasonable to consider that these foci have arisen from cells differing fundamentally from all the remainder. Since they were presumably not originally different in either a pathological or embryonic sense (The Cohnheim-Ribbert doctrine being no longer tenable, as shown by Nicholson, 1950), the subsequent change may be due to the action of the carcinogen on a few cells in a particular and comparatively rare physiological state such as, for example, cell division. The mitotic rate is enormously increased following exposure of the liver to any substance which causes non-fatal damage, and a few of these dividing cells may undergo a particular mutation, bringing them to a precancerous phase. Such cells, exposed to the intermittent action of gonadotrophic hormones, may slowly differentiate through subsequent cell generations to true neoplastic cells no longer subject to the bonds of controlled physiological growth.

This is, of course, not an entirely new concept. It does, however, dispense with the cell-rest hypothesis, even now occasionally invoked to account for the focal nature of some tumours ; and it does embrace the various factors of extrinsic stimulus, chronic irritation, compensatory fibrosis, increased rate of cell division and hypertrophy, mutation, the pre-cancerous cell and intrinsic hormonal influence—all undoubtedly concerned in the initiation of the neoplastic state. Even the latent symbiotic " virus " as conceived by Rous (1936) could fit into the scheme. Lastly, it helps to explain why such simple organic substances such as chloroform or carbon tetrachloride are liver carcinogens or such unlikely inorganic compounds as zinc chloride or sulphate are capable of causing teratomata to develop from the pluripotential germ cells in the testes of cockerels, and why successful induction is subject to seasonal influence (Michalowsky, 1928, 1929), or the simultaneous administration of gonadotrophic hormones subsequent to the spring months (Bagg, 1936).

SUMMARY.

Multiple primary epithelial tumours were obtained in 3 out of 5 fowls more than 4 years subsequent to a period of oral administration of 2-acetamidofluorene in sesame oil. These were hepatomata and carcinomata of the oviduct and ovary. Some of the tumours were propagated for a short time on the chorioallantoic membrane of fertile eggs, but none were successfully maintained in young chickens. The remaining two fowls had cystic kidneys and liver cirrhosis when killed at the termination of the experiment. Controls dosed with sesame oil alone remained normal. The significance of the focal origin of tumours after exposure of tissues to a carcinogen is discussed, and a synthesis of known factors in carcinogenesis is suggested to account for the sequence of events leading to the formation of a tumour.

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REFERENCES.

BAGG, H. J.—(1936) *Amer. J. Cancer*, **26**, 69.
BEVERIDGE, W. I. B. AND BURNETT, F. M. (1946) Spec. Rep. Ser. med. Res. Coun., Lond., No. 256.
BIELSCHOWSKY, F. AND GREEN, H. N.—(1945) *Nature*, **156**, 780.
CAMPBELL, J. G.—(1945) *J. comp. Path.*, **55**, 308.—(1949) *Brit. J. Cancer*, **3**, 198.
GREENWOOD, A. W., BLYTH, J. S. S. AND CARR, J. G.—(1948) *Ibid.*, **2**, 135.
MICHALOWSKY, I.—(1928) *Virchows Arch.*, **267**, 27.—(1929) *Ibid.*, **274**, 319.
NICHOLSON, G. W. DE P.—(1950) 'Studies on Tumour Formation.' London (Butterworth & Co.).
OLSON, C. AND BULLIS, K. L.—(1942) *Bull. Mass. agric. Exp. Sta.*, 391
PEACOCK, P. R. AND PEACOCK, A.—(1949) *Brit. J. Cancer*, **3**, 289.—(1954) *Ibid*, **8**, 147.
ROUS, P.—(1936) *Amer. J. Cancer*, **28**, 233.
WILSON, R. H., DE EDS, F. AND COX, A. J.—(1941) *Cancer Res.*, **1**, 595.

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An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort ("Senecio jacobæa" Linn.), with special reference to the Induction of Liver Tumours with Seneciphylline

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VI.—An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort (*Senecio jacobæa* Linn.), with special reference to the Induction of Liver Tumours with Seneciphylline.*

By **J. G. Campbell,** British Empire Cancer Campaign Unit at the Poultry Research Centre, West Mains Road, Edinburgh, 9. *Communicated by* Dr A. W. GREENWOOD, C.B.E. (With Four Plates.)

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SYNOPSIS

The alkaloids of Ragwort (*Senecio jacobæa* Linn.) are known to be hepatotoxic to farm animals and man. Survivors invariably show permanent impairment of the liver, manifested by cirrhosis and its sequelæ. *Senecio* infusions may be consumed in small amounts in Britain as a herbal remedy, and in South Africa the Bantu frequently incorporate them in native medicines. It has been shown that these tribes have a high incidence of cirrhosis and primary liver cancer, and there is good evidence that their deficient diet may play an important part in sensitizing the liver to toxins. In the present study, the ragwort alkaloid seneciphylline has been administered by injection to fowls maintained on both adequate and deficient diets, and further fowls received the dried plant in their food. A significant proportion of these birds subsequently developed primary liver tumours. Although there was a higher mortality rate in both treated and controls fed the deficient diet, there was no evidence of a greater tendency to develop liver tumours compared with the birds on the balanced diet.

It is also suggested that the condition known as "cavernous angioma" of the liver of cattle and sheep in Britain may be due to ingestion of ragwort in sub-lethal amounts.

INTRODUCTION

The Senecios *as Poisonous Plants*

THE genus *Senecio* has a large number of species, probably in the region of 1250, and is widely represented throughout the world. These Compositæ have been of great interest to veterinarians, pathologists, chemists and pharmacologists ever since Gilruth (1903) drew attention to a hepatic cirrhosis in horses and cattle in New Zealand following the ingestion of *S. jacobæa*, or Ragwort. His article was rapidly followed by a report from South Africa by Chase (1904), who gave detailed notes on the clinical and pathological pictures in cattle, and these were augmented by Pethick

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(1906) in a report on Pictou disease, which he reproduced in feeding experiments, and by Robertson (1906) and Verney (1911), who described cirrhosis of the liver in cattle and horses of Cape Colony produced by two species of *Senecio*. Cushny (1910–11) followed with some experimental observations on the toxicology of *Senecio* alkaloids, and related his findings to those reported cases of hepatic cirrhosis in cattle variously known as Pictou disease (Nova Scotia), Molteno disease (South Africa) and Winton disease (New Zealand). Later, Cushny and Watt (1920) recorded an outbreak of ragwort poisoning in cattle in England. Other synonyms for seneciosis are Walking disease (N.W. Nebraska), Sirasyke (Norway) and Schweinsberger's disease (Germany).

Theiler (1918*a*) worked extensively on a disease known as Dunsiekte in horses in South Africa, and although he reproduced a condition closely resembling it by feeding a *Senecio* species, he remained unconvinced that they were etiologically identical. A number of reports followed in rapid succession, culminating in the classic work of De Koch, Du Toit and Steyn (1931), who reconciled Theiler's findings with their own undoubted experimental cases of *Senecio* poisoning in horses.

In Britain, the offending plant is *S. jacobæa*, or Ragwort, and cases of livestock poisoning have been recorded in veterinary literature by Cartwright (1936), Reynolds (1936), Bisset (1936), Ferguson (1940) and Lofthouse (1949). Both fresh and dried plants are toxic, but ensilage destroys the toxic factors (Vardiman, 1952). The symptoms and pathology of ragwort poisoning in horses and cattle are admirably detailed by Forsythe (1954), who comments that "when farmers and others can be brought to realize that ragwort alone probably causes more annual loss to the livestock industry than all the other poisonous plants together, then, and only then, will there be any diminution in the enormous quantities which grow in pastures throughout the country." This, despite the fact that ragwort is classed as a noxious weed, and landowners are obliged by law to eradicate it or at least to keep it under control.

The only reference in the literature to experimental administration of *Senecio* spp. to birds is by Dahme and Müller (1955), who fed young ducks on a normal diet + 10 per cent fresh *S. vulgaris* and *S. jacobæa*. This was a short-term experiment, and showed that the latter plant is more acutely toxic to the avian liver than *S. vulgaris* or groundsel. In man, outbreaks of poisoning or "seneciosis" were recorded in the Cape Province in 1920 and in Cape Town in 1931 (Selzer and Parker 1951). In both instances contamination of flour with *Senecio* spp. due to inadequate winnowing and sifting were thought to be the cause. Schoental (1954) believes there may be some connection between Kwashiokor in the Gold

Coast and the habit of drinking "bush tea"—a brew made from a variety of herbs possibly including *Senecio* spp. It is certain that the Bantu use *Senecio* infusions both internally and externally to treat a variety of conditions (Watt and Breyer Brandwijk 1932), and dried *S. jacobæa* is obtainable in Britain from many herbalists in the form of "Ragwort Tea" (Cook, Duffy and Schoental 1950), although inquiries in Edinburgh showed that the demand for this is small.

Since the end result of chronic poisoning with *Senecio* spp. in livestock and in man is cirrhosis, and liver cancer is so prevalent in the Bantu tribes (Berman 1941), it is not unreasonable to suppose there might be a common cause. Working on this hypothesis, some evidence has already been obtained by Cook, Duffy and Schoental (1950) and Schoental, Head and Peacock (1954) that certain of the *Senecio* alkaloids can be carcinogenic for the liver of the rat, and it is the purpose of this paper to confirm and extend these observations to the fowl.

Most of the early work on the pathological effects of *Senecio* alkaloids was comparatively short term, *i.e.* very few observations were extended beyond a period of three months. In veterinary medicine, animals developing symptoms of ragwort poisoning either die rapidly as a result of acute liver necrosis, or are slaughtered as soon as it is obvious that liver cirrhosis and ascites have developed. The only reported cases in man appear to have been rather acute, with a high mortality; present clinical data are not likely to indicate any relationship between chronic hepatitis due to eating the plants and the ultimate development of liver cancer.

The Alkaloids of Senecio jacobæa

Leonard (1950) lists 37 "*Senecio*" alkaloids derived from not only Compositæ but also from other families, *e.g.* Boraginacæa and Leguminosæ, whose alkaloids are very similar. Some of these alkaloids have since been shown to be identical. The genus *Senecio* is, however, the most important from the point of view of distribution, and numerous species are associated with livestock poisoning. *S. jacobæa* is plentiful as a pasture weed in parts of Australia, where Bradbury and Culvenor (1954 *a*) have isolated five alkaloids from it. These, in common with all other *Senecio* alkaloids, contain one nitrogen atom and usually eighteen carbon atoms. They are alkamine esters which, upon hydrolysis, give rise to a "necine" and a "necic acid", neither of which, according to Rose, Finck, Harris and Chen (1945), is hepatotoxic. One of the *S. jacobæa* alkaloids (Jaconine) is unique in that its molecule contains a chlorine atom (Bradbury 1954).

The five alkaloids are Seneciphylline (syn. Jacodine), Jaconine, Jacobine, Jacozine and Jacoline, and of these, Jacobine (Harris, Anderson and Chen 1942) is the only alkaloid which has been studied for toxic effects in pure form. Schoental, Head and Peacock (1954) used a mixture of alkaloids derived from *S. jacobæa*, probably mixed with *S. vulgaris* or groundsel, the chief alkaloid of which is Senecionine (Barger and Blackie 1937).

Materials and Methods

(A) *Extraction Procedure*

Ragwort (*S. jacobæa*) was harvested in the flowering stage in July and August every year from 1950 onwards, dried, usually in a room with heated floor, milled, and stored in boxes for further use. In the course of drying there was an 83 per cent loss of moisture. Usually the whole plant less the root system was milled, but various portions were occasionally extracted separately, *e.g.* stems and leaves, flower heads and roots, in order to obtain some idea of the distribution of the alkaloids. In one group of experiments the whole plant (less roots), after milling, was used in feeding experiments, whilst the remaining trials were with the principal alkaloid. After numerous trials a satisfactory method of extraction was developed, the following being a typical example.

(1) 4·65 kg. dried, milled ragwort, whole plant less the roots, collected in August and stored two years, was damped with 930 ml. of 10 per cent aqueous citric acid and extracted by continuous reflux with 96 per cent ethanol for five hours.

(2) The volume of the extract was reduced by removal of the ethanol by distillation under reduced pressure.

(3) An equal volume of N sulphuric acid was added, and solids (gums and resins) removed by filtration twice through paper pulp.

(4) The acid filtrate was thrice extracted with chloroform.

(5) The acid aqueous layer was rendered alkaline (pH 10) with ammonia.

(6) The precipitate was filtered off and dried over phosphoric oxide under reduced pressure.

(7) The alkaline filtrate was thrice extracted.

(8) The bulked chloroform washings were twice extracted with N sulphuric acid.

(9) The bulked acid extracts were made alkaline (pH 10) with ammonia, and the solid collected, dried and united to (6). By this method 4·028 g. crude mixed alkaloids were obtained.

(10) The crude alkaloids were stirred with cold ethanol (99 per cent), the residue was collected on a Buchner funnel, washed with small amounts of ethanol and dried over phosphoric oxide in a vacuum desiccator. The final yield was 1·9442 g. (0·042 per cent). The presence of alkaloids in the various extracts was continually checked by Mayer's reagent (mercuric potassium iodide), which gives a cream-coloured flocculent precipitate.

Once a satisfactory extraction was established, a large quantity (860 kg.) of ragwort was harvested and dried to a final weight of 147 kg. No other plants were included. This was extracted in an industrial extractor and the mixed alkaloids separated. The percentage yields (based on dry weight) from different parts of the plant were as follows: Flowers, 0·05 per cent), stems and leaves 0·014 per cent, roots 0·0038 per cent. The yield of alkaloids from dried ragwort (not roots) purchased from a herbalist was 0·02 per cent. On the grounds that the alkaloids may occur in the plants partially as N oxides, which are insoluble in chloroform and hygroscopic, Koekemoer and Warren (1951) detailed a method for alkaloid extraction giving much improved yields for various *Senecio* species (not *S. jacobæa*). This was tried, but failed to give a better yield. Bradbury and Culvenor (1954 *b*) have since reported their failure to find N oxides in ragwort.

Properties of the Alkaloids

The mixed alkaloids were obtained in small colourless hexagonal plates (Pl. I, fig. 1). Analysis gave an empirical formula of $C_{18}H_{25}O_6N$, m.pt. 218°–219° C. in a preheated bath (decomp.), and a similar result with slight decomposition in an evacuated tube. $(\alpha)_D$ was $-91°$ in 0·98 per cent solution in chloroform, and the R_F value 0·61 using 5 per cent acetic acid water/butanol as the mobile phase and iodine vapour as the printing agent. The hydrochloride gave sagitate bundles of spicular crystals. Table I gives comparisons with authentic specimens of jacobine and seneciphylline.

TABLE I

Alkaloid	Empirical Formula	M.pt. °C.	R_F Value	$(\alpha)_D$
Mixed	$C_{18}H_{25}O_6N$	218–219	0·61	− 91°
Jacobine . . .	$C_{18}H_{25}O_6N$	228	0·39	− 40°
Seneciphylline . .	$C_{18}H_{23}O_5N$	217	0·52	−139°

Mixed with an authentic sample of jacobine, the m.pt. was 215°–216° C. From these figures it seems clear that the sample was mainly seneciphylline (Bradbury, personal communication). This has been confirmed by X-ray

crystallographic studies, in which practically identical patterns were obtained from the mixed alkaloid and seneciphylline, but some marked differences were found when compared with jacobine (Beevers, personal communication). Most of the experiments to be detailed were, therefore, performed with seneciphylline admixed with a small amount of jacobine. The alkaloid was tested out for pharmacological effects on an anæsthetized cat (chloralose). The hydrochloride was given intravenously in molar solution, pH 6·8. In doses of 1, 7 and 17 mg. no effect was observed on the blood pressure. It did not antagonize the effect of adrenaline even after the cumulative dose of 25 mg. Higher dosage was not attempted.

(B) *Histological Methods*

Tissues were fixed routinely in Susa, except where frozen sections for the demonstration of fat were required. These were fixed in 10 per cent formal saline. After embedding in wax in the usual way, sections were cut at 5–8 μ and stained with hæmatoxylin and eosin. Masson's trichrome stain was used to demonstrate connective tissue, and Sudan IV was used on frozen sections to study the distribution of fat. Hæmosiderin deposits were stained by the potassium ferricyanide-HCl method, and the sections counterstained with safranin.

Toxicity Tests on Chicks

These were performed on 6-week-old chicks using an "Agla" micrometer syringe for the intravenous injection of an approximately molar solution of the alkaloids in N/10 hydrochloric acid (pH 6·8). The alkaloids are soluble with difficulty at this concentration but dissolve on gentle warming. Since 80 mg. jacobine per kilogram has been reported as fatal to mice (Harris, Anderson and Chen 1942), the dosages chosen ranged between 20 and 80 mg./kg.

From the results shown in Table II, although the numbers are small due to necessity in conserving material, it seems probable that the median lethal dose is about 40 mg./kg. live weight for young chickens.

Symptoms and Pathology of Acute Poisoning due to S. jacobæa *Alkaloids*

Within a few seconds of intravenous injection at the level of 80 mg./kg. the bird commences a distressed cheeping, followed by increased oral respiration. A fine muscular tremor develops, the bird shakes its head,

TABLE II.—TOXICITY TESTS WITH SENECIPHYLLINE HYDROCHLORIDE

No.	Sex	Wt. (g.)	Dose (mg.)	Result at 14 Days
Group A (20 mg./kg. intravenously)				
Z. 5322	♂	251	5·02	Survived
Z. 5272	♀	258	5·16	,,
B. 1311	♀	250	5·00	,,
B. 1320	♀	259	5·18	,,
Group B (40 mg./kg.)				
Z. 8781	♂	264	10·56	,,
B. 1245	♀	250	10·00	,,
B. 1313	♀	252	10·08	Died
B. 1288	♀	241	9·64	,,
Group C (80 mg./kg.)				
B. 1289	♂	265	21·20	,,
B. 1239	♀	246	19·68	,,
B. 1277	♀	248	19·84	,,
B. 1284	♀	239	19·12	,,

which then droops forward with eyes partially closed. At this stage the bird defæcates, and partly recovers, but remains in a crouching position, exhibiting intermittent colonic spasms affecting the legs and wings, causing it to jerk backwards. There is a temporary exaggerated response to stimuli such as noise or vibration. The pupil response is unaffected. Complete recovery occurs in 10 minutes. Usually within 36–48 hours the bird becomes lethargic and has a tense sensitive abdomen. Death follows rapidly after a period of coma. Post-mortem, such birds show pale, swollen firm livers, with no gross abnormality of other organs. Histological examination shows cloudy swelling, some necrobiosis of liver cells and swelling of the reticulo-endothelium. Other organs appear microscopically normal.

Short-term Tests with Sub-lethal Doses

For the purpose of these tests, six 6-week-old male chickens were divided into 3 groups, receiving intravenous injections of 35, 40 and 45 mg./kg. alkaloid as the hydrochloride, given at weekly intervals, until death or inanition terminated the experiment. Table III summarizes the results.

The preliminary trials having shown promise, the next experiment was planned to ascertain the effects of the prolonged administration of the alkaloid on birds maintained on adequate and deficient diets respectively. The particular deficiencies chosen were proteins and choline, on the

TABLE III.—PATHOLOGICAL CHANGES IN SUB-ACUTE POISONING WITH SENECIPHYLLINE HYDROCHLORIDE

No.	Sex	Wt.	Dose (mg.)	Date	Result
Group A (35 mg./kg.)					
B. 8690	♂	363	12·7	19/1/54	Firm liver—pale subcapsular area. Histology: marked unorganized bile-duct proliferation. Degeneration of liver cells, with compensatory hyperplasia. Sinusoidal congestion. Areas of necrosis. Testes: atrophy of interstitial cells, no spermatogenesis.
		442	15·4	26/1/54	
		479	16·7	2/2/54	
		577	20·0	10/2/54	
		Died 13/8/54. Total dose: 64·8 mg.			
B. 8848	♂	315	11·0	19/1/54	Liver and other organs appear normal. Histologically there is a marked bile-duct hyperplasia. Liver cell hypertrophy and regeneration especially around central veins. Dilated sinusoids. Testes atrophied but hyperplasia of interstitial cells. Other organs normal.
		396	13·8	26/1/54	
		443	..	1/2/54	
		Killed 1/2/54. Total dose: 24·8 mg.			
Group B (40 mg./kg.)					
B. 8570	♂	272	10·8	19/1/54	Ascites. Swollen "nutmeg" liver, with fibrinous exudate on capsule. Venous congestion. Undeveloped testes. Histology: massive interstitial hæmorrhage —breakdown of sinusoids. Liver cells — cytolysis, hypertrophy. Testis undeveloped. Sertoli cell hyperplasia.
		332	13·2	26/1/54	
		382	15·2	2/2/54	
		472	18·8	10/2/54	
		Died 12/2/54. Total dose: 58·0 mg.			
Z. 8191	♂	285	11·4	19/1/54	Pale swollen liver. Slight atrophy adrenals and testes. Histology: liver cell hypertrophy, swollen endothelial cells. Adrenal: lymphoid foci. Testis: tubular atrophy with interstitial cell hyperplasia.
		352	14·0	26/1/54	
		375	..	1/2/54	
		Killed 1/2/54. Total dose: 25·4 mg.			
Group C (45 mg./kg.)					
B. 8739	♂	257	11·3	19/1/54	Ascites. Firm yellow-brown puckered liver. Undeveloped testes. Histologically the liver shows very active bile-duct hyperplasia and hypertrophy of liver cells, nuclei and nucleoli (Pl. I, fig. 2). Nodular hyperplasia—cytolysis —hæmorrhages.
		278	12·5	26/1/54	
		286	12·8	2/2/54	
		312	14·0	10/2/54	
		Died 11/2/54. Total dose: 50·6 mg.			
B. 8806	♂	270	12·2	19/1/54	Congested firm liver. Marked atrophy of adrenals and testes. Histologically the liver shows venous congestion, liver cells hypertrophied. The testes show interstitial atrophy and tubular degeneration. Small hæmorrhages in Virchow-Robin space surrounding the cerebral vessels (Pl. I, fig. 3).
		315	14·2	26/1/54	
		331	..	1/2/54	
		Killed 1/2/54. Total dose: 26·4 mg.			

assumption that similar inadequacies are an important factor in development of liver cirrhosis (Gillman, Gillman, Mandelstam and Gilbert 1954).

The experiment was commenced on forty 7-week-old chickens, with equal numbers of each sex. Those in Group I were fed the normal balanced ration of the Centre, whilst those in Group II received the following formula:

		Vitamin and Mineral Supplements	
Maize meal	50·0 parts	Potassium iodide	0·6 mg./100 g.
Wheat bran	10·0 ,,	Manganese sulphate	0·0 ,,
Grass meal	5·0 ,,	Riboflavin	0·4 ,,
Bone meal	2·5 ,,	Calcium (d) pantothenate	0·5 ,,
Ground limestone	0·5 ,,	Vitamin B_{12}	1·0 g./100 g.
Salt	0·5 ,,	Choline chloride	60·0 mg./100 g.
Casein	2·5 ,,	Vitamin A	530 I.U./100 g.
Dextrin	28·0 ,,	Vitamin D	90 I.U./100 g.

The composition of this diet is as follows:

		Normal Requirements
Crude protein	8·7 per cent.	20·0 per cent.
Calcium	1·0 ,,	1·0 ,,
Phosphorus	0·6 ,,	0·6 ,,
Riboflavin	5·5 p.p.m.	4 p.p.m.
Pantothenic acid	11·6 p.p.m.	11 p.p.m.
Choline	0·88 mg./g.	1·54 mg./g.

Half the birds in Group I, *i.e.* 5 males and 5 females, received weekly intravenous injections of alkaloid at the rate of approximately half the median lethal dose for periods up to 8 weeks or until death. Where death occurred early in the experiment, other birds were substituted. The remainder served as controls. The birds in Group II were similarly treated. At the end of the period of treatment the survivors were placed under observation for an indefinite period. The alkaloids were administered initially at the rate of 35 mg./kg., but the dose was reduced to 20 mg./kg. after the second injection since it was apparent that the birds were reacting too severely. The results are detailed below.

Results

Group 1 Adequate Diet. Males

M. 130; duration 20 days; 3 weekly injections = 48·0 mg. total wt. alkaloids; wt. gain 106 g. *Autopsy.* Ascites; atrophied liver; prominent or cystic ducts. Histologically, several small nodules composed entirely of bile-duct epithelium (Pl. I, fig. 4), also generalized bile-duct hyperplasia and cholangiectasis.

M. 97; duration 13 days; 2 weekly injections = 32 mg. total wt. alkaloids; wt. loss 20 g. *Autopsy.* Liver small, yellow-brown, congested veins. Histologically, subacute liver atrophy.

B. 9725 (replacement); duration 32 days; 5 weekly injections = 70·6 mg. total wt. alkaloids; wt. gain 256 g. *Autopsy.* Atrophied liver, wt. of bird 1260 g., wt. of liver 28 g. Histologically, fairly normal apart from high cellularity, rather small liver cells.

C. 275; duration 67 days; 6 weekly injections (one week missed because of small weight gain) = 91·2 mg. total wt. alkaloids; wt. gain 425 g. *Autopsy.* Ascites, atrophied liver. Small pale mass present at hilum of gall-bladder. Hypertrophied heart, general venous congestion. Histologically, small hepatomata, each surrounded by pseudo-capsule of compressed liver cells; tumour adjacent to gall-bladder apparently a bile-duct adenocarcinoma (Pl. I, fig. 5); some regions show profound periportal fatty change, and numerous extravasculæ accumulations of mononuclear cells.

C. 984 (replacement); duration 24 days; 3 weekly injections = 28·4 mg. alkaloids. *Autopsy.* Small accessory tag of liver tissue attached to capsule of right liver lobe; liver slightly swollen and pale. Histologically, cloudy swelling of liver parenchyma, with early necrotic changes.

Females

C. 255; duration 20 days; 3 weekly injections = 48·4 mg. total wt. alkaloids; wt. gain 141 g. During experiment ascites developed, 50 ml. clear yellow fluid withdrawn from abdomen; anæmia; blood transfusion, 5 ml. normal whole blood + 2 ml. 6·0 per cent dextrose on two consecutive days prior to death. *Autopsy.* Ascites, severely atrophied liver. Histologically, acute liver atrophy and necrosis; emboli of necrotic cells present in the central veins; thickened liver capsule.

C. 234; duration 84 days; death hastened by accidental starvation; 6 weekly injections (one week omitted) = 82·5 mg. alkaloid; wt. gain 404 g. at time of last injection. *Autopsy.* Liver atrophied, firm and mottled; small (6 mm.) pale nodule present in apical portion of left liver lobe. Histologically, rapidly growing cholangioma (Pl. I, fig. 6 and Pl. II, fig. 1); also advanced portal cirrhosis (Pl. II, fig. 2). Other regions of liver show several small relatively poorly differentiated hepatomata; emboli apparently viable hepatoma cells in central veins.

C. 304; duration 45 days; 4 weekly injections (1 week omitted = 61·7 mg. alkaloid. Wt. gain at last injection 236 g. followed by rapid loss in weight and death. *Autopsy.* Firm brown atrophied liver, prominent ducts. Histologically, hypertrophy of liver cells, biliary hyperplasia.

At the time of preparation of this report, there are in this Normal diet group 5 survivors (3 male and 2 female), which are being kept under observation; 4 of these have had 6 weekly injections, and the remaining male 4 injections.

Group II Deficient Diet. Males

C. 269; duration 79 days; 5 weekly injections = 88·4 mg. alkaloids (1 week omitted); wt. gain 395 g. *Autopsy.* Ascites; multiple nodular liver tumours; anterior portions both lobes swollen, firm and pale, with prominent subscapular vessels (Pl. II, fig. 3); no extrahepatic metastases. Histologically, multiple hepatomata characterized by great variation in nuclear sizes (Pl. II, fig. 4). Some areas dividing cells with features typical of the more malignant hepato-cellular carcinomata.

C. 246; duration 8 days; 2 weekly injections = 36·9 mg. alkaloid; wt. gain 6 g. *Autopsy.* Atrophied, mottled liver; gall-bladder distended with colourless bile. Histologically, liver showed acute atrophy and cellular infiltration.

C. 297; duration 234 days; 5 weekly injections over 6 weeks = 71·1 mg. alkaloid, wt. gain 272 g. over injection period. Killed, undersized but apparently healthy. *Autopsy.* Atrophied, firm liver exhibiting mottled irregularly shaped slightly raised areas; gall-bladder small; testes under-developed; right ventricle of heart hypertrophied. Histologically, numerous hepatomata and large foci of hæmorrhagic necrosis.

C. 303; duration 231 days; 6 weekly injections over 7 weeks = 90·5 mg. alkaloid; wt. gain 242 g. Killed *in extremis.* *Autopsy.* Emaciated, various skeletal deformities; atrophied liver showing widely disseminated dark red areas; large full gall-bladder; testes under-developed. Histologically, liver shows many foci of widely dilated sinusoids containing large basophilic cells and red blood cells in various stages of development. Similar foci in kidneys.

C. 280; duration 14 days; 2 weekly injections = 30·4 mg. alkaloid; wt. loss 22 g. *Autopsy.* Yellowish red-flecked atrophied liver; gall-bladder distended with colourless fluid. Histologically, considerable foci of pure duct hyperplasia; caudal extremity of left liver lobe completely transformed into cholangiomatous tissue (Pl. III, fig. 1).

B. 9739 (replacement); duration 26 days; 2 weekly injections = 25 mg. alkaloid; wt. gain 1 g. Killed in emaciated condition. *Autopsy.* Yellow-coloured atrophied liver and undeveloped testes. Histologically, fatty change and acute necrosis of liver.

C. 990 (replacement); duration 3 days; 5·4 mg. alkaloid. *Autopsy.* Cloudy swelling, liver.

Females

C. 302; duration 69 days; 6 weekly injections over 7 weeks = 67·7 mg. alkaloid; wt. gain 262 g. *Autopsy*. Fatty liver. Histologically, profound fatty change, liver cell necrosis and cytolysis, interstitial infiltration with macrophages and other mononuclear cells; kidney similar.

C. 256; duration 174 days; 5 weekly injections over 6 weeks = 74·7 mg. alkaloid; wt. gain 251 g. *Autopsy*. Liver atrophied, yellowish and firm; nephritis. Histologically, fibro-reticular hyperplasia with heavy infiltration eosinophils, macrophages and mononuclears, sufficiently intense in one lobe to simulate myeloid leukæmia; leucocythæmia; reticulum-celled hyperplasia spleen, with many myeloblasts and myelocytes; tubular nephritis.

C. 300; duration 15 days; 3 weekly injections = 34·7 mg. alkaloid; wt. gain 84 g. Death partly due to cæcal coccidiosis. *Autopsy*. Ascites, firm yellow patchily congested liver. Histologically, extreme sinusoidal congestion. Some sinusoids dilated, others ruptured or coalesced with those adjacent to form blood "lagoons"; also duct hyperplasia.

Summarizing the results of these injection experiments, it will be seen that in the group receiving an adequate diet, out of the 12 birds treated, 8 subsequently died, 5 male and 3 female, and of these 2 male and 1 female had primary liver tumours. In the group fed a deficient diet, out of 12 birds, 10 died, 7 male and 3 female, and of these, 3 males developed tumours.

Of the controls, none died in the adequate diet group and two died in the deficient diet group, one showing symptoms of paralysis which could not be accounted for by post-mortem examination, and the other was emaciated but with histologically normal organs. None of the birds thrived on the deficient diet, some developing swollen joints and overgrown and malformed beaks and claws, but they made remarkable progress when finally placed on the normal ration.

Feeding Experiment.—Dried and milled ragwort (whole plant except the roots) was incorporated in the standard chick mash used at the Centre. The proportion originally chosen was 7·5 lb. of plant to 100 lb. of mash, which was considered, on the basis of extraction studies, to provide the bird with roughly 1 mg. alkaloids/day/bird. However, this was found to be too toxic, and after a month the proportion was reduced by one-half. For the purposes of the experiment, a total of 43 six- to eight-week-old chickens, containing approximately equal numbers of both sexes, were fed the mash for about 14 weeks. All birds dying during and subsequent to the period of administration were submitted to post-mortem and histological examinations, the results of which are detailed below.

Males

C. 7783; duration 21 days. General venous congestion, hydrops pericardii. Histologically, congestion and hæmorrhages in liver and kidneys.

C. 7817 and C. 7789; duration 24 days. Both birds had general venous congestion and hydrops pericardii; C. 7789 also had ascites. Hæmorrhagic necrosis, liver; tubular nephritis; degeneration, adrenal medulla; ulcers in horny lining of gizzards.

C. 7771; duration 47 days. Buried under litter, when found was unfit for examination.

C. 7768; duration 52 days. Discrete pale, slightly raised mass near right liver lobe apex (Pl. III, fig. 2); kidneys congested; petechiæ in intestinal mucosa; gizzard ulcers. Histologically, nodular hyperplasia, bizarre liver cells, compression of hyperplastic bile-ducts and coarse connective tissue trabeculæ between nodules; swollen sinusoids, forming blood "lagoons"; areas of fatty change and necrosis; hæmosiderin pigment fairly plentiful. The apical liver lesion consists of a hyaline mass in the thickened capsule, covering a hepatoma composed of branching columns of closely packed cells, with no detectable ducts (Pl. III, fig. 3).

C. 7755; duration 53 days. Atrophied liver, distended gall-bladder; general venous congestion, mucoid enteritis, and petechiæ at œsophagus-proventriculus junction. Histologically, massive necrosis of one liver lobe; remainder of organ congested, cloudy swelling, and fibro-reticular hyperplasia. Pancreas, advanced patchy necrosis; degenerative changes in adrenal medulla.

C. 8406; duration 91 days. Ascites; swollen leukæmic-looking liver; blood picture normal. Histologically, advanced fatty change of liver cells, which exhibit pleomorphism and great variation in nuclear and nucleolar size. Ducts moderately hypertrophied.

C. 7739; duration 91 days. Killed, ascites. Histologically, thickened capsule and advanced hyperplasia of ducts, which varies from apparently undifferentiated structures to cholangiectatic cysts. Sinusoids swollen, often disrupted to form blood "lagoons"; liver cells fatty, hypertrophied and pleomorphic.

C. 7792; duration 96 days. Ascites; sero-fibrinous exudate covering atrophied right liver lobe. Histology similar to the preceding case, plus large eosinophilic inclusions in liver-cell cytoplasm.

C. 8741; duration 147 days. Firm and nodular liver, thickened capsule. Histologically, several solid ductless hepatomata, compressing closely packed cords composed of smaller liver cells.

M. 1824; duration 155 days. Firm atrophied liver, hydrops pericardii with adhesions; yellow tumour (?) at caudal pole of right testis. Histologically, advanced hyperplasia of ducts; bile-stained liver cells adjacent to main ducts; nodular hyperplasia of liver cells verging on true hepatoma but still containing ducts. Right testis showed massive necrosis (infarct?) of one extremity; slight seminiferous activity in remainder of gland.

C. 8376; duration 157 days. Cirrhotic liver containing one small encapsulated hepatoma.

Females

C. 7819; duration about 42 days. Buried in litter, unfit for examination.

C. 7763; duration 59 days. Atrophied liver, thickened ligaments, distended gall-bladder. Bile-stained fluid in abdomen; mucoid enteritis; gizzard and proventriculus atonic. Histologically, hyaline degeneration thickened liver capsule; liver cells pleomorphic with loss of normal structure, and only few ducts; parenchymatous nodular hyperplasia in parts.

C. 8630; duration 68 days. No evident abnormality apart from distended crop; liver histology normal.

C. 7735; duration 69 days. Atrophied liver with areas of necrosis. Post-mortem change prevented critical examination.

C. 7795; duration 96 days. Ascites; atrophic right liver lobe, containing small greyish nodules; thickened capsule. Histology, pleomorphic hyperplastic liver cells, fatty change and eosinophilic inclusions in cytoplasm. Moderate duct proliferation. Blood "lagoons" from coalescent distended sinusoids and scattered foci lymphoid cells in sections of right lobe.

C. 8394; duration 124 days. Gross intraparenchymatous liver hæmorrhages, hæmosiderin-laden cells surrounding blood "lagoons".

C. 7798; duration 147 days. Normal-sized liver, containing numerous tumours (Pl. III, fig. 4). Histologically, distinct areas of liver-cell (Pl. IV, fig. 1) and bile-duct carcinoma (Pl. IV, fig. 2); tumorous liver cells heavily laden with hæmosiderin (Pl. IV, fig. 3) and necrotic in one region; zones of fatty change, small patches of lymphoid infiltration; portal tracts fibrosed (Pl. IV, fig. 4).

C. 8384; duration 166 days. Atrophied liver; small fusiform body resembling adrenal tissue attached to splenic vein. Liver histology, venous congestion, cloudy swelling, fatty change; extravascular accumulations of plasma cells and lymphocytes. The small body was an accessory pancreatic nodule, very rich in islet tissue.

C. 8369; duration 184 days. Killed *in extremis*. Thickened liver capsule; left lobe normal apart from numerous foci lymphoid cells; right lobe, biliary hyperplasia, hypertrophied pleomorphic liver cells; foci of eosinophils and lymphocytes.

Reviewing the results of the feeding experiment, 21 birds have died in a total of 43 which received the ragwort/chick mash mixture. The dead birds included 12 males and 9 females, and of these 3 males and 1 female developed primary liver tumours. Of the 12 males, 3 died soon after the experiment began, from acute liver damage due to the ragwort-mash ratio being too high.

A summary of all the results for both administration of alkaloid in the pure form and in the dried plant is given in Table IV.

TABLE IV

Injection of Alkaloid	Total treated	Deaths		Liver Tumours	Controls Surviving
		Male	Female		
Group A (normal diet) .	12 (7 ♂, 5 ♀)	5	3	2 ♂, 1 ♀	10/10
Group B (deficient diet) .	12 (7 ♂, 5 ♀)	7	3	2 ♂, 1 ♀	8/10
Feeding experiment .	43 (23 ♂, 20 ♀)	12	9	3 ♂, 1 ♀	..

SUMMARY AND CONCLUSIONS

The hepatotoxic property of the alkaloids of ragwort (*S. jacobæa*) is, of course, well known and needs no further comment except to remark that the pathological features of poisoning in the fowl prove to be essentially similar to those in mammals. True cirrhosis, however, is not such a common finding in the avian liver as, *e.g.*, in the horse, cattle or man. This may simply be due to the normal scanty fibro-reticular framework of the avian liver rather than a species difference in reactivity of the connective tissue to irritants, since the capsule, which is normally quite well defined, does become thickened in cases of chronic hepatitis. Apart from this variation in liver reaction to toxins, which incidentally is shared with mice but apparently not rats (Chen *et al.* 1940; Cook *et al.* 1950), the main pathological changes in fowls receiving repeated sub-lethal doses are fatty change, necrosis, sinusoidal congestion and disruption of the endothelium forming hæmorrhages and "blood lagoons". These latter appear to be essentially similar to the lesions described by Theiler (1918 *b*) and others in Dunsiekte, and by Harris, Anderson and Chen (1942) in small laboratory

animals. The foregoing sequence is followed by regeneration, with the formation of hyperplastic liver-cell nodules and a very intense bile-duct proliferation. Such changes, in a percentage of cases, merge imperceptibly into true neoplasia. The marked proliferation of endothelial cells in the liver, as observed by Davidson (1935) in the rat, and confirmed by Selzer *et al.* (1951) and Schoental and her colleagues (1954) with other *Senecio* alkaloids, was not apparent in the fowl. Bull (1955) suggests that the typical histopathology of the liver in natural cases of poisoning with pyrrolizidine alkaloids in horses and sheep may be pathognomic. He bases this on the findings of hepatic cell megalocytosis, and the presence of inclusion globules in the cytoplasm of such cells. In the opinion of the present author, however, such findings are common during regeneration in any liver which has been damaged by toxins. In all cases, males were more susceptible than females to the effects of the alkaloid, and exhibited a higher rate of mortality. They also showed a greater tendency to develop ultimate liver tumours.

Males did not stand up so well to the low protein and low choline diet as females, but birds on this diet, plus the alkaloid, did not show any greater tendency to develop liver tumours than those maintained on a normal diet plus treatment with the alkaloid. The deficient diet alone did not appear to cause liver damage.

Of the two methods of administration of the alkaloid, that in which it was given intravenously as hydrochloride was the most successful in the induction of liver tumours.

Hæmosiderin deposits were only found in birds fed dried ragwort admixed with the diet. In two of these cases, one male and one female, tumours were present; in the third case, a female, the deposits were unassociated with neoplastic change.

All birds, dosed by whatever route, failed to attain sexual maturity, and it appeared that the male gonads were more severely affected than those of the female. The occurrence of adrenal lesions in several of the treated birds is of interest in view of the report by Sapeika (1952) that pterophine, a *Senecio* alkaloid, produced a decrease of ascorbic acid in that gland in rats, although carbon tetrachloride, another hepatotoxic agent, did not.

Attention must be drawn to the extraordinary speed of development of benign bile-duct tumours in male birds M. 130 and C. 280. These received the alkaloid by injection, the first on the adequate and the second on the deficient diet. The experiment was terminated by death after only three and two injections respectively, and both showed small purely cholangiomatous masses which had developed in 20 and 14 days. In the

remainder of the birds developing tumours, three, occurring in female C. 7798 and males C. 269 and C. 275, could be classified on histological criteria as malignant, but even in these cases metastases could not be found in organs other than the liver. The remainder were either hepatomata or cholangiomata, although female C. 234 had numerous emboli of tumour cells within the central veins, possibly indicating early intrahepatic metastases.

In birds killed several months after the termination of injections or the cessation of feeding, the livers show little tendency to return to normal. It is therefore hoped that some of the survivors may eventually develop an extrahepatic metastasising liver tumour.

Finally, the carcinogenic properties of several *Senecio* alkaloids have been amply confirmed by the present observations and those of the Glasgow team of workers. Liver tumours have been reported in rats following the administration by drinking water of the mixed alkaloids of *S. jacobæa*, by retrorsine and isatidine from South African *Senecio* spp., and by monocrotaline, a pyrrolizidine alkaloid obtained from *Crotallaria* sp. (Cook, Duffy and Schoental 1950; Schoental, Head and Peacock 1954; Schoental and Head 1955). The present observations add a further alkaloid, possibly two, namely seneciphylline, containing traces of jacobine, isolated from *S. jacobæa*. In the light of these findings, serious consideration must be given to the possibility that the intermittent consumption of *Senecio* alkaloids as a constituent of medicinal infusions may be a dangerous practice, causing eventual liver damage, and occasionally primary cancer of that organ. From the veterinary aspect the hepatotoxic properties are well recognized, but there is a possibility that liver cancer in cattle, and particularly in sheep, may be a sequel to sub-lethal doses of the alkaloids in the form of ragwort-contaminated pasture, and that the fairly common cavernous angiomata in the livers of cattle may be a manifestation of ragwort poisoning comparable to the "blood lagoons" as described in Dunsiekte.

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References to Literature

Barger, G., and Blackie, J. J. (1937). *J. Chem. Soc.*, 584.
Berman, C. (1941). *S. Afr. J. Med. Sci.*, **6**, 11.
Bisset, H. N. (1936). *Vet. Rec.*, **48**, 1488.
Bradbury, R. B. (1954). *Chem. and Ind.*, 1022.
Bradbury, R. B., and Culvenor, C. C. J. (1954 *a*). *Chem. and Ind.*, 1021.
——, —— (1954 *b*). *Aust. J. Chem.*, **7**, 378.
Bull, L. B. (1955). *Aust. Vet. J.*, **31**, 33.
Cartwright, C. W. (1936). *Vet. Rec.*, **48**, 817.
Chase, W. H. (1904). *Rep. Chief Vet. Surg. C.G.H.*, **G41**, 19.
Chen, K. K., Harris, P. M., and Rose, G. L. (1940). *J. Pharmacol.*, **68**, 130.
Cook, J. W., Duffy, E., and Schoental, R. (1950). *Brit. J. Cancer*, **4**, 405.
Cushny, A. R. (1910–11). *J. Pharmacol.*, **2**, 531.
Cushny, A. R., and Watt, H. E. (1920). *Lancet*, ii, 1089.
Dahme, E., and Müller, B. (1955). *Berl. Münch. Tierärztl. Wschr.*, **68**, 101.
Davidson, J. (1935). *J. Path. Bact.*, **40**, 285.
De Koch, G., Du Toit, P. J., and Steyn, D. G. (1931). *Rep. Vet. Res. S. Afr.*, **17**, 617.
Ferguson, H. N. (1940). *Vet. Rec.*, **52**, 758.
Forsythe, A. A. (1954). "British poisonous plants", *Bull. Minist. Agric. Lond.*, **161**, 47.
Gillman, J., Gillman, T., Mandelstam, J., and Gilbert, C. (1945). *Brit. J. Exp. Path.*, **26**, 67.
Gilruth, J. A. (1903). *Rep. Dep. Agric. N.Z.*, **11**, Appendix V, p. 228.
Harris, P. N., Anderson, R. C., and Chen, K. K. (1942). *J. Pharmacol.*, **75**, 78.

KOEKEMOER, M. J., and WARREN, F. L. (1951). *J. Chem. Soc.*, 66.
LEONARD, N. J. (1950). *The Alkaloids:* I, p. 107. (Editors, MANSKE, R. F., and HOLMES, H. L.) Academic Press, New York.
LOFTHOUSE, F. (1949). *Vet. Rec.*, **61**, 115.
PETHICK, W. H. (1906). *Special Report on Pictou Cattle Disease.* (Dep. Agric., Ottawa, Canada.)
REYNOLDS, A. (1936). *Vet. Rec.*, **48**, 1407.
ROBERTSON, W. (1906). *J. Comp. Path. Ther.*, **19**, 97.
ROSE, C. L., FINCK, R. D., HARRIS, P. N., and CHEN, K. K. (1945). *J. Pharmacol.*, **83**, 265.
ROSENFELD, I., and BEATH, O. A. (1945). *Amer. J. Clin. Path.*, **15**, 407.
SAPEIKA, N. (1952). *Brit. J. Exp. Path.*, **33**, 223.
SCHOENTAL, R. (1954). *Brit. Med. J.*, i, 335.
SCHOENTAL, R., and HEAD, M. A. (1955). *Brit. J. Cancer*, **9**, 229.
SCHOENTAL R., HEAD, M. A., and PEACOCK, P. R. (1954). *Brit. J. Cancer*, **8**, 458.
SELZER, G., and PARKER, R. G. F. (1951). *Amer. J. Path.*, **27**, 885.
SELZER, G., PARKER, R. G. F., and SAPEIKA, N. (1951). *Brit. J. Exp. Path.*, **32**, 14.
THEILER, A. (1918 *a*). *Rep. Dir. Vet. Res. S. Afr.*, **5/6**.
—— (1918 *b*). *Rep. Dir. Vet. Res. S. Afr.*, **7/8**.
VARDIMAN, P. H. (1952). *J. Amer. Vet. Med. Ass.*, **121**, 397.
VERNEY, F. A. (1911). *J. Comp. Path. Ther.*, **24**, 226.
WATT, J. M., and BREYER BRANDWIJK, M. C. (1952). *The Medicinal and Poisonous Plants of South Africa*, p. 198. E. & S. Livingstone, Edinburgh.

DESCRIPTION OF PLATES

PLATE I

Fig. 1. Seneciphylline crystals. Partly polarized light. ×20.
Fig. 2. B. 8739 ♂ Biliary hyperplasia. H. & E. ×300.
Fig. 3. B. 8806 ♂ Hæmorrhage into Virchow-Robin space. Cerebrum. H. & E. ×300.
Fig. 4. M. 130 ♂ Cholangioma. H. & E. ×300.
Fig. 5. C. 275 ♂ Bile-duct adenocarcinoma. H. & E. ×60.
Fig. 6. C. 234 ♀ Rapidly growing cholangioma (carcinoma?). H. & E. ×45.

Plate II

Fig. 1. C. 234 ♀ Higher magnification of Pl. I, fig 6. Note mitotic figures. H. & E. ×60.

Fig. 2. C. 234 ♀ Portal cirrhosis, with small islands of surviving liver cells H. & E. ×60.

Fig. 3. C. 269 ♂ (Deficient diet.) Liver showing multiple hepatomata and possibly liver cell carcinoma. Ten months after last injection. × .

Fig. 4. C. 269 ♂ Section from above, showing carcinomatous appearance. H. & E. ×550.

Plate III

Fig. 1. C. 280 ♂ (Deficient diet.) Cholangiomatous tissue after only 14 days. Masson's trichrome stain. ×120.

Fig. 2. C. 7768 ♂ Liver. (Feeding experiment.) Natural size.

Fig. 3. C. 7768 ♂ Section of hepatoma shown above. H. & E. ×550.

Fig. 4. C. 7798 ♀ Liver. (Feeding experiment.) ×⅔.

Plate IV

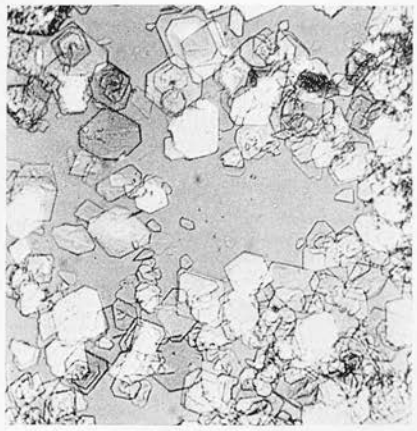
Fig. 1. C. 7798 ♀ Showing liver cell carcinoma. H. & E. ×300.

Fig. 2. C. 7798 ♀ Another region showing bile-duct carcinoma. H. & E. ×300.

Fig. 3. C. 7798 ♀ Hæmosiderin deposits in liver cells. H. & E. ×300.

Fig. 4. C. 7798 ♀ Portal cirrhosis showing connective tissue encircling small nodules of liver cells. Masson's trichrome stain. ×300.

(*Issued separately May* 31, 1956)



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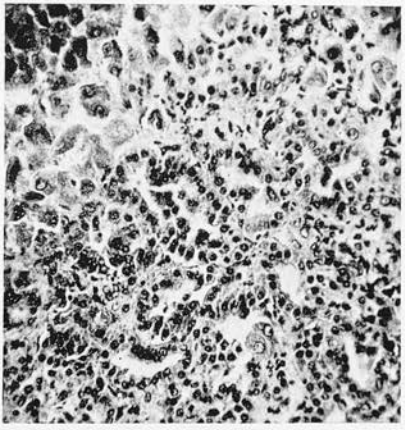

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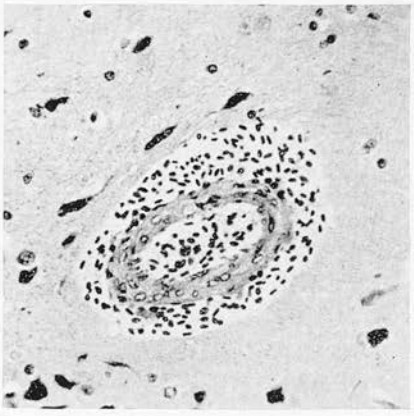

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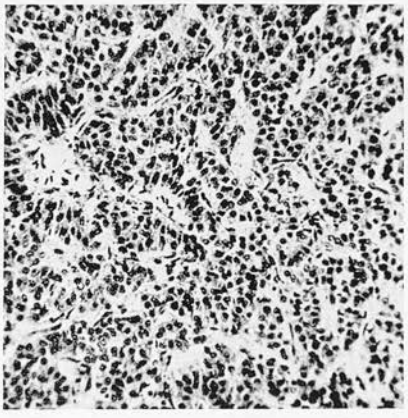

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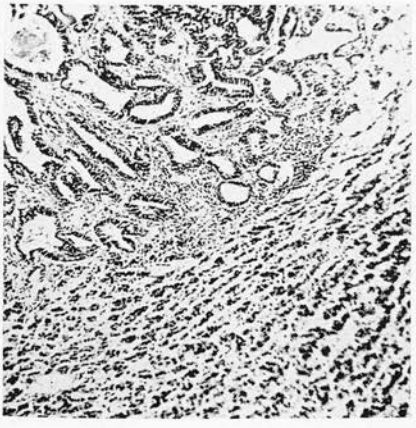

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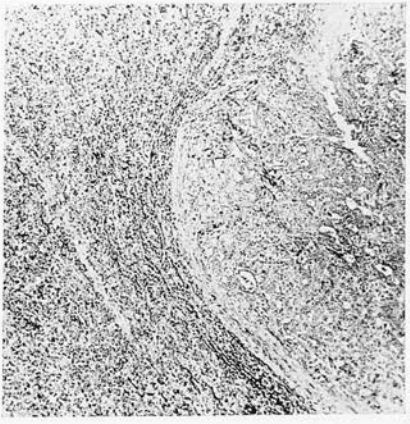

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FIGS. 1–6

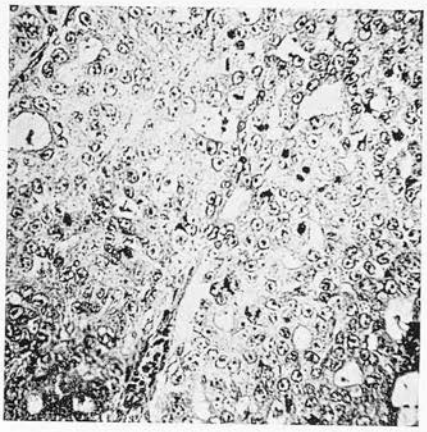


FIG. 1

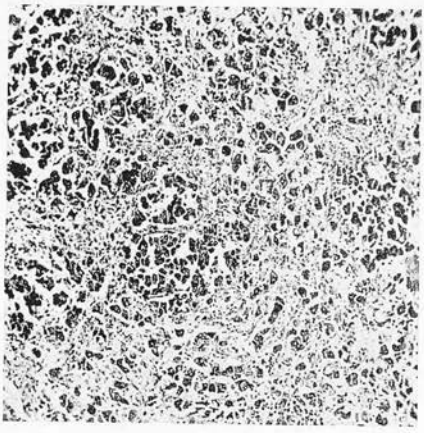


FIG. 2

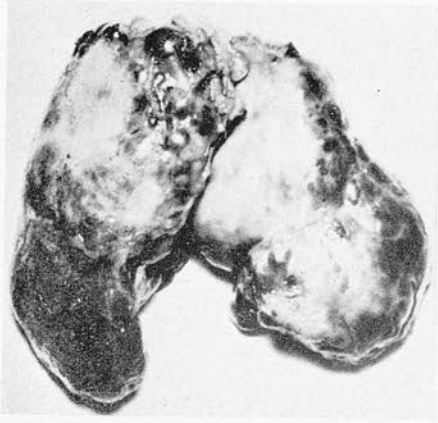


FIG. 3

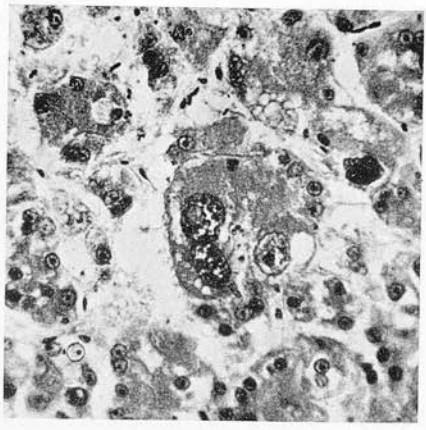
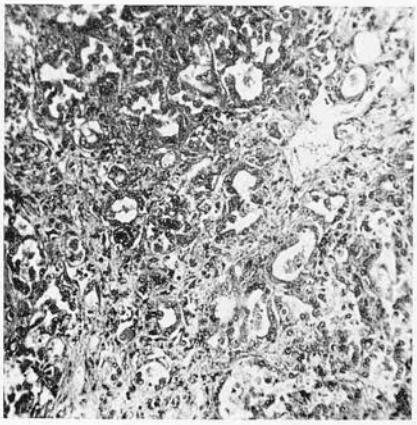


FIG. 4



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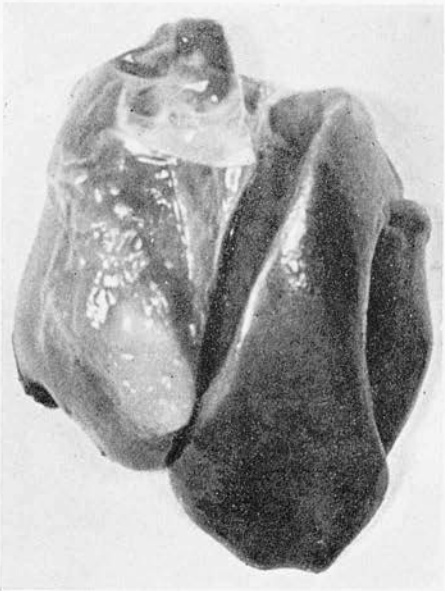

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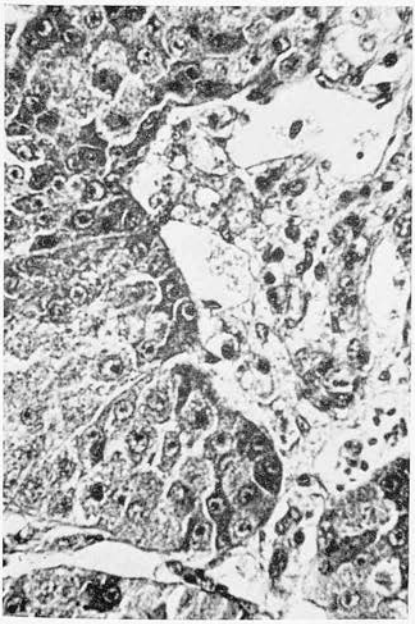

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FIGS. 1–4

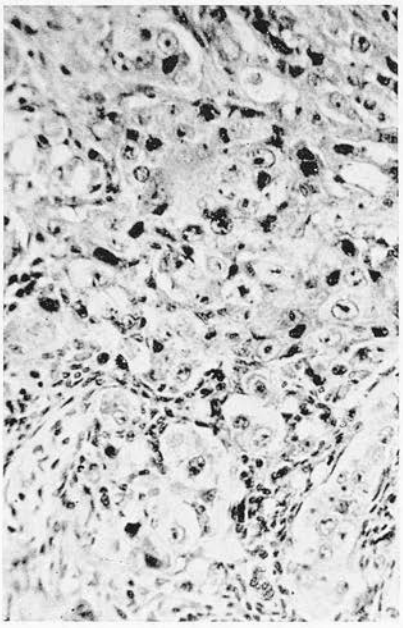

FIG. 1

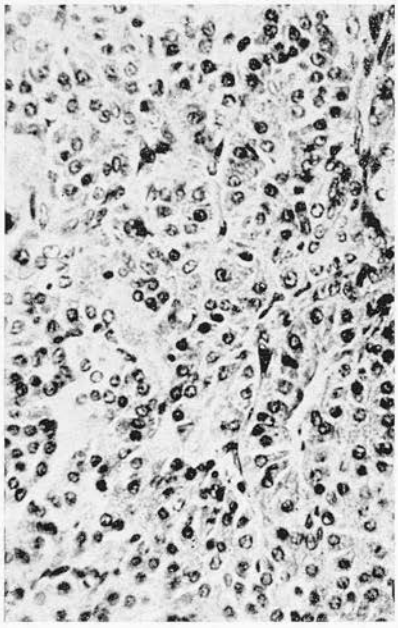

FIG. 2

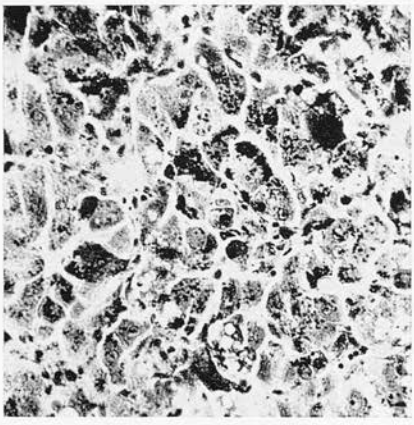

FIG. 3

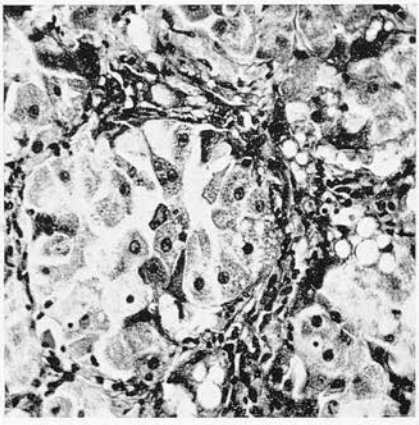

FIG. 4

Leucosis and Fowl Paralysis Compared and Contrasted

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THE leucotic complex of diseases in the fowl has long been the subject of controversy among pathologists, who find themselves in acute difference over the question of the inclusion of fowl paralysis within the complex. A brief glance at the history of these diseases will help to understand how this divergence of opinion has arisen. When fowl paralysis was first recorded in Britain by Galloway (1929) it was described as a disease entity characterised primarily by paralytic symptoms in immature fowls associated with typical infiltrations of various peripheral nerves by cells of the lymphoid series. This was in accord with reports of the disease from various parts of the world, and the name neurolymphomatosis gallinarum was proposed and generally adopted.

It was known from the outset that nerve lesions were not the only manifestation, and the terms visceral lymphomatosis and ocular lymphomatosis were given to the other forms.

It is as well to realise that long before the recognition of fowl paralysis in its various guises, the conditions now known as the leucoses were familiar to pathologists. These conditions, now classified as lymphoid leucosis, myeloid leucosis and erythroleucosis, were not found to be associated with nerve lesions, although later studies have shown that on rare occasions an invasion of nerves with cells of these different series may occur. In view of the undoubted malignancy of the various leucoses, this is not surprising.

The form of fowl paralysis called visceral lymphomatosis bears some gross resemblance to the discrete form of lymphoid leucosis, and it is this similarity which caused various workers, especially in the United States of America, to include all forms of fowl paralysis in the leucotic complex. Because of this, visceral lymphomatosis and lymphoid leucosis were believed by some to be synonymous, and by implication had a common aetiology. Many workers on this side of the Atlantic accepted this as a working classification, tending to overlook certain discrepancies. Indeed, it must be admitted that there was no great harm apparent in this from the viewpoint of the poultry farmer and the busy diagnostic laboratories. But it was obvious to some that this classification meant that lymphoid leucosis, whether discrete (lymphocytoma) or diffuse (lymphosarcoma), was synonymous with not only the visceral form of fowl paralysis, but with the neural and ocular forms also, none of these being neoplastic conditions. Not only this, but the other forms of leucosis were also included, and a tendency became evident to report all these conditions simply as fowl paralysis.

The situation was not clarified as research into the aetiology of these various conditions continued, and it became apparent that myeloid and erythroleucosis were associated with a closely similar if not identical filterable agent or "virus," comparable, but not apparently identical with the "virus" Rous fibromyxosarcoma of fowls, so well-known to cancer research workers all over the world. Lymphoid leucosis, on the other hand, has not been shown to have an associated filterable agent except by Burmester and his colleagues, and some doubt has been raised in the minds of a few British workers that the Burmester transmissible lymphoid tumour is in fact comparable with lymphoid leucosis, but is rather an atypical erythro or myeloid leucosis.

If, in fact, the visceral form of fowl paralysis (visceral lymphomatosis) and lymphoid leucosis are distinct conditions with a different aetiology and mode of transmission, the controversy is resolved, since it is evident that the rival schools are reporting results for the particular condition with which they happen to be working. It is the purpose of this paper to present evidence that this may be the case, and that the various forms of fowl paralysis should be considered apart from the leucotic complex.

It is obviously not possible in the short time at our disposal to go fully into the pros and cons of this controversy, but the reader may find most of the necessary data in Biester & Schwarte (1952) and in a paper by Campbell (1954).

Seasonal and Age Difference

Attention has already been drawn to the fact that the leucoses were known in Britain for a considerable time prior to the first recorded case of fowl paralysis. On the whole, fowl paralysis affects a younger age group of birds than leucosis, and in fact it shows a significant seasonal variation, which leucosis does not. The peak months for fowl paralysis are August to October, and since the majority of fowls are spring hatched, and the available evidence shows that the birds are infected during the first week or so in life, this peak gives us a guide to the incubation period before clinical paralysis or other associated lesions appear, *i.e.* about five to eight months. This is, in fact, the age when most cases occur, although there now seems to be a tendency for even younger birds to develop symptoms. Leucosis, on the other hand, usually affects an older age group, when the birds are already in full production.

Differences in Pathology

The leucoses are neoplastic conditions affecting haematopoietic tissue. As such, they are divisible into lymphoid, myeloid, and erythroleucosis. Each of these may be sub-divided into diffuse and discrete forms, in descending order of malignancy. In addition, the diffuse forms may show a true leucaemia, though this is usually terminal. The exception is erythroleucosis, which is nearly always leucaemic and only rarely occurs as aleucaemic diffuse or discrete forms. These leucoses show all the accepted criteria of malignancy such as rapid proliferation, uniformity of cell type with undifferentiated or relatively immature cells, infiltrations and destruction of invaded tissues. In addition, at least two of the

leucoses, namely erythro and myeloid leucosis, are associated with a filterable agent or "oncogenic virus," whilst the third, or lymphoid leucosis, is only doubtfully so associated, as we have already seen.

In the neural form of fowl paralysis, the early lesion consists of oedema of the nerve, followed by an infiltration with typical chronic inflammatory cells, mainly mature lymphocytes and plasma cells, which may be so severe as to destroy all normal nerve structure. A somewhat similar process occurs in the development of the visceral lesions, although usually the earliest sign is a minute focus of fibrinous necrosis, followed by an infiltration with lymphocytes, plasma cells, and even cells of the granular series. At this stage the lesion closely resembles an early pullorum disease focus. The mature lymphocytes rapidly aggregate at the site and finally a "tumour" or rather, a lymphogranuloma, is produced. This lesion bears a gross resemblance to the lymphocytoma of discrete lymphoid leucosis, but does not typically behave in a malignant manner. Figs. 1 to 8 compare fowl paralysis lesions with similar organs affected by lymphoid leucosis.

Although these lymphogranulomatous "tumours" are usually associated with nerve lesions in any particular case, they occasionally occur in the apparent absence of neural infiltration. That these cases can with practice be differentiated from lymphoid leucosis tumours is well shown in a study by J. E. Wilson and myself comparing results obtained from independent diagnosis based on gross *post-mortem* findings with the results of histopathological examinations. In a large series of *post-mortem* examinations, several such cases have been found, with excellent agreement as to their nature. It would of course be extraordinary if an exception to a general rule never occurred in any biological study, and on rare occasions a malignant transformation of fowl paralysis lesions has been observed, terminating in frank lymphosarcomata. This is not unexpected, however, being simply an example of the occasional conversion of a chronic inflammatory lesion to a true neoplasm. The fundamental difference in their beginnings, however, probably constitutes the best evidence for separation of fowl paralysis and associated conditions from the leucoses, which are neoplastic diseases from the outset.

Other points of difference may briefly be detailed, such as the absence of marrow or blood changes in fowl paralysis, or the practically invariable absence of nerve involvement in the leucoses. The production of fowl paralysis following inoculation with material from a leucosis case has occasionally been reported, also the converse, but the possibility of spontaneous disease confusing the issue was not by any means eliminated. The great majority of workers, and the writer is one, have never succeeded in transmitting fowl paralysis in any form following the injection of many different tissues or filtrates derived therefrom into fowls of widely different ages, whereas it is common experience that the leucoses may be transmitted in this way, although most would agree that lymphoid leucosis is difficult to transmit by cell-free material.

To sum up. It is held that fowl paralysis is typically a chronic disease associated with inflammatory infiltrations in the nerves and viscera, and a progressive though not neoplastic accumulation of cells of the lymphoid series which eventually produce tumour-like lymphogranulomata. There is no blood or marrow involvement, and no conclusive evidence that leucosis has ever resulted following attempted transmission experiments with fowl paralysis material or vice versa. The epidemiology of fowl paralysis shows a number of significant differences when compared to the leucoses, which are true neoplastic diseases of haemotopoietic tissue.

References

BIESTER, H. E., & SCHWARTE, L. S. (1952). Diseases of Poultry. Iowa State College Press. 3rd Edn.

CAMPBELL, J. G. (1954). Tenth World's Poultry Congress. Sect. C. 1.

GALLOWAY, I. A. (1929). *Proc. Roy. Soc. Med.* xxii, 1167.

WILSON, J. E., & CAMPBELL, J. G. (1956). Unpublished data.

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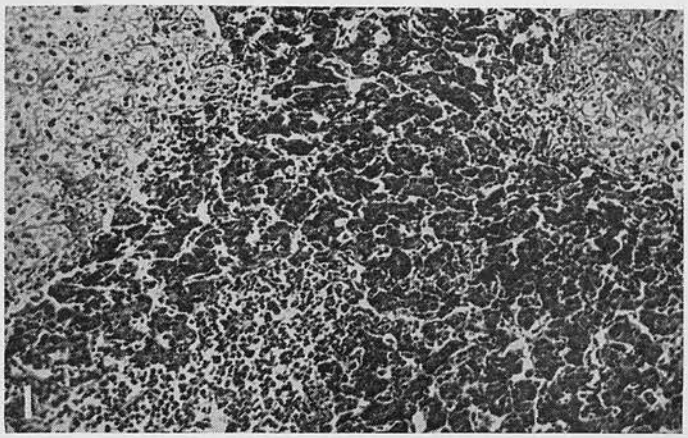

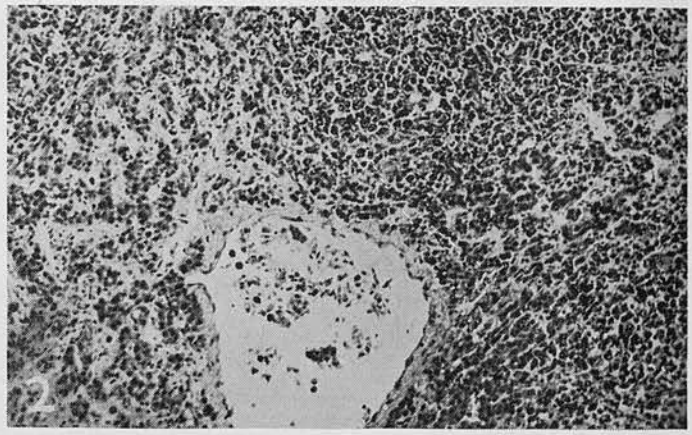

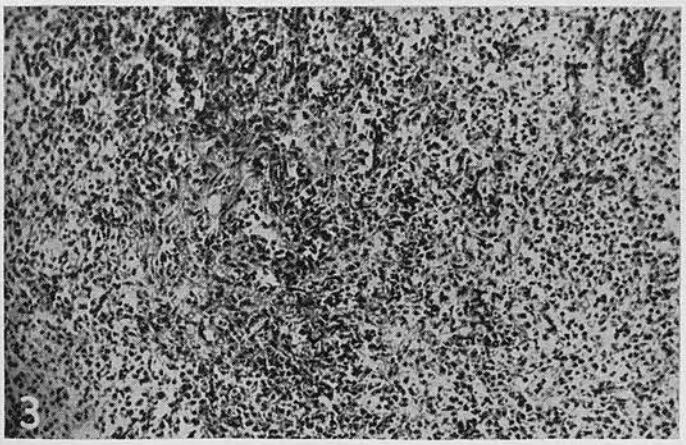

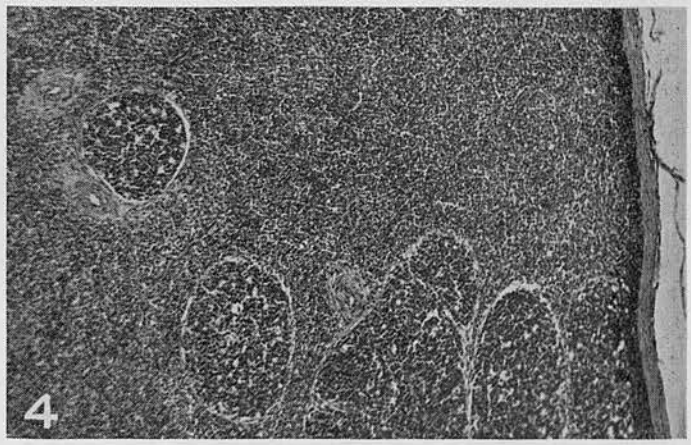

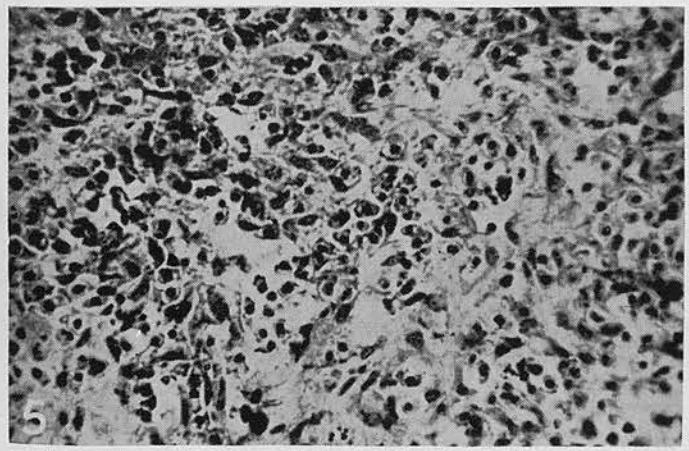

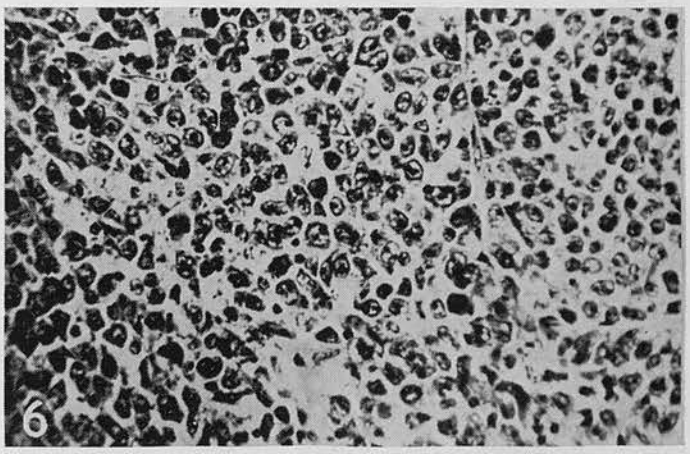

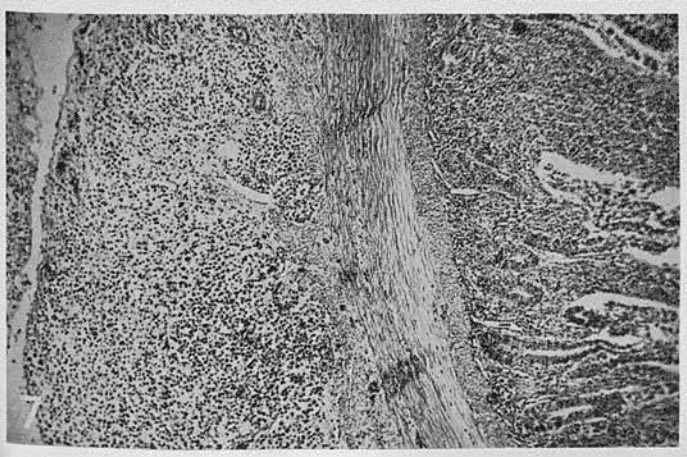

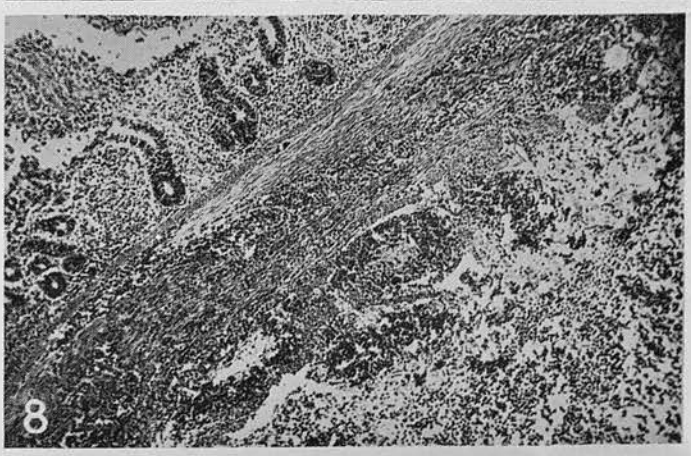

FIG. 1.—Early visceral fowl paralysis, liver. Two regions of fibrinous necrosis in upper part of photograph, and infiltration of lymphoid cells into an older lesion below. H. & E. × 130.

FIG. 2.—Diffuse lymphoid leucosis, liver. Note terminal leucaemia. H.&E. ×130.

FIG. 3.—Early fowl paralysis lesion, spleen. H. & E. × 130.

FIG. 4.—Early lymphoid leucosis, spleen. Explosive multi-centric growth in follicles. H. & E. × 110.

FIG. 5.—Typical early lymphogranuloma, fowl paralysis, ovary. H.&E. ×325.

FIG. 6.—Lymphoid leucosis, ovary. H. & E. × 325.

FIG. 7.—Lymphogranulomatous deposits on serosa of intestine. There is no tendency to invade the underlying musculature. H. & E. × 110.

FIG. 8.—Lymphoid leucosis. Tumour deposits on serosa of gut, showing invasion of muscle coats. H. & E. × 110.

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TWO CASES OF RHABDOMYOSARCOMA IN THE FOWL

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PRIMARY tumours of striated muscle are extremely rare in domesticated animals judging from the scarcity of case reports in veterinary pathology. A substantial proportion of these, seven cases in all, were found in the fowl, and it is the purpose of this paper to review these cases briefly and to describe two further examples of this interesting neoplasm.

The earliest record appears to be by Peyron and Blier (1927) who described a transplantable rhabdomyoma originating in the leg of a cockerel. This was followed by Babic (1931) who gave a brief account of a rhabdomyoma in the pectoral muscle of a young pullet. Makower (1931) cited a case recorded by Reitsma in which multiple tumours occurred in the muscles of the maxilla, thorax and abdomen of a young cockerel. Eber and Malke (1932) described multiple rhabdomyomata in the breast muscle of a hen. Meyer, cited by Feldman (1932), reported a multiple tumour arising in the pectoral muscle of a young cockerel, and finally Olson and Bullis (1942) gave an account of two such tumours in the leg muscles of an eight-month-old male and a ten-month-old pullet respectively. All these tumours occurred in the skeletal muscles of young fowls, a reversal of the situation in the human subject, where rhabdomyomata of such muscles are extremely rare. The two cases to be described conform to the previous reports of these tumours in fowls in that they also had their origins in skeletal muscle. They represent the first instance of this tumour in over 5000 spontaneous cases of neoplasia examined to date, a relative incidence of 0·04 per cent. The tumours were found in two out of a group of three young (14 week) chickens sent from the same source to a routine diagnostic laboratory for report. Material, consisting of breast muscle, ovary, segment of duodenum and pancreas, liver, spleen, kidney, bone marrow and various portions of peripheral nerves, was fixed in formal-saline and sent to the writer for microscopical examination and report. Gross examination of the tissues as received showed in the first case tumorous thickening of the duodenal wall, pancreatic involvement, multiple ovarian tumours, and several portions of muscle containing tumours. On section, one of the latter, which measured 25 × 15 × 15 mm., was seen to be a firm, greyish ovoid mass whose cut surface showed an appearance suggestive of fibroma. The second case was represented by duodenum and pancreas, liver, spleen, kidneys, marrow and portions of nerves, but only the gut and pancreas showed visible abnormality, similar in appearance to that from the first chicken. Blocks from all this material were post-fixed in susa, sections cut at 8μ, and stained with H. & E., a Picro-Mallory method, and Mallory's phosphotungstic acid haematoxylin. Glycogen stains were, of course, not possible by the time the nature of the tumour was appreciated.

Microscopic structure

The muscle tumours consisted of irregularly disposed interlacing bundles of elongated cells with eosinophilic cytoplasm and large strap-like nuclei containing finely particulate chromatin. Cross sections of the cells showed the nuclei to be mainly peripherally situated. A certain degree of cellular polymorphism was apparent, and the terminal cytoplasmic margins often had a ragged or flame-like appearance. Anastomosis between cells was occasionally noted. Although not evident in the H. & E. preparations many of these cells possessed cross striations, not always completely traversing the cell, which were plainly visible in the P.T.A.H. preparations (Fig. 1). Many of the cells also showed a fine fibrillary structure of the cytoplasm, or occasionally a lattice-like appearance. Cells cut transversely showed radially arranged striae enclosing a paler centre. Intermixed with these elements were regions of fairly dense collagenous tissue or cellular tissue with a mesenchymatous appearance. The whole tumour was richly supplied by blood capillaries. At the periphery, mature muscle formed a pseudo-capsule.

The tissue ensheathing the wall of the gut was composed of less differentiated polymorphic cells, having cytoplasmic spurs and processes, pale nuclei and prominent basophilic nucleoli. The fibrillary nature of the cytoplasm was again evident especially in transverse sections (Fig. 2). These cells lay in a matrix of rather dense connective tissue and were actively invading the pancreas and the muscular coats of the intestine (Fig. 3).

The ovarian tumour showed similar cytology, with many binucleate cells and occasional giant cells with hypertrophic nuclei. Mitotic figures were rare (Fig. 4).

In the second case, the tumour was only represented in the preparation from the duodenum and pancreas, the remaining organs being normal. However, despite the absence of a primary tumour, the histology is exactly comparable to that of the first case, with fibrillary cytoplasm, marginal hatching or fully developed radial striae in cross sections of the invading cells.

DISCUSSION

It must be assumed that the probably small primary tumour of the skeletal muscle was missed in the second case. The site of the primary in the first case is interesting, since skeletal muscle origin is extremely rare in man, the most common sites being bladder, prostate, vagina, spermatic cord, epididymis and palate, where striated muscle is normally absent (Willis, 1953). In view of this, and the almost invariable confinement of these tumours to young subjects, together with the presence in such tumours of mesenchymal tissue, Willis con-

EXPLANATION OF PLATE

FIG. 1.—Tumour in breast muscle, showing cross striations in the cells. Phosphotungstic acid-haematoxylin. × 550.

FIG. 2.—Rhabdomyoblasts lying in connective tissue matrix in the tumour investing the duodenum. Note pleomorphism and the peripheral beading of the cells cut transversely. P.T.A.H. × 550.

FIG. 3.—Rhabdomyosarcoma cells invading the circular muscle coat of the duodenum. H. & E. × 60.

FIG. 4.—Structure of the ovarian tumour, showing pleomorphic and binucleate rhabdomyoblasts. H. & E. × 550.

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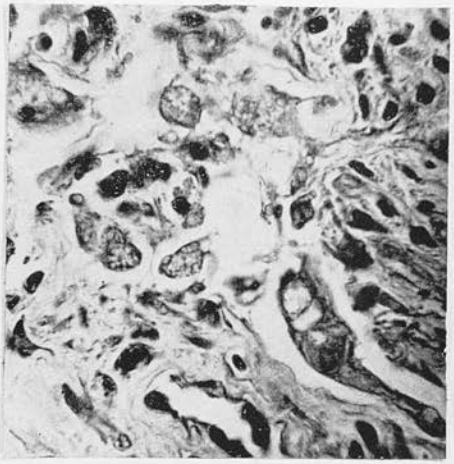

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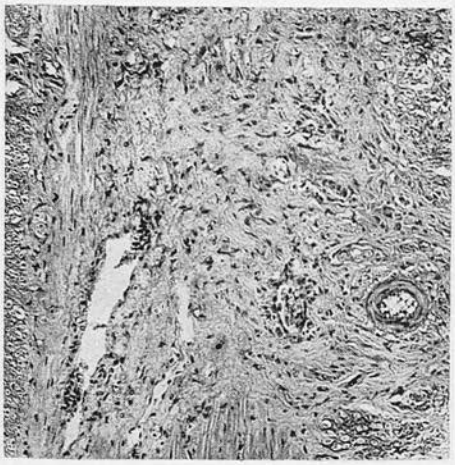

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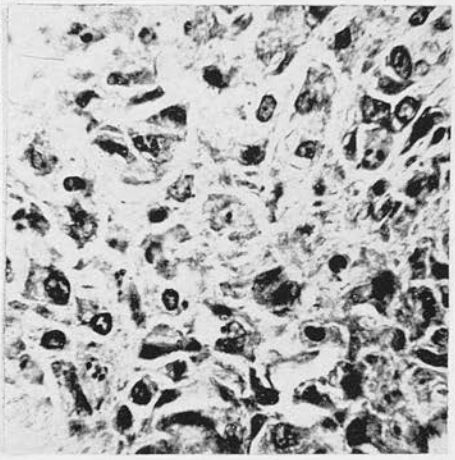

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cludes that the usual source is not adult muscle tissue, but either embryonic myogenic cells or undifferentiated mesenchyme with aberrant striated muscle producing tendencies. This suggests that such cells are almost exclusively confined to the sites listed above in the young human subject. However, in the cases under consideration the known site of origin of one tumour was skeletal muscle, and the site in the second case was probably similar, in view of the invariably reported skeletal muscle origin in the earlier literature. It may therefore be concluded that cells with rhabdomyoblastic potencies occur occasionally in the breast or leg muscles of the young fowl. In this connection it would be interesting to compare the regenerative capacity of avian voluntary muscle after injury, with that in mammals, where the muscle cell has lost its power of regeneration and any breach is made good with fibrous tissue. Comparative pathologists are familiar with the phenomenon of persistence of embryonic characteristics in avian tissues well beyond the first few days of life. Hepatic extra-medullary haematopoiesis, the occurrence of mixed tumours such as histiocytic and myeloblastic sarcoma, or erythroblastoma and fibro-sarcoma, the not uncommon embryonal tumours of the kidney and carcino-sarcoma of the female reproductive tract, are well known examples. Another instance, recently discovered in this Unit, is that under certain conditions, erythroleukaemia virus can initiate renal sub-capsular papillary adenomata if the chick is not older than twelve days when infected (J. G. Carr, 1956). This too, can be reasonably explained by the assumption that in the mesodermally derived kidney, embryonic elements with a wide capacity for differentiation persist for a short while after hatching.

SUMMARY AND CONCLUSIONS

Two cases of rhabdomyosarcoma in the fowl are reported, bringing the total in the literature to nine. All these cases arose in skeletal muscle. In view of the undoubted embryonic nature of the tumour it is concluded that mesenchymal tissue with myoblastic potentiality sometimes persists in the breast and leg muscles of young fowls. It is indicated that this is a further example of avian tissues retaining in immediate post-embryonic life an embryonic capacity for wider than normal differentiation, and that such undifferentiated tissue may give rise, under certain stimuli, to neoplasia.

REFERENCES

Babic, I.—(1931) *Vet. Archiv.*, **1**, 158.
Carr, J. G.—(1956) *Brit. J. Cancer*, **10**, 379.
Eber, A. and Malke, E.—(1932) *Z. Krebsforsch.*, **36**, 178.
Meyer, cited by Feldman, W. H.—(1932) 'Neoplasms of Domesticated Animals'. Philadelphia (W. B. Saunders Co.)
Olson, C. and Bullis, K. L.—(1942) *Bull. Mass. agric. exp. Sta.*, No. 391, 36.
Peyron, A. and Blier, J.—(1927) *Bull. Ass. franç., Cancer*, **16**, 516.
Reitsma, cited by Makower, L.—(1931) *Rev. Path. comp.*, **31**, 703 *et seq.*
Willis, R. A.—(1953) 'Pathology of Tumours', p. 757. London (Butterworth & Co. Ltd.)

NOTE ADDED IN PRESS.

A further case, occurring in the cervical muscles of a pullet, and bringing the total to 10, is detailed in "Tumeurs Spontaneés des Animaux de Laboratoire," p. 141 and Plate 103, by M. Guerin (1954). Amédée Legrand & Cie., Paris.

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STUDIES ON THE INFLUENCE OF SEX HORMONES ON THE AVIAN LIVER

I. SEXUAL DIFFERENCES IN AVIAN LIVER CLEARANCE CURVES

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(*Revised manuscript received 25 February* 1957)

SUMMARY

1. A modified method of determining the avian liver clearance of intravenously administered sodium bromosulphthalein (BSP) is described.
2. Using this test, a sexual difference has been observed in the clearance gradients over the periods 5–10 and 10–15 min after injection of BSP. This appears to be determined by sex hormones, in that males caponized by stilboestrol possess a female type of clearance.
3. Surgically caponized birds appear to occupy an anomalous position which can be variously interpreted as showing female or male trends, according to the particular clearance gradients selected. Androgen, however, causes a clear reversion to the male type of excretion.
4. High initial BSP concentrations in the plasma of certain females have been correlated with a relatively smaller blood volume in proportion to body weight increase.

The various methods of evaluating liver function by clearance tests do not appear to have been applied to the fowl. One of these tests is based on the capacity of the liver cell to extract sodium bromosulphthalein (BSP) from the blood and excrete it into the bile, and was introduced by Rosenthal & White [1925]. During the course of experiments to investigate the action of oestrogen in cases of acute and chronic liver damage caused by ragwort (*Senecio jacobaea* L.), it became desirable to study the impairment of liver function, but it was found necessary to modify the BSP method for this purpose. The object of this article is to describe these modifications and to present details of preliminary results obtained in the application of the test to normal mature male, female and castrated fowls.

MATERIALS AND METHODS

The birds used were healthy first-year Brown Leghorns from the flock maintained at this Centre. Sodium BSP (L. Light and Co. Ltd.) in distilled water was injected intravenously, and blood was withdrawn at definite intervals into a heparinized syringe. It was found that the dose of 2–5 mg/kg body weight, recommended in human clinical medicine, did not yield a sufficiently high concentration to enable dependable plasma readings to be obtained. Eventually, a dose of 20 mg/kg was established as satisfactory, the dye concentration being 20 mg/ml. water.

5 ml. of blood was withdrawn from the brachial vein for each estimation, performed on most occasions at 5, 10, 15 and 25 min subsequent to the injection of dye. According

to Pino, Weiss & Sturkie [1951], complete mixing of a substance introduced into the blood stream of the fowl takes at least 2 min, and so the first readings were made at 5 min in order to eliminate the possibility of that particular error. Blood samples were placed in graduated centrifuge tubes and spun at 1200 *g* for 15 min, and the plasma was pipetted into 3 ml. glass cuvettes for the determination of plasma blanks. These usually remained constant in individual fowls over the period of the experiment, indicating absence of haemolysis. Colour was developed by adding 4 drops of N-NaOH to each 3 ml. plasma, and readings were made with a Unicam S.P. 400 absorptiometer set at 580 mμ, previously determined on a standard prepared by dissolving 35·3 mg BSP in 100 ml. plasma, and adding NaOH to 3 ml. as above. This represents approximately the concentration of the dye (20 mg/kg) at zero time if thoroughly mixed with the blood in a fowl weighing 1·3 kg (having a plasma volume of about 85 ml.).

Laying hens sometimes yield an opalescent plasma due to lipo-proteins which may interfere with the test. Usually, fasting overnight overcame this difficulty. Capons were produced either surgically by removal of the testes or chemically by the implantation of diethylstilboestrol pellets (50 mg) at the base of the skull. Such birds, too, sometimes gave opalescent plasma which could often be cleared by fasting. A similar implantation technique was used for methyl testosterone pellets.

RESULTS

(1) *Normal fowls*

Fig. 1 shows that the typical male and female BSP clearance curves have a different shape. The male curve has a steep gradient, which levels off fairly abruptly at about 10 min. The female type, on the other hand, is characterized by a less steep gradient,

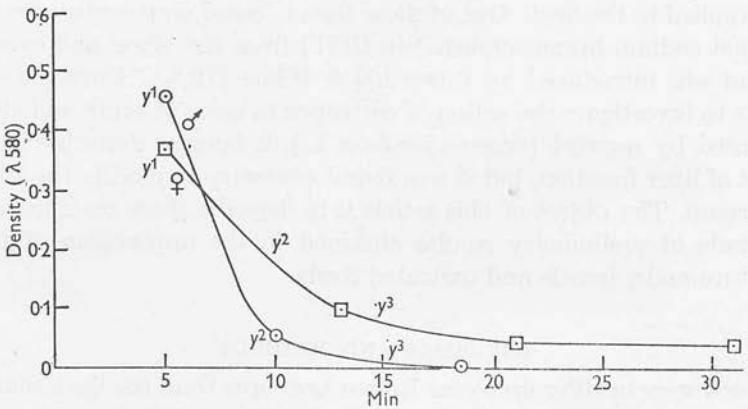

Fig. 1. Liver clearance of sodium BSP in the normal male and female fowl.

which levels off less rapidly. For the purposes of statistical examination, gradients were computed for the clearance curves of four males and five females, over two periods, 5–10 and 10–15 min, on a simple straight line basis, i.e. the gradients of the straight lines joining the two points of observation. Since the time intervals are equal, the gradients can be taken simply as the difference between the ordinates of the curves at the particular points 5 min (y^1) to 10 min (y^2), and 10 min (y^2) to 15 min (y^3).

Thus gradient 5–10 min $= y^1 - y^2$ and gradient 10–15 min $= y^2 - y^3$. In most cases observations were taken at 5–10–15 min, so the gradients were computed from the actual observations. In the exceptions, the ordinates at 5–10–15 min were interpolated from the graphs. Table 1 sets out the results which are corrected for plasma blanks.

The differential behaviour of males and females can be subjected to an analysis of variance, the gradients being used as the variable (18 values). Table 1 also gives the results of this analysis.

Table 1. *Sex difference in liver clearance gradients of sodium bromosulphthalein (BSP) in fowls*

	Min after BSP				Gradients	
Males	5	10	15	25	5–10	10–15
(1)	0·685	0·195	0·10	0·09	−0·490	−0·095
(2)	0·515	0·11	0·06	0·02	−0·405	−0·050
(3)	0·63	0·14	0·125	0·08	−0·490	−0·015
(4)	0·49	0·14	0·115	0·095	−0·350	−0·025
					Av. −0·434	−0·046 = 89 % change
Females						
(1)	0·37	0·175	0·075	0·05	−0·195	−0·100
(2)	0·69	0·21	0·055	0·04	−0·480	−0·155
(3)	0·38	0·17	0·11	0·08	−0·220	−0·060
(4)	0·81	0·46	0·29	0·23	−0·350	−0·170
(5)	0·41	0·10	0·03	0·005	−0·310	−0·070
					Av. −0·311	−0·111 = 64 % change

	Analysis of variance of gradients	
	D.F.	Mean square
(*a*) Difference between series	1	0·003738
(*b*) Difference between periods	1	0·361250
(*c*) Interaction series and periods	1	0·039062
(*d*) Difference between birds of same sex	7	0·008309
(*e*) Error	7	0·003066

The mean square for interaction (*c*), which is of particular interest, is highly significant at the 1 % level, and confirms that there is a characteristic excretion pattern for males and females. The insignificance of mean square (*a*) indicates that the average gradients for males and females over the whole 15 min are not significantly different. Mean square (*d*) is also insignificant, showing that there are no significant differences between birds of the same sex as regards average gradients.

As the number of tests rose, it became apparent that there was often a considerable variation between individual females in the initial dye concentration in the blood, despite the standard dosage estimated on a body weight basis. According to Newell & Shaffner [1950], the ratio of blood vol. to body weight increases almost linearly in female birds (of the New Hampshire breed) between 400 and 1200 g. Thereafter there is a smaller proportional increase in blood vol. The packed cell vol. is not influenced materially by either age or weight, remaining at a little below 30 %. This being the case, of two birds given a standard intravenous dose of a drug, the heavier bird will have a higher initial concentration in the blood, since it has a proportionately smaller blood vol. than the lighter one. A comparison of three graphs of normal liver

clearance tests, performed on females weighing 800, 1092 and 1642 g respectively, showed progressively higher initial plasma dye concentrations at 5 and 10 min, although all received 20 mg/kg BSP. A test was therefore made in which two birds, A and B, one approximately twice the weight of the other, received the standard dose of 20 mg/kg of BSP, resulting in the usual variation in initial plasma dye concentration. Nine days later the heavier bird was given a dose of 16 mg/kg, calculated from Newell & Schaffner's [1950] regression curve of the body weight/blood vol. relationship, in order to give an approximately equal initial concentration as obtained in the lighter bird. The discrepancy was then observed to have virtually disappeared (Fig. 2).

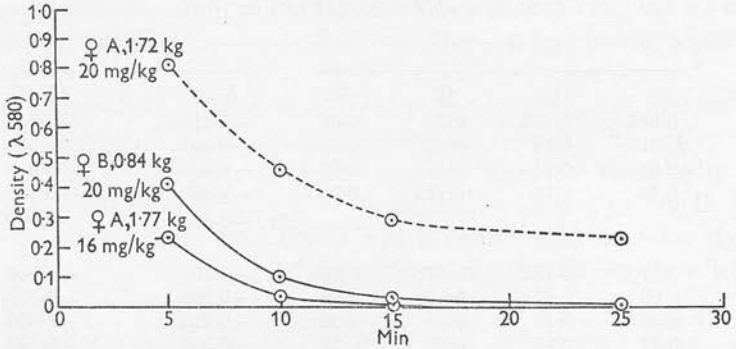

Fig. 2. Comparison of the effects of dosing, (1) according to body weight (---), and (2) according to estimated blood volume (——).

Table 2. *BSP clearance in 11-month-old male fowls, 4 weeks after implantation of stilboestrol (chemical capons)*

	Min after BSP				Gradients	
Fowls	5	10	15	25	5–10	10–15
1	0·50	0·30	0·13	0·02	−0·20	−0·17
2	0·52	0·33	0·20	0·06	−0·19	−0·13
3	0·50	0·28	0·09	0·04	−0·22	−0·17
4	0·52	0·36	0·12	0·02	−0·16	−0·24
5	0·46	0·24	0·10	0·04	−0·22	−0·14
					Av. −0·198	−0·170 = 14 % change

(2) *Effects of caponization*

(*a*) *Chemical caponization.* Fig. 3 shows a typical BSP clearance curve of a chemically castrated male (referred to below as chemical capon), with normal male and female curves for comparison. Table 2 gives details of this experiment.

The curves appear flatter and change less in gradient than do those of normal females. The average gradient for chemical capons changes only by 14 %, whereas females show a 64 % change. Repeating the previous analysis, a comparison of normal males with chemical capons shows a very highly significant differential behaviour. (Interaction mean square = 0·143600, error mean square = 0·002039, $F = 70{\cdot}4$ for 1 and 7 D.F.). There is also a highly significant interaction in the comparison of chemical capons versus normal females ($F = 12{\cdot}82$ for 1 and 8 D.F., significant at the 1 % level).

(*b*) *Surgical caponization.* The clearance curves for surgical capons are shown in Fig. 4, together with the result of treating one of these birds with androgen. Table 3 sets out the results.

The average gradient of these year-old birds, castrated for 11 months, shows a 22 % change. In a comparison of surgical capons and normal females (64 % change) the mean square is significant at the 5 % level ($F = 7{\cdot}20$ for 1 and 6 D.F.), i.e. in the

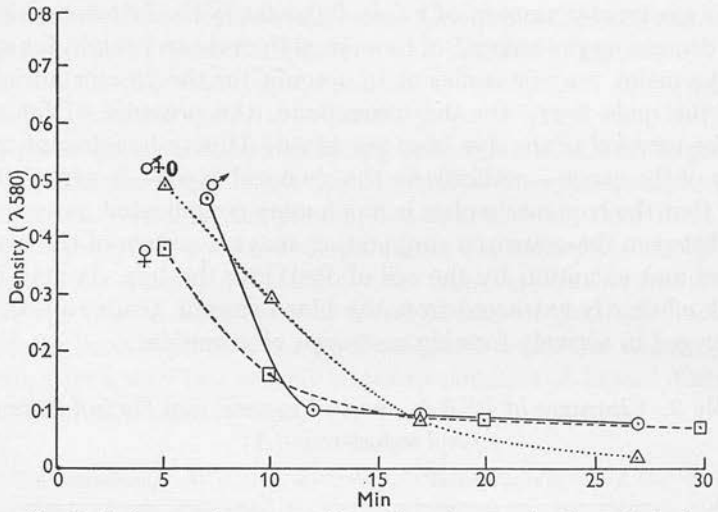

Fig. 3. Influence of oestrogen (**0**) on liver clearance in the male fowl.

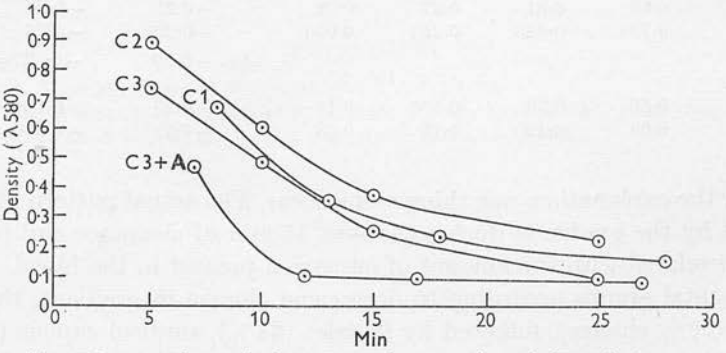

Fig. 4. Liver clearance in surgical capons and restoration of the male type excretion by administration of methyl testosterone (A).

same direction but not so marked as in chemical capons. It follows that surgical capons are also significantly different from normal males (89 % change). There is no significant interaction in a comparison of the two types of capon—in fact the interaction mean square is smaller than that for error. With the limited data so far obtained it is not possible to attach significance to the reversion of the androgen-treated birds to the male type of clearance, but they fit into the same pattern, as seen from birds (1) and (3) in Table 3, before and after androgen treatment. The sharper change with androgen is only significant at the 20–10 % level.

DISCUSSION

It has not been possible to suggest any straightforward explanation of the sex difference in the liver clearance curves. It is obviously associated with the oestrogen-androgen balance, and it would seem that the liver cell exposed to excess of oestrogen is somewhat slower than the androgen-exposed cell in the initial stages of dye excretion from the blood. The only detectable histological difference between the male and female liver is the greater amount of intracellular fat in the latter, and it is possible that this, by decreasing the amount of functional liver tissue (weight for weight compared with the male), may be sufficient to account for the greater initial excretory efficiency of the male liver. On the other hand, the presence of fat may be an obstacle to the removal of the dye from the blood. It may be relevant to note here that the liver of the capon—particularly the chemical capon—is very fatty. It may be suspected that the true mechanism is much more complicated, possibly involving competition between the oestrogen conjugating enzyme system of the liver cell, and the absorption and excretion by the cell of BSP into the bile. It may be that the dye, although efficiently extracted from the blood stream, tends to leak back from liver cells engaged in actively forming oestrogen glucuronides.

Table 3. *Clearance of BSP in surgical capons, and the influence of methyl testosterone* (***A***)

Surgical capons	Min after BSP				Gradients		
	5	10	15	25	5–10	10–15	
(1)	0·87	0·53	0·29	0·16	−0·34	−0·24	
(2)	0·89	0·61	0·37	0·22	−0·28	−0·24	
(3)	0·735	0·485	0·255	0·095	−0·25	−0·23	
					Av. −0·29	−0·237 = 22 %	change
+A							
(1)	0·55	0·30	0·175	0·17	−0·25	−0·125	
(3)	0·68	0·19	0·10	0·08	−0·32	−0·085	

Whatever the explanation, one thing seems clear. The actual pattern of excretions as indicated by the gradients during the first 15 min of clearance can to a certain extent be correlated with the amount of oestrogen present in the blood. Arranging the experimental groups according to decreasing change in gradient, the order is: males, first (89 % change), followed by females (64 %), surgical capons (22 %) and, last, chemical capons (14 %, i.e. approximately a straight line excretion). Omitting surgical capons, this suggests an inverse relationship between the magnitude of change in gradient and the level of oestrogen in the blood, since normal males may be assumed to have least oestrogen and heavily 'stilboestrolized' males most, with normal females occupying an intermediate position. However, such an arrangement would imply that surgical capons (22 % change) have more oestrogen than normal females (64 %), which is obviously not the case, as indicated by the failure of the plumage of surgical capons to become female in type. A possible explanation for this anomaly may be that if an average gradient is calculated over the entire 15 min, surgical capons show an average gradient of 0·26, compared with 0·18 for chemical capons for the same period (normal females 0·22, and normal males 0·24). This difference,

showing a more rapid overall decline for surgical capons, indicates a trend towards 'maleness' and is highly significant. Obviously this is a point needing further study.

The observed variability of initial plasma dye concentration in females when dosed according to body weight indicates a possible source of error when administering drugs intravenously to hens on that basis. It may easily happen that a much higher blood level of, for example, a barbiturate, overdosage with which is not easily tolerated by birds, may be reached in a heavy bird, when a fowl of half the weight would receive a full anaesthetic but safe dose. The position in the case of male birds is not so clear. Newell & Schaffner [1950] found that the blood vol. and body weight bore a linear relationship at all ages, but that the packed cell vol. tends to increase with approaching sexual maturity, relative to total blood vol., so that in (New Hampshire) males between 1800 and 2800 g it becomes almost 50 % greater than at lower weights. It seems possible that this could result in an essentially similar relative decrease of plasma vol. as in the female, but in actual fact very little difference is noticeable when the initial dye concentrations are determined on a body weight basis in males of different weight. Fluctuations are observed, but do not show a regular grading as in the case of females. One is led to suspect that other factors may be involved, not the least important being the relatively avascular abdominal and other fat in the hen, which may or may not be independent of an actual decrease of total blood vol. in older and heavier birds.

All expenses connected with this investigation were borne by the British Empire Cancer Campaign. I am indebted to Dr R. Osborne of this Centre for help in the statistical examination of the data.

REFERENCES

Newell, G. W. & Schaffner, C. S. [1950]. *Poult. Sci.* **29**, 78.
Pino, J. A., Weiss, H. S. & Sturkie, P. D. [1951]. *Proc. IXth World's Poult. Congr.* **3**, 102.
Rosenthal, S. M. & White, E. C. [1925]. *J. Amer. med. Ass.* **8**, 1112.

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STUDIES ON THE INFLUENCE OF SEX HORMONES ON THE AVIAN LIVER

II. ACUTE LIVER DAMAGE IN THE MALE FOWL, AND THE PROTECTIVE EFFECT OF OESTROGEN, AS DETERMINED BY A LIVER FUNCTION TEST

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(*Revised manuscript received 25 February* 1957)

SUMMARY

1. Liver function in male fowls as assessed by a modified sodium bromosulphthalein clearance test, is noticeably impaired 1–1½ hr after a sublethal dose of the hepatotoxic alkaloid, seneciphylline, and is greatly impaired at 4–4½ hr. Between this period and 24 hr, the clearance curve returns to more normal values, but is now of the female type, and this alteration in the mode of excretion of the dye persists for at least 5 days after a single administration of the alkaloid.

2. Previous treatment with diethylstilboestrol protects the male bird's liver to a considerable degree against the hepatotoxic effect of the alkaloid.

3. On the evidence of liver clearance curves, male birds receiving repeated weekly sublethal doses of seneciphylline return to normal much more quickly if previously treated with stilboestrol than control birds not so treated; they also gain weight at a faster rate.

4. It is suggested that within reasonable limits of liver damage, the accumulation of oestrogen, through the inability of the liver to excrete it and its retention in inflamed cells, may be beneficial in that it helps to protect against further damage, and also may encourage repair processes.

In the preceding paper [Campbell, 1957] a modified method of assessing liver function in the fowl by means of sodium bromosulphthalein (BSP) was described. A significant sex difference in the clearance gradients of the dye during the first 15 min after injection was detected, and it was shown that sex hormones played a major part in determining this difference. The present paper describes experiments on male birds suffering from acute liver damage caused by seneciphylline, one of the pyrrolizidine alkaloids of ragwort (*Senecio jacobaea* L.), and the effect of diethylstilboestrol in protecting the liver against it. For a full account of the hepatotoxic and carcinogenic effect of ragwort alkaloids in the fowl, see Campbell [1956].

MATERIALS AND METHODS

The birds used were young male Brown Leghorns chosen arbitrarily from the several inbred lines maintained at the Poultry Research Centre. Acute but not fatal liver damage was caused by the intravenous injection of seneciphylline hydrochloride in molar solution. This has a pH of 6·8, and the dose used was 20 mg/kg. Liver clearance tests were performed at different stages of liver damage by determining the

concentration of BSP in the plasma at intervals subsequent to its intravenous injection at the rate of 20 mg/kg. The effects of oestrogenization were determined by the subcutaneous implantation of a 50 mg pellet of diethylstilboestrol.

RESULTS

In order to establish how quickly liver function, as determined by the clearance test, was impaired following the injection of seneciphylline hydrochloride, a series of estimations was made at intervals of 13 min, 1 hr, 4 hr and 24 hr. Fig. 1 shows that 13–23 min after administration of the alkaloid liver clearance was unimpaired, the excretion curve being that of a normal male fowl with a sharp gradient decrease at 10 min. At 1–1½ hr an impairment is noticeable, and at 4–4½ hr this has become very

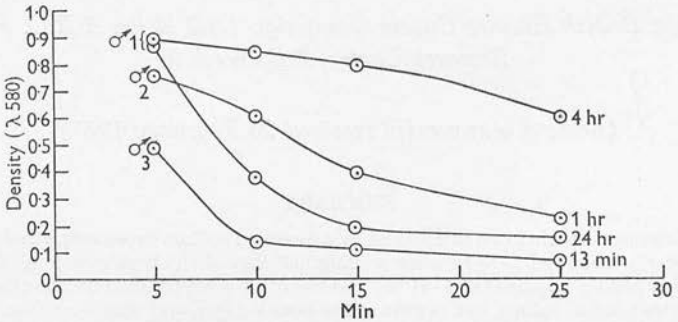

Fig. 1. Male liver clearance at various intervals up to 24 hr subsequent to the administration of seneciphylline hydrochloride.

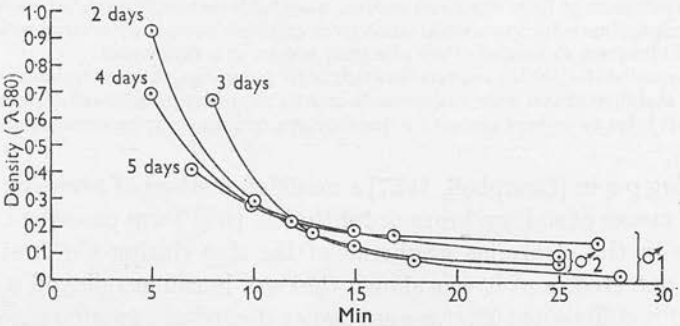

Fig. 2. Male liver clearance 2–5 days after administration of seneciphylline.

pronounced. At 24 hr, the clearance curve has assumed the typical female form with a smaller gradient change, but apart from this, the function of the liver as estimated by the excretion rate has returned to within normal limits. The shorter period observations were made, of necessity, on different birds, but of comparable age and weight.

Fig. 2 gives the clearance curves for some of the same birds at 2, 3, 4 and 5 days subsequent to liver damage, and it will be noticed that there is no tendency to revert to the male type of excretion curve within that period.

Because of certain observations on the effects of oestrogen on the chronically damaged avian liver, reported in the following communication, and also because of

the sex difference in the susceptibility of the mouse kidney to damage from various nephrotoxic agents [Eschenbrenner & Miller, 1945; Kerr, Campbell & Levvy, 1949], it was decided to try the effects of a previous period of oestrogenization on the male fowl's liver before administration of the alkaloid. As a 4–4½ hr period after the injection of alkaloid gave maximal impairment of liver function as measured by the test, this was the period selected, and Fig. 3 shows two typical results. It is apparent that stilboestrol, in this case implanted a week previously, has protected the liver against damage to a marked degree. This straight-line type of clearance was obtained regularly in oestrogenized males 4 hr after inflicting liver damage. The time taken

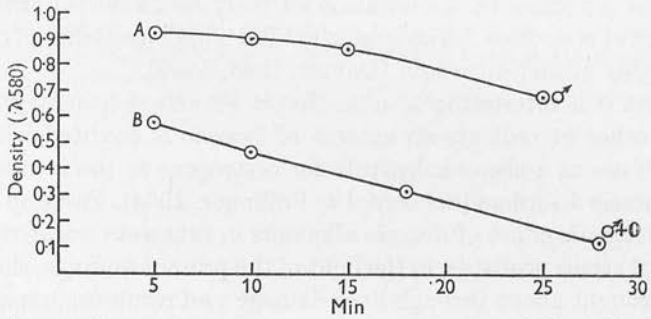

Fig. 3. Liver clearance in a male (*A*) 4 hr after dosing with seneciphylline, (*B*) the same but in a previously stilboestrolized fowl (+**0**).

Table 1. *Weights (g) of (A) young untreated, and (B) young oestrogenized males receiving similar weekly doses of seneciphylline hydrochloride*

(Age at commencement 6–7 weeks)

	Controls (A)		Oestrogen protected (B)	
Weeks	1	2	3	4
1	398	473	363	380
2	473	563	465	415
3	501	573	502	520
4	—	—	605	658
5	761	709	790	800
6	894	677	942	932
7	917	643	1158	1085
Total weight gain	519	170	795	705

for the clearance curves to revert to the normal male type was not determined. In the course of these experiments it was interesting to note that the protected fowls in the period immediately following injection did not exhibit such severe reactions to the alkaloid, in this way resembling the modified response of females compared with males as previously reported by Campbell [1956].

The effect of repeated sublethal doses of seneciphylline is to damage the male bird's liver to such an extent that even 20 months after the last of six weekly doses, liver clearance is still of the female type, as will be shown in the next paper. In two cases, however, 6- to 7-week-old males implanted with stilboestrol 2 days before the first of the alkaloid injections, showed that the clearance curve had resumed its male character at the end of 16 weeks, and a comparison of the weekly weight gains of the two birds with the weights of two controls during the course of the injections showed greater gains in the oestrogenized males even after one extra dose (see Table 1).

DISCUSSION

Oestrogen inactivation in the liver is probably confined to the liver cell, with the reticulo-endothelial system playing little or no part [Israel, Meranze & Johnston, 1937; Zondek & Sklow, 1941]. It is therefore not surprising that parenchymatous damage should result in an impaired ability to excrete oestrogens [Talbot, 1939]. The phenomena of gynaecomastia and testicular atrophy in advanced cases of liver cirrhosis in the human male [Glass, Edmondson & Soll, 1940; Klatskin, Salter & Humm, 1947] are due to accumulated oestrogens, and it seems possible that the same factor causes the tendency to feminization of body and skeletal structure seen in a high proportion of male East Africans who exhibit a high incidence of liver disorders, which are probably of dietetic origin [Davies, 1948, 1949].

In this context it is interesting to note that a French proprietary medicine containing among other ingredients an extract of *Senecio* is credited with oestrogenic effects, and is in use as a cheap substitute for oestrogens in the treatment of menopausal and prostatic disorders [Schoental & Pullinger, 1954]. Tests by these workers for a direct oestrogenic effect of *Senecio* alkaloids in rats were negative for the small doses tried, but it seems probable, in the light of the present findings, that a secondary effect may be brought about through liver damage and resultant impaired ability to inactivate endogenous oestrogens.

It has been shown [Singher, Kensler, Taylor, Rhoads & Unna, 1944; Hertz, 1948] that the loss in the oestrogen-inactivating ability of the liver parallels the decrease of riboflavin and thiamin in that organ, and that it is possible to protect the liver by oestrogen when on a cirrhosis-producing diet. The latter observation is in agreement with the results reported in the present paper. In a subsequent communication it will be shown that stilboestrol can bring about a substantial regeneration in the chronically damaged avian liver, and these facts, coupled with Brunelli's [1935] observation that oestrogens accumulate and are retained in inflamed tissues, make it seem not unlikely that the inability of the damaged liver to excrete oestrogen is in fact beneficial in that it helps not only to protect the liver against further damage but also encourages repair. Further evidence to support this will be given in the subsequent communication. Finally, the observation that an outbreak of chronic hepatitis in Danish women was almost entirely confined to the post-menopausal period, and that liver cirrhosis is much more common in men than in women [Hertz, 1948], also lends support to the view that oestrogens help to protect the liver against damage, and indeed, as mentioned earlier, the same mechanism may be at work in the protection of other organs, showing a sex difference in susceptibility to damage by toxins, as for example, the mouse kidney.

All expenses connected with this investigation were borne by the British Empire Cancer Campaign.

REFERENCES

Brunelli, B. [1935]. *Arch. int. Pharmacodyn.* **49**, 295.
Campbell, J. G. [1956]. *Proc. roy. Soc. Edinb.* (B) **66**, 111.
Campbell, J. G. [1957]. *J. Endocrin.* **15**, 339.
Davies, J. N. P. [1948]. *Lancet*, **2**, 474.
Davies, J. N. P. [1949]. *Brit. med. J.* **2**, 676.
Eschenbrenner, A. B. & Miller, E. [1945]. *Science*, **102**, 302.
Glass, S. J., Edmondson, H. A. & Soll, S. N. [1940]. *Endocrinology*, **27**, 749.
Hertz, R. [1948]. *Recent Progr. Hormone Res.* **2**, 172.
Israel, S. L., Meranze, D. R. & Johnston, C. G. [1937]. *Amer. J. med. Sci.* **194**, 835.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. [1949]. *Biochem. J.* **44**, 487.
Klatskin, G., Slater, W. T. & Humm, F. D. [1947]. *Amer. J. med. Sci.* **213**, 19.
Schoental, R. & Pullinger, B. D. [1954]. *Brit. Empire Cancer Campaign*, 32nd Annual Report, p. 300.
Singher, H. O., Kensler, C. J., Taylor, H. C. Jr., Rhoads, C. P. & Unna, K. [1944]. *J. biol. Chem.* **154**, 79.
Talbot, N. B. [1939]. *Endocrinology*, **25**, 601.
Zondek, B. & Sklow, J. [1941]. *Proc. Soc. exp. Biol., N.Y.*, **46**, 276.

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STUDIES ON THE INFLUENCE OF SEX HORMONES ON THE AVIAN LIVER

III. OESTROGEN-INDUCED REGENERATION OF THE CHRONICALLY DAMAGED LIVER

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(*Revised manuscript received* 25 *February* 1957)

SUMMARY

1. In the male fowl spontaneous repair of the liver, damaged by repeated doses of the alkaloids of *Senecio jacobaea* L., takes place extremely slowly, if at all.
2. Females subjected to the same treatment tend to recover.
3. In both sexes, but particularly in the male, massive doses of stilboestrol continuously absorbed from pellet implants initiate and speed up liver regeneration.
4. The hypothesis is advanced that the impaired ability of the damaged liver to conjugate and inactivate oestrogen is advantageous in so far as the raised oestrogen level stimulates repair processes in that organ.

At the termination of an investigation into the hepatotoxic and carcinogenic effects of the pyrrolizidine alkaloids of ragwort (*Senecio jacobaea* L.) in the fowl [Campbell, 1956], about thirty surviving male and female fowls with presumed chronic liver damage were set aside for long-term study. Several of these died soon afterwards from liver dysfunction, one developing cholangiectasis and a benign hepatoma. The remainder provided material in which it was planned to try the effects of heavy oestrogenization on the liver, as it was thought that this might stimulate pre-neoplastic liver and bile-duct epithelium to tumour formation. The actual results were unexpected and are the subject of this communication.

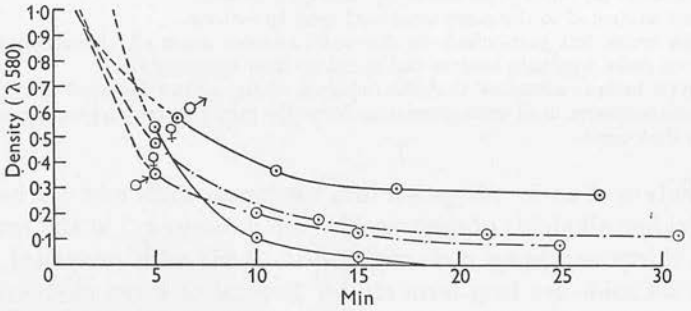
MATERIALS AND METHODS

Twelve male and twelve female Brown Leghorns, 21–22 months old with suspected chronic liver damage of 20 months' duration in the form of cirrhosis, biliary hyperplasia and interstitial granulocytic infiltration induced by *Senecio* alkaloids, were selected from the survivors. Of these, six males and six females received a subcutaneous implant of a 50 mg pellet of stilboestrol, and after 4 weeks the clearance of sodium bromosulphthalein (BSP) by the liver [Campbell, 1957*a*] was determined on the controls and on the stilboestrol-treated birds; three of each were then killed and the livers and gonads taken for histological examination. Tissues were fixed in Susa's fluid, formol saline and 80% methanol; sections were cut at 8μ after paraffin wax embedding, or examined as frozen sections; the stains used were haematoxylin and eosin, Best's glycogen stain, the Prussian blue method for haemosiderin and Sudan IV.

RESULTS

The treated birds, especially the males, showed signs of improvement when compared with the controls, as judged by external appearance and behaviour. It should be emphasized that in the males there were no signs of improvement up to 20 months following the last administration of alkaloids, whereas most of the females started to improve in appearance and vigour in about 12–14 months, so that in their case oestrogen was given to birds already on the way towards recovery.

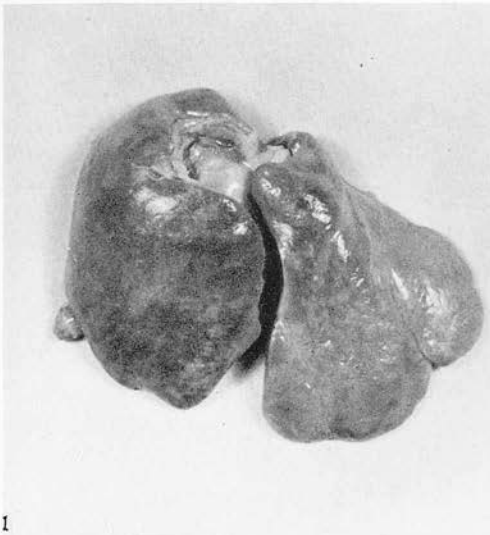
Text-fig. 1 (⊙—⊙) shows the liver clearance of BSP in typical male and female controls. It will be seen that the excretion curve of the dye is normal in the case of the hen, if allowance is made for the high initial plasma concentration of the dye (determined by extrapolation), and which is due to the relative decrease in the blood vol./body weight ratio with increasing age and weight. This variation in initial plasma concentration of an intravenously administered substance, despite a standard dose calculated on a body weight basis, is described in the first paper in this series [Campbell, 1957*a*]. The male excretion curve, however, is not normal, in that it indicates a somewhat retarded 'female' mode of clearance. Post-mortem examination showed this bird



Text-fig. 1. BSP clearance in controls with liver damage of 20 months' duration (⊙—⊙). BSP clearance of fowls with chronic liver damage, 1 month after stilboestrol implantation (⊙—·—⊙).

to have a small, firm and mottled liver (Pl. 1, fig. 1) and atrophied testes. Pl. 1, fig. 2, shows the histology of the liver. There was disorganization of the parenchyma, bile-duct proliferation, cirrhosis, heavy infiltration of granulocytes, and a large amount of intracellular haemosiderin. The hen's liver was normal, and the ovary was active.

Text-fig. 1 (⊙—·—⊙) also shows typical clearance curves for two of the oestrogen-treated birds. The female curve is much the same as in the control, but the male shows a very efficient excretion which, if extrapolated to zero time (about 1·3 on the density ordinate with the dose of BSP used), gives a sharp gradient over the first 5 min, thus conferring a somewhat male type of configuration to the curve. Pl. 1, fig. 3, shows the liver of one of the males 4 weeks after stilboestrol implantation and 1 day after the clearance test. The left lobe is largely replaced by a new mass of liver tissue. The other two males examined a few days later showed similar liver regeneration, though not so well defined macroscopically. As shown in Pl. 1, fig. 4, this consists of columns of new liver cells with a rather fatty cytoplasm. The testes were small and flaccid. The hens had plentiful abdominal fat, the livers were fatty but otherwise normal, and the ovaries and oviducts were inactive.



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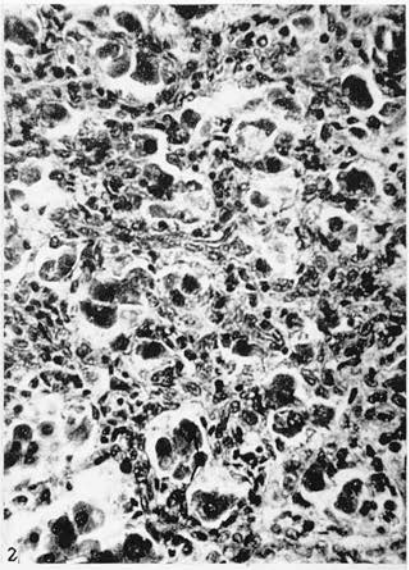

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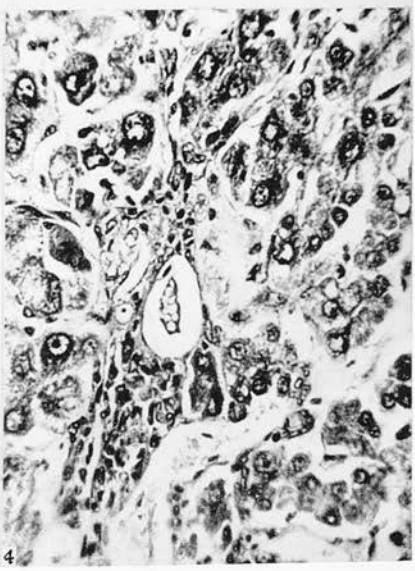

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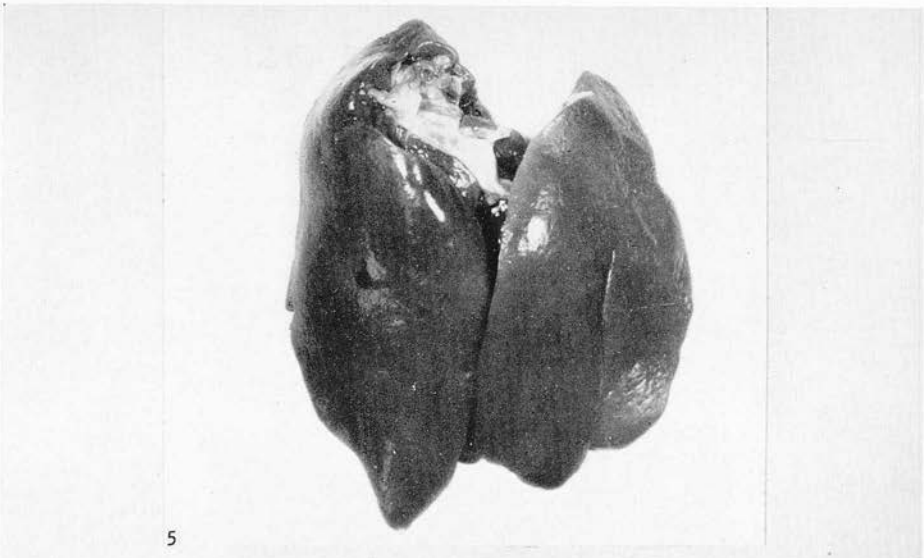

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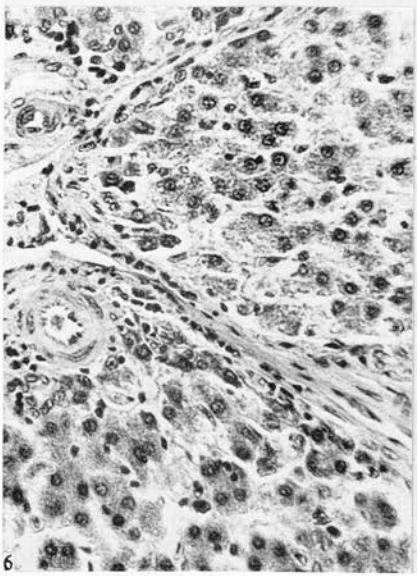

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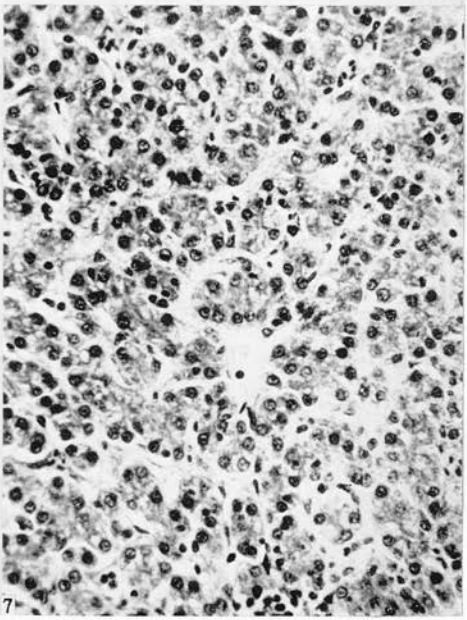

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Virtual completion of liver regeneration is shown in Pl. 2, figs. 5 and 6, from a male 3 months after oestrogenization (Pl. 2, fig. 7, illustrates the histology of a normal liver for comparison). The control, killed at the same time, showed no sign of regeneration. However, both birds still exhibited the female type of clearance when tested before being killed. About 5 months after the implantation of stilboestrol, and at the age of 26–27 months, the remaining treated males began to show sexual activity and a male type clearance curve, while the controls were still inactive, had a rather dull plumage, were lighter in weight, and maintained the female type of liver clearance. Surviving females, on the other hand, whether controls or oestrogen treated, were all laying eggs.

DISCUSSION

It has already been shown [Campbell, 1957*b*] that acute liver damage in the male fowl produces a 'female type' of liver clearance curve, and this was ascribed to accumulated oestrogen. The present observations on birds with chronically impaired livers show that males are much slower than females in returning to normal, as evidenced by the liver clearance test and subsequent post-mortem and histological examination. It is probable that the females did not initially sustain such severe liver damage as the males by virtue of the protective effect of their natural oestrogens, and that the higher concentration of oestrogen in the intervening months before treatment hastened the processes of repair. This view gains support from the demonstration that by subjecting the damaged male liver to massive doses of oestrogen, repair is speeded up, if not actually induced, where it would otherwise not have occurred. Moreover, such treated males show every sign of returning to normal, even after prolonged chronic impairment of the liver.

The question naturally arises, how these reparative processes are brought about. As a working hypothesis, the following sequence of events is suggested. After the liver is damaged, its ability to inactivate oestrogen [Israel, Meranze & Johnston, 1937] is impaired [Talbot, 1939]. This inactivation is brought about by conjugation, resulting in an oestrogen glucuronide [Crépy, 1947]. It is postulated that the impaired liver can no longer synthesize glucuronides from glycogen through the mediation of uridine triphosphate and the glucuronyl transferase system discovered by Dutton & Storey [1951, 1954], and unconjugated oestrogen tends to accumulate, especially at the site of damage [Brunelli, 1935]. This accumulation has been found to be associated with an increase of both liver glycogen and blood sugar in mice [Bullough, 1946], and this increased glucose stimulates cell division [Bullough, 1950]. Such increased β-glucuronidase activity in regenerating liver [Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950] may therefore be a function of the restoration of the balance between glucuronide synthesis and hydrolysis in that organ, and of its returning capacity to conjugate oestrogens. This hypothesis would help to explain, among other things, the observations of McDonald & Odell [1947] and Odell & McDonald [1948], that women in pre-eclampsia show abnormally high serum β-glucuronidase activity, assuming that in such cases the liver is already in a state of early damage coexistent with attempts at repair. It is suggested that in fowls both stilboestrol and natural oestrogens have a direct protective and reparative effect on the liver exposed to hepatotoxic agents. Further, it is suggested that

β-glucuronidase, which shows an increased activity in the regenerating liver, is an index of that organ's returning ability to conjugate and inactivate oestrogens.

All expenses connected with this investigation were borne by the British Empire Cancer Campaign.

REFERENCES

Brunelli, B. [1935]. *Arch. intern. Pharmacodyn.* **49**, 295.
Bullough, W. S. [1946]. *Phil. Trans.* B, **231**, 453.
Bullough, W. S. [1950]. *J. Endocrin.* **6**, 340, 350.
Campbell, J. G. [1956]. *Proc. Roy. Soc. Edinb.* B, **66**, 111.
Campbell, J. G. [1957*a*]. *J. Endocrin.* **15**, 339.
Campbell, J. G. [1957*b*]. *J. Endocrin.* **15**, 346.
Crépy, O. [1947]. *Arch. Sci. physiol.* **1**, 427.
Dutton, G. J. & Storey, I. D. E. [1951]. *Biochem. J.* **48**, xxix.
Dutton, G. J. & Storey, I. D. E. [1954]. *Biochem. J.* **57**, 275.
Israel, S. L., Meranze, D. R. & Johnston, C. G. [1937]. *Amer. J. med. Sci.* **194**, 835.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. [1949]. *Biochem. J.* **44**, 487.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. [1950]. *Biochem. J.* **46**, 278.
Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. [1948]. *Biochem. J.* **42**, 462.
McDonald, D. F. & Odell, L. D. [1947]. *J. clin. Endocrin.* **7**, 535.
Odell, L. D. & McDonald, D. F. [1948]. *Amer. J. Obstet. Gynec.* **56**, 74.
Talbot, N. B. [1939]. *Endocrinology*, **25**, 601.

DESCRIPTION OF PLATES

Plate 1

Fig. 1. Male liver, with seneciphylline-induced chronic hepatitis of 20 months' duration. ($\times \frac{4}{5}$.)
Fig. 2. Histology of the same, showing extreme biliary hyperplasia and cellular infiltration. (H. & E., $\times 308$.)
Fig. 3. Massive regeneration of left liver lobe from a similar male, 4 weeks after stilboestrol implantation. ($\times \frac{2}{3}$.)
Fig. 4. Histology of regenerating lobe, showing rather fatty but actively growing liver cell cords. (H. & E., $\times 308$.)

Plate 2

Fig. 5. Almost complete regeneration of a male liver, 3 months after stilboestrol implantation. ($\times \frac{4}{5}$.)
Fig. 6. Histology of preceding liver, showing essentially normal structure. Almost complete disappearance of bile-duct hyperplasia and inflammatory cells. Slender bands of connective tissue still present. (H. & E., $\times 308$.)
Fig. 7. Histology of normal male liver for comparison. (H. & E., $\times 308$.)

THE DETECTION AND IDENTIFICATION OF AVIAN GONADAL TUMOURS BY MEANS OF A LIVER CLEARANCE TEST.

by

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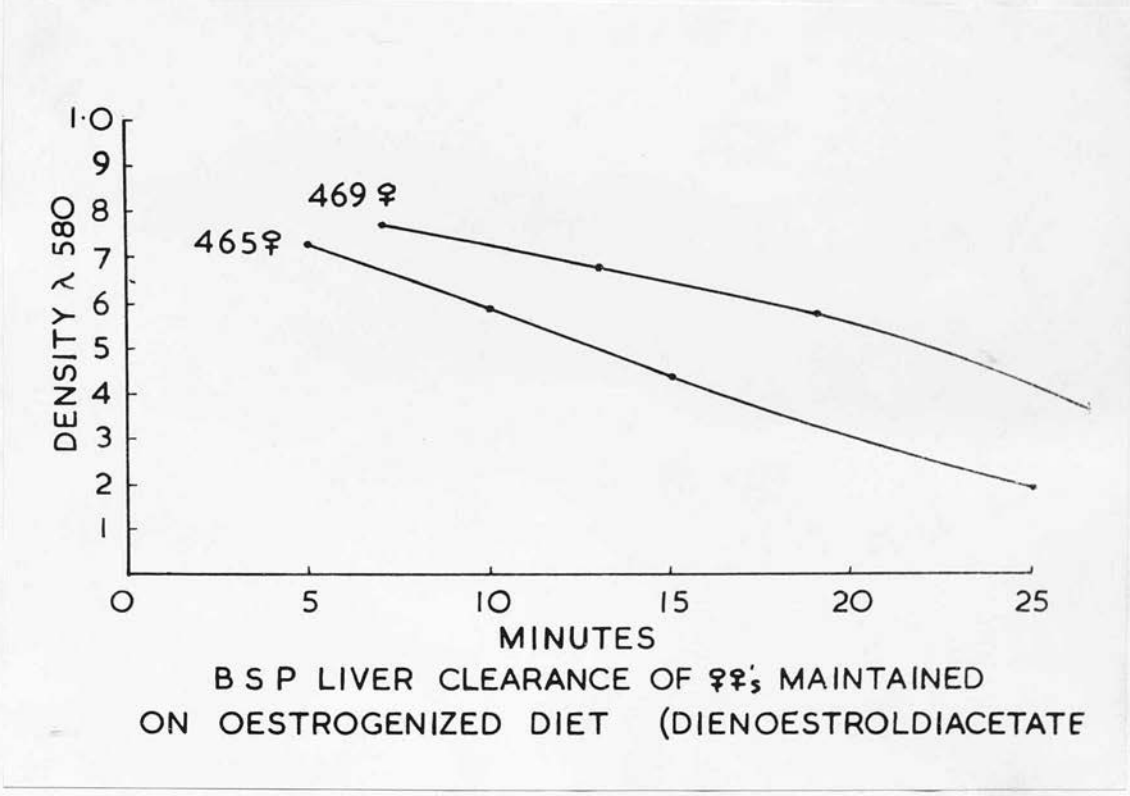
Introduction :-

It has been shown in fowls that a sex difference in the rate of clearance from the circulation of intravenously administered sodium bromosulphthalein is dependent on the amount of oestrogen in the blood at the time of the test (Campbell, 1957a). In this communication details are given of the relationship between the liver clearance gradient and the subsequent pathology of birds showing some aberration in their sexual physiology such as a failure to ovulate or the prolonged cessation of ovulation in an otherwise apparently normal bird. In a study of this nature it is inevitable that more females than males come to examination, since most cockerels are not kept until they reach an age when testicular tumours are likely to develop, and in any event such tumours appear to be rare (Campbell, 1951).

Materials and Methods :-

All birds used in this investigation, with the exception of the intersex "half-sider", were Brown Leghorns belonging to the inbred flock maintained at this Centre. The methods for estimating liver clearances by means of BSP. and the calculation of gradient differentials have been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B S P LIVER CLEARANCE OF ♀♀'s MAINTAINED
ON OESTROGENIZED DIET (DIENOESTROLDIACETATE

Text-fig.1

detailed in a previous publication. The normal gradient change in the periods 5-10 and 10-15 minutes after injection of the dye are 64% for the mature female, and 86% for the mature male respectively (Campbell, 1957a). Mature hyperoestrogenized females (produced either by feeding dienoestroldiacetate 2 oz./ton of food, or by the subcutaneous implantation of diethylstilboestrol, 25 mg.) which were tested 2-3 weeks after the commencement of treatment, show a negligible gradient change in the same periods, i.e. they have a straight line type of clearance (See Text Fig. 1). All the birds tested were subsequently killed, usually immediately after the result of the test was known, but some were followed for several months and through 2 or 3 tests. At post-mortem examination tissues taken for histology included gonads, various endocrines, liver and kidney, and were fixed in Susa, formal saline or formal ammonium bromide. Both paraffin and frozen sections were submitted to microscopical examination, after haematoxylin and eosin, Mallory's trichrome or periodic acid Schiff stains, or, in the case of frozen sections, after staining with Sudan IV and haematoxylin, for the detection of the lipid.

Results :-

Table 1 sets out the main findings in the birds investigated. These will be described in more detail below.

(1) A $2\frac{1}{2}$ year old hen 4799, one of a group in a feeding experiment, had not laid for 13 months. It showed no plumage abnormalities but had a moderately well developed comb. Just prior to testing this bird produced a very small egg. The clearance curve indicated a gradient change of 86.7% i.e. a marked deviation to the male type clearance. Post-mortem examination was made 2 days after the test. The bird was in good condition, but there was a tumour

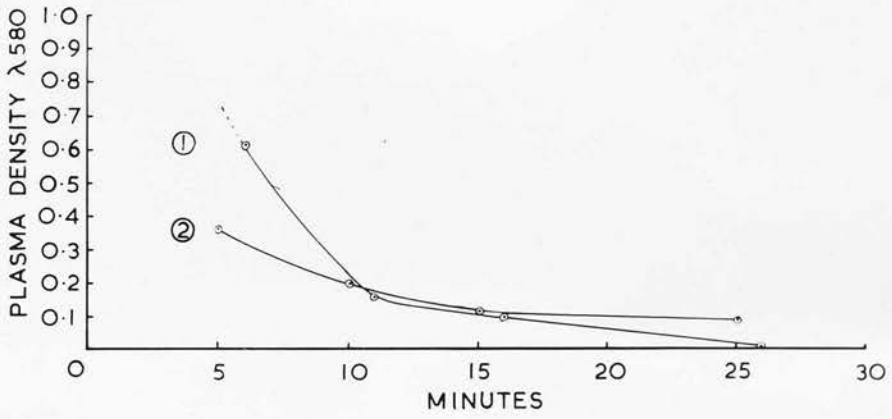
No.	Sex.	History.	Plasma density λ 580. (Corrected) 5	10	15 mins.	Gradients. 5–10	10–15	% change.	Histology.
1	♀	Not laid for 13 mths.	0.61	0.16	0.10	0.45	0.06	86.7	Arrhenoma
2	♀	Non-layer. Ascites.	0.36	0.20	0.12	0.16	0.08	50	Granulosa and thecal-cell tumour
3	♀	18 mth. old nonlayer	1.10	0.95	0.85	0.15	0.10	33.4	Sertoli-cell tumour
4	♀	18 mth. old nonlayer	0.99	0.55	0.49	0.44	0.06	86.4	Arrhenoma
5	♂ ?	Predominantly ♂ ½ sider changing to ♀	A. 0.62	0.20	0.12	0.42	0.08	81	Ovo-testis
			B. 0.3	0.10	0.02	0.2	0.08	60	↓
			C. 0.95	0.74	0.54	0.21	0.2	2	Androblastoma
6	♀	Suspected abdom. malignancy	0.43	0.23	0.12	0.20	0.11	45	Thecal cell tumour + oviduct adeno-carcinoma
7	♀	Nonlayer	A. 0.84	0.49	0.36	0.35	0.13	63	Normal. "Grape-cluster" ovary
			B. 0.92	0.57	0.296	0.35	0.274	23	
8	Castrate ♀	Development of comb. Palpable abdom. mass	0.65	0.40	0.355	0.25	.045	82	Arrhenoma
[	♀♀	Castrates	0.64	0.28	0.06	0.36	0.22	39	Normal castrates ("poulards")]
			0.36	0.255	0.175	0.105	0.08	25	
			0.5	0.31	0.159	0.19	0.151	21	
9	♀	Nonlayer	0.715	0.55	0.39	0.185	0.16	13.5	"Folliculoma malignum." Granulosa tumour + oviduct adenocarcinoma.

Table I.

(BSP. administered i/v. at 20 mg./Kg. All plasma density readings corrected by deduction of plasma blanks.)

attached by fibrous strands to the middle part of the oviduct. This was irregular in shape, of a yellowish pink colour, firm in some parts, spongy in others. A dwarf shelled egg was present in the uterus. The ovary was active and grossly normal (Pl. I, Fig. 1). Histological examination of the oviduct tumour showed it to be a fibro-leiomyoma, with unusually plump, well defined smooth muscle cells. Sections of the ovary revealed what appeared to be small ova converted to a solid mass of epithelial cells, some forming acini, especially at the periphery, and lying in a matrix of fibrous tissue. These were diagnosed as arrhenomata (Pl. I, Figs. 2 & 3).

(2) Another 2½ year old hen, 4499, from the same feeding experiment, was treated for ascites on 2 occasions by the withdrawal of several hundred mls. of fluid. A clearance test showed a 50.4% gradient change. Just prior to killing, and subsequent to the test, a solitary egg was laid, the first for many months. Post-mortem examination showed a fat bird with several large but not mature ova, and a number of atretic ova in the ovary. Some turbid blood-stained fluid was in the abdomen. There was a peritoneal carcinomatosis resulting in schirrous induration and contraction of the mesentery. Scattered pearly tumours covered the intestine, pancreas, oviduct and oviduct ligament. The ovary showed a fairly extensive cyst-adenocarcinoma (Pl. II, Fig. 4) with a softish hilar tumour of a brownish red shading to cream colour. Histologically this was a typical mixed thecal and granulosa-cell tumour (Pl. II, Fig. 5) showing luteinization of the granulosa "nests". Some regions in the granulosa component showed a cylindromatous appearance, others showed a pseudo-adenomatous structure, with palisaded nuclei. Hyalinization of the connective tissue stroma, and arterio-sclerosis of the ovarian vessels were other features of this tumour. The remainder of



① MALE TYPE OF CLEARANCE IN A LAYING FEMALE ASSOCIATED WITH A SMALL OVARIAN ARRHENOBLASTOMA

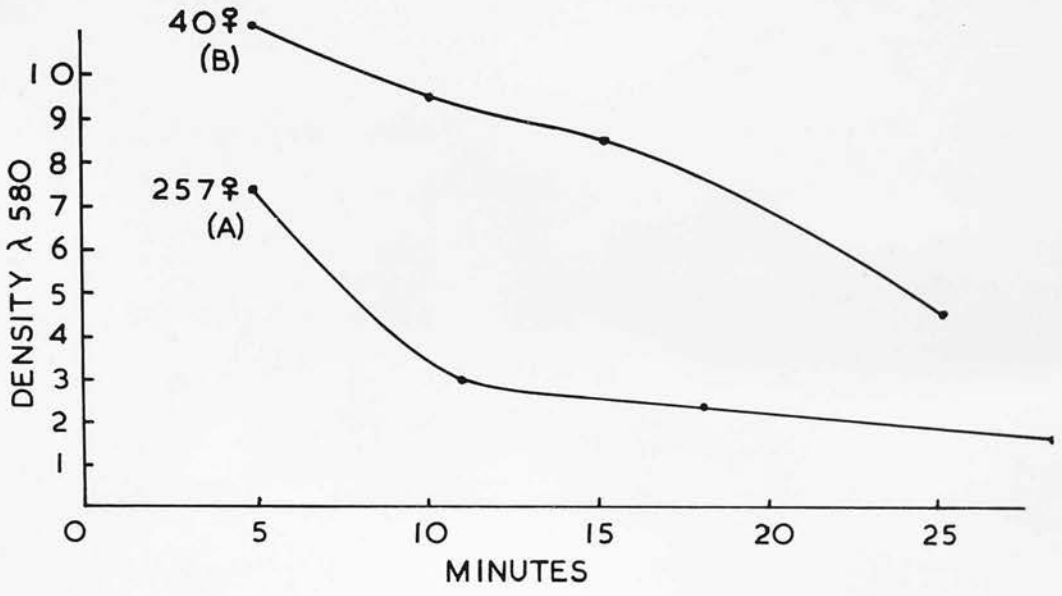
② HYPEROESTROGENIZED TYPE OF CLEARANCE IN A NON-LAYING FEMALE ASSOCIATED WITH A MIXED THECAL CELL & GRANULOSA CELL OVARIAN TUMOUR

Text-fig.2

the ovarian tumour was typical of a cyst-adenocarcinoma, and all the secondaries showed a similar structure. Sections of adrenal showed a marked cortical hyperplasia. The clearance curves for these 2 cases are shown in Text Fig. 2.

(3) Hen 40, aged 18 months, had never laid. At the time of testing it was noted that the plasma was very opalescent, necessitating clearing by means of the addition of alcohol, cooling in the refrigerator and centrifuging prior to evaluation of the samples. The clearance curve, corrected for the dilution, shows a small gradient change (33%) indicating hyperoestrogenization. Post-mortem examination revealed a large amount of abdominal fat, a small fatty liver and an inactive ovary. The oviduct was similar in size to that occurring in a hen in full production, but had small soft nodules protruding externally from the wall of the magnum. Histological examination of the ovary revealed the presence of a substantial Sertoli-cell tumour involving the medulla adjacent to the hilus. A small nodule of arrhenomatous tissue was also present, but the Sertoli-cell part was greatly predominant (Pl. II, Fig. 6). The oviduct nodules were composed of hyperplastic glandular tissue, often in the form of cysts.

(4) Hen 257, a bird showing all the external signs of laying but in fact a non-layer, was found to have a gradient change of 86.4% to the BSP. test. The bird was killed and examined. It weighed 2.28 kg. and was excessively fat. The ovary was active, but the oviduct was small and non-functional. The liver was fatty, and there was a pronounced lipaemia. Liver, ovary, oviduct, adrenals and pituitary were taken for histological examination ; of these, the only organ showing abnormality was the ovary, which had a small tumour composed of cords of epithelial cells apparently arising from

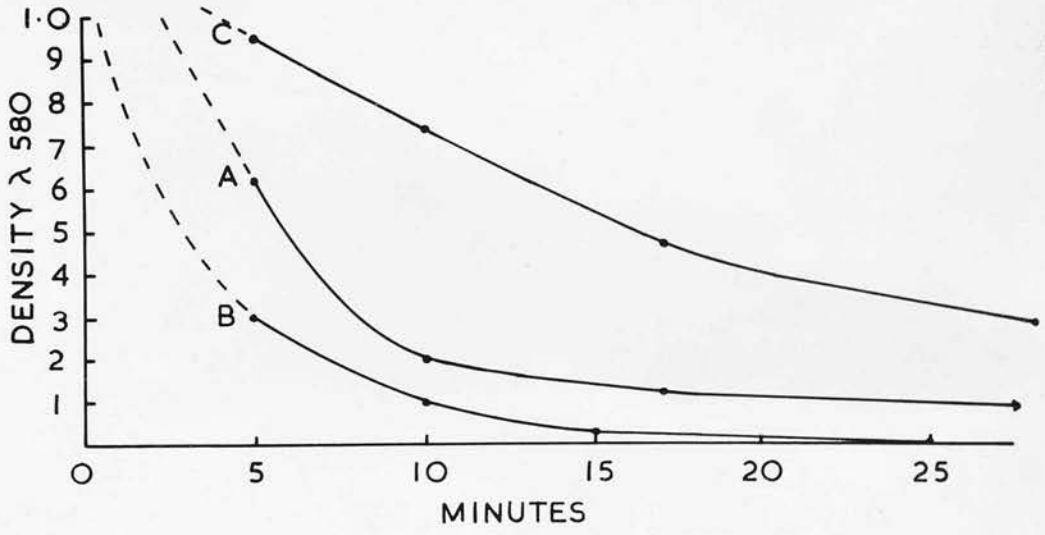


BSP LIVER CLEARANCE OF Ⓐ♀ WITH OVARIAN ARRHENOMA, & Ⓑ♀ WITH OVO-TESTIS AND SERTOLI CELL TUMOUR

Text-fig.3

germinal epithelium. This was diagnosed as a well-differentiated arrhenoma (Pl. II, Fig. 7). The clearance curves for these 2 cases are illustrated in Text Fig. 3.

(5) Text Fig. 4 shows a series of 3 clearance curves obtained from a cross-bred cockerel (Br. L. ♂ x L. S. ♀) sent to this Centre for observation and examination. This bird was an example of that rare combination, a gynandromorph exhibiting bilateral asymmetry involving feather colour, skin and eye pigmentation, size of wattles and length of long bones. Similar examples have been previously described by Greenwood & Blyth (1951). An exploratory laparotomy performed a few weeks before the first test showed a right testis and what appeared to be a left ovary. At the time of the first test, though a male in physical and behavioural characteristics, the bird showed some slight feminization of plumage on the right side of the body. The clearance gradient change was 81% (A). Three months later, the female type of plumage was more marked on the right side, and the cockerel had ceased to crow. The gradient change was 60% (B). After a further interval of 5 months, the gradient change was 2.0%, indicating an excess of circulating oestrogen. The bird was killed after the last test. It was in excellent condition, and showed a female type of plumage most marked on the right of the midline. The right wattle was shorter than the left, and the comb lopped to the right. The right shank was white, the left yellow, while the right eye showed a paler iris than the left. No copulatory organ could be detected but glands were present each side of the penis site. A moderate amount of fat was in the abdominal cavity. The irregularly shaped left gonad, measuring 4 x 3 x 2 cms. and weighing 11.5 gms. resembled an immature ovary, and had a protuberant spherical yellow tumour 8 mm. in diameter at its caudal extremity. The



B S P LIVER CLEARANCE IN AN INTER-SEX HALF-SIDER, ORIGINALLY PREDOMINANTLY MALE, SHOWING PROGRESSIVE CHANGE TO THE HYPEROESTROGENIZED TYPE OF EXCRETION

Text-fig.4

right gonad resembled an atrophic testis, and was 2.5 cms. in length and weighed 2 gms. It showed constricting bands due to capsular contraction. A moderately developed left oviduct was present, measuring 30 cms. (Pl. III, Fig. 8). The only other gross abnormalities consisted of extreme atrophy of the thyroids and the presence of persistent lobules of thymus tissue.

Histologically the right testis, although macroscopically atrophied, showed considerable spermatogenesis. The left gonad, despite its gross resemblance to an undeveloped ovary, was also mainly composed of active testicular tubules. However, scattered in the subcapsular region, and particularly localized as the yellow tumour already mentioned, were groups of haphazardly arranged cells with irregular intensely basophilic nuclei lying embedded between trabeculae composed of a hyaline eosinophilic substance. Furthermore, small ova occurred in wedge-shaped areas in the subcapsular region (Pl. III, Figs. 9, 10). Frozen sections showed these areas to be rich in lipoid.

(6) Hen 4048 was a bird used for the production of Rous anti-serum. Palpation of a swelling abdomen revealed firm tumorous nodules in the viscera. A liver clearance test gave a gradient change of 45%, and the bird was immediately killed. Post-mortem examination showed an ovarian tumour, and tumours involving the oviduct, mesentery and liver. The ovarian tumour was a well developed thecoma (Pl. IV, Fig. 11) and the oviduct had a primary adenocarcinoma, with secondaries involving the mesentery and liver. The dorsal aorta was atherosclerotic, and the kidneys showed glomerulo-nephritis and arteriosclerotic changes.

(7) A non-layer (22) aged 18 months was tested and found to have a clearance gradient change of 63%. Two months later she was retested and this time

gave a change of 23%. The bird was killed and post-mortem examination showed a multiovular ovary ("grape-cluster") with many ova all at the same stage of development (Pl. IV, Fig. 12). The oviduct was similar to a normal functional organ. The adrenals were enlarged and the liver was fatty. Histological examination showed adrenal cortical hyperplasia. All the other organs were normal apart from the presence of a ciliated cyst in the anterior pituitary, of common occurrence in fowls.

(8) The "normal" ovariectomized hen has a clearance gradient change of under 40%, indicating hyperoestrogenization (See Table I). This may at first seem surprising until it is recalled that the adrenals of female mice ovariectomized at birth later enlarge and produce large amounts of oestrogen, sufficient to cause hypertrophy of the uterus and extensive development of the mammary ducts (Wooley, Fekete & Little, 1941). In the case to be recorded a clearance gradient of 82% was found in an ovariectomized hen (poulard 3030) which began to show certain male characteristics in spur and comb growth and in behaviour some 18 months after operation. At post-mortem examination, a friable pinkish tumour measuring 8.5 x 5.5 x 5 cms., bosselated and with large ramifying subcapsular vessels, was found occupying the site of the rudimentary right gonad. There were secondary tumours in the liver, lungs and spleen, the latter being cystic and almost entirely replaced by tumour tissue. Histological examination showed a fairly well differentiated arrhenoma (Pl. IV, Fig. 13), and the metastases had a similar structure.

(9) ♀ 311, a very fat non-layer, had a clearance gradient of 13.5%, an almost straight line or hyperoestrogenized type of clearance. Post-mortem examination showed it to have a moderate degree of ascites. The liver

was rather fatty and friable. There was a large number of small firm tumours scattered over the serosa of the oviduct, which was of normal size for a laying hen, and the mesentery, intestines and pancreas showed a more diffuse involvement with similar tumours. The ovary contained a number of atretic follicles. Histologically, the oviduct tumours, and those involving the mesentery and intestine were adeno-carcinomata of oviduct origin. Sections of the ovary however showed a very good example of the "folliculum malignum" type of granulosa tumour (von Kahlden, 1895) surrounded by a zone of extremely hyperplastic thecal cells.

Discussion :-

In birds, it is easy to distinguish a BSP. liver clearance influenced by the level of blood oestrogen, from the clearance of an acutely impaired liver (Campbell, 1957b). The gradient change in the early part of the clearance is significantly different in males and females (Campbell, 1957a) and shows no sex difference in immature birds. Moreover the total clearance in the 30 minute period after injection of the dye is efficient and usually practically complete in all these cases. This holds true even for a hyperoestrogenized bird, where the curve may have a zero change in gradient, i.e. it is a straight line clearance. Acute liver damage, on the other hand, results in a very retarded clearance, often characterized by a convex curve, whilst birds with very fatty livers frequently produce a sigmoid clearance curve.

While it is true that male birds with chronic liver damage often have a female type of clearance by reason of the inability to inactivate and excrete oestrogen (Campbell, 1957c), and that these curves are indistinguishable from those produced by oestrogen from other sources, yet usually the poor general condition of a bird with chronic liver

damage will aid differential diagnosis.

This being so, it should be possible to detect quite small hormone-secreting gonadal tumours through their influence on the clearance gradient change characteristic for normal adult birds of either sex. The results detailed here amply justify this hypothesis.

Burrows (1952) writes of human gonadal tumours that it is not safe to draw conclusions on histological grounds alone, regarding the nature of the hormone secreted by a particular tumour. Of two morphologically identical tumours, one may secrete oestrogen, the other androgen, as witness Teilum's androblastoma (1950). Where the amounts of hormone secreted are in quantities sufficient to cause changes in the secondary sex characteristics of the host, no problem arises regarding the correlation of the tumour histology with the type of hormone secreted, but early cases, merely manifested by some disturbance in the normal sex physiology, are not easy to diagnose or to classify as oestrogenic, androgenic or inactive.

As it happens, all the tumours encountered in this series show a straight-forward correlation between histology and activity, as shown by the type of liver clearance demonstrated by the host. For example, all the hyperoestrogenized birds with one exception proved to have either Sertoli, thecal or granulosa tumours, all of which may secrete oestrogen (Campbell, 1951 ; Siller, 1956). Similarly those showing smaller amounts of oestrogen than normal with resultant increase in the gradient change in the BSP. clearance curve proved to have arrhenomata. The exception was ♀ 22 (No. 7) which showed a progressive change to a state of hyperoestrogenization, confirmed by oviduct and adrenal cortical hyperplasia and suppressed ovulation. No tumours were detected, and in this case it seems that the

ovary was responding normally to gonadotrophins, but the ova were all held up at about the same degree of development. The reasons for this are not clear, but excessive oestrogen could be the result of impaired liver inactivation or be of adrenal origin. The liver was certainly fatty, but showed no other histologically detectable abnormality. Here is an example of the difficulty of differentiating cause and effect, since one cannot be certain whether the fatty liver was due to hyperoestrogenization or the cause of it.

Willis (1953) stresses the close relationship between thecal, granulosa and cystic ovarian tumours in the human subject, and the occurrence of all three types in Case 2 (♀ 4499) provides a striking example of the fundamental similarity between avian and human ovarian tumours.

The case of the gynandromorph bilaterally asymmetrical "cockerel" is of special interest. A clear-cut sex-reversal over a period of 8 months was followed by means of the test with complete agreement between the results and the physical changes affecting the bird. The ultimate detection of an ovo-testis with an associated tumour corresponding to Teilum's androblastoma (1950) is an example of the way the test could be useful in identifying the nature of the hormones secreted by an undifferentiated avian gonadal tumour, although of course in this case the diagnosis was facilitated by secondary sex changes unaccountably confined more or less to one side (the right) of the body.

Other findings suggesting hyperoestrogenization are the occurrence of "fibroids" in the oviduct ligament, hyperplastic proliferation of oviduct mucosa, adrenal cortical hyperplasia and heavy lipoproteinaemia. It is tempting to speculate that even the adeno-carcinomata involving the

oviduct in cases 6 and 9 may have been the end result of the actions of excessive oestrogen on a sensitive target organ. Again, case 4 showed a male-type clearance associated with arrhenomata, and the oviduct was atrophic, suggesting lack of oestrogen stimulation. On the other hand, case 1, although showing a male-type clearance associated with an arrhenoma, had a well-developed oviduct with an attached "fibroid", an obvious contradiction, and suggesting in fact an oestrogen action. This could be explained by assuming that the long period of 13 months without ovulation was due to hyperoestrogenization of liver origin, and that this had resolved itself, but not before pituitary gonadotrophins, in an effort to restore the hormonal balance, had stimulated the development of androgen-secreting cells from the indifferent cells of the ovarian cortex.

Summary :-

Following the observation that the rate of clearance, expressed as a gradient change, of sodium bromosulphthalein by the avian liver is a sensitive index to the amount of oestrogen in the blood, it has been found possible to detect early hormone secreting gonadal tumours in either sex, even before any secondary sexual changes become apparent. As a corollary to this, the relation between tumour histology and its oestrogenic or androgenic activity can be readily determined.

All expenses connected with this investigation were borne by the British Empire Cancer Campaign.

REFERENCES

Burrows, H. (1952). Oestrogens and Neoplasia. Chap. XVII. Blackwell, Oxford.

Campbell, J.G. (1951). Brit. J. Cancer, 5. 69.

Campbell, J.G. (1957a). J. Endocrinol. 15. 339.

Campbell, J.G. (1957b). J. Endocrinol. 15. 346.

Campbell, J.G. (1957c). J. Endocrinol. 15. 351.

Greenwood, A.W. & Blyth, J.S.S. (1951). Heredity, 5. 215.

Kahlden, C. von (1895). Zbl. Path. 6. 257. Cited by E. Novak : "Gynecologic and Obstetric Pathology." 3rd edit. (1953), p.408. W.B. Saunders Co.

Siller, W.G. (1956). J. Endocrinol. 14. 197.

Teilum, G. (1950). "Progress in Gynaecology." II. 48.

Willis, R.A. (1953). "Pathology of Tumours." 2nd edit. p.510. Butterworth & Co. Ltd.

Wooley, G.W., Fekete, E. and Little, C.C. (1941). Endocrinology. 28. 341.

PLATE LEGENDS

Plate I

Fig. 1 ♀4799. The ovary appears normal. The oviduct tumour practically obscures that organ. x $\frac{3}{4}$.

Fig. 2 Ovarian arrhenoma from same case. Mallory. x 70.

Fig. 3 The same. x 300.

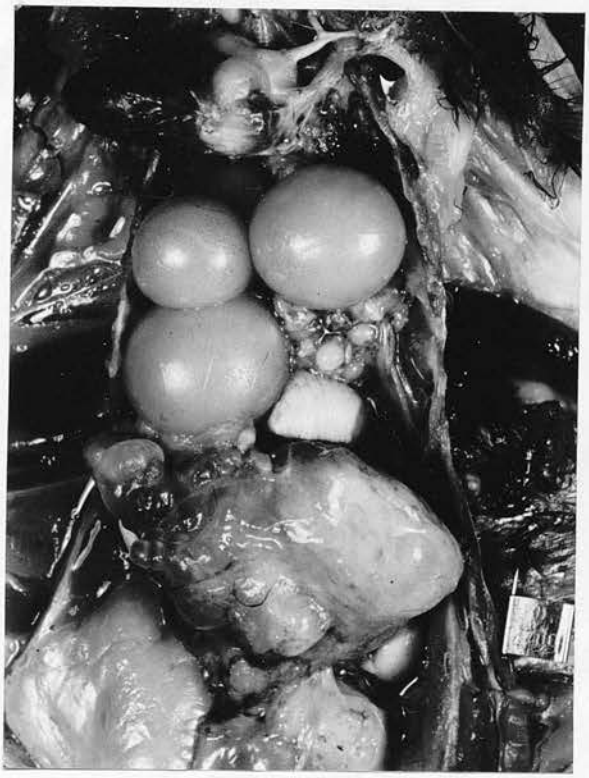


Fig.1

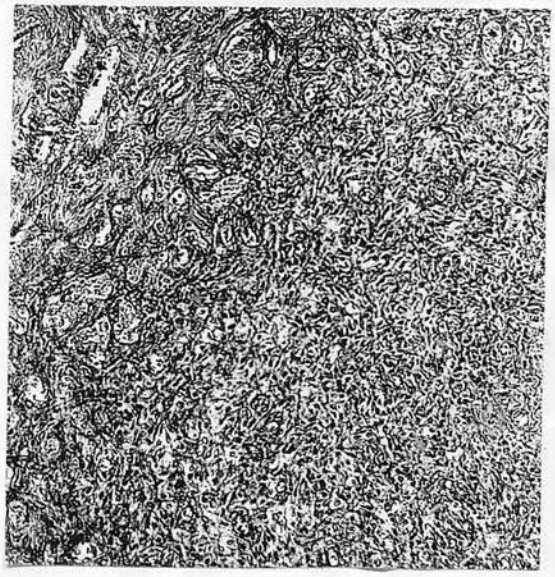


Fig.2

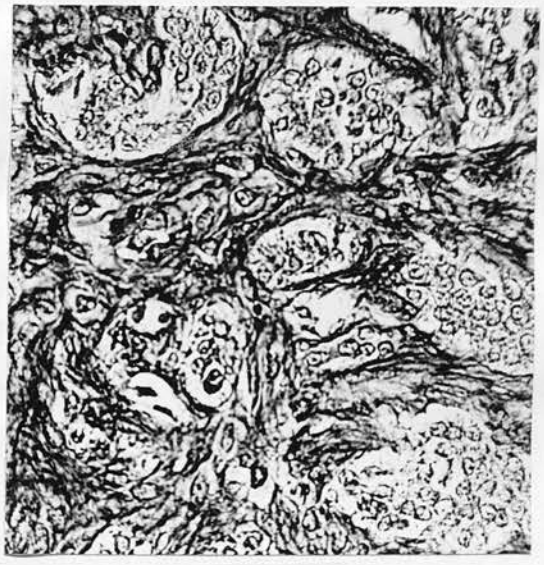


Fig.3

Plate III

Fig. 8 Testis and ovo-testis of the gynandromorph. A well-developed oviduct is present. x $\frac{3}{4}$

Fig. 9 Left gonad of above case, showing a small ovum surrounded by hyalinized thecal tissue, and testicular tubules. H&E x 150.

Fig. 10 Histology of the yellow tumour of left gonad, corresponding to an "androblastoma". H&E x 150.

Fig. 11 ♀4048. Thecoma. H&E x 200.

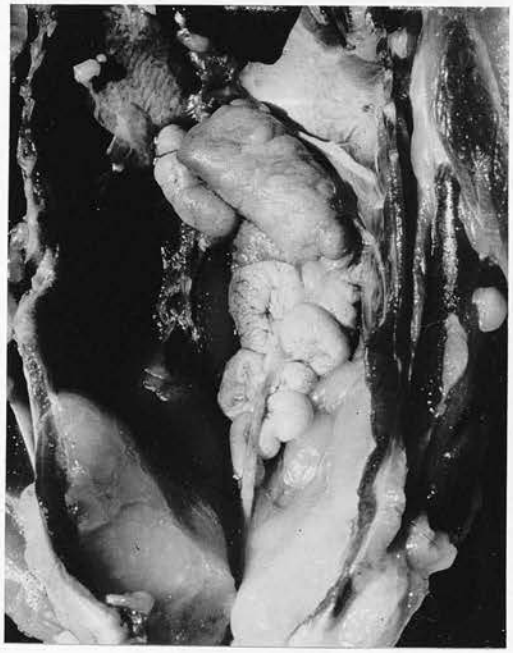


Fig.8

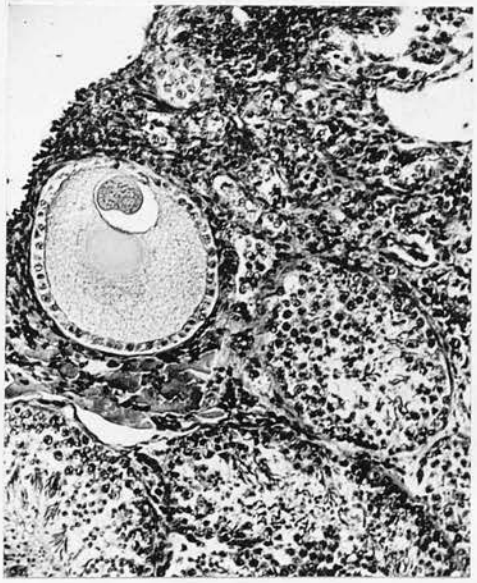


Fig.9

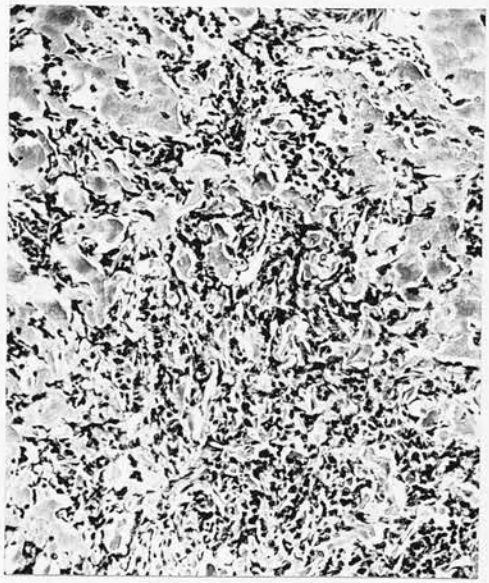


Fig.10

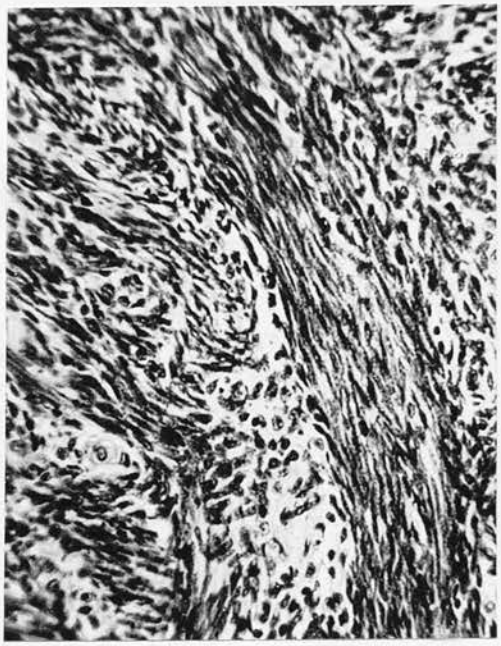


Fig.11

Plate IV

Fig. 12 ♀22. Note the fully developed oviduct in association with the multiovular ovary. x $\frac{3}{4}$

Fig. 13 ♀3030. Structure of an arrhenoma growing at the site of the rudimentary right gonad in an ovariectomized hen. H &E x 300.

Fig.12

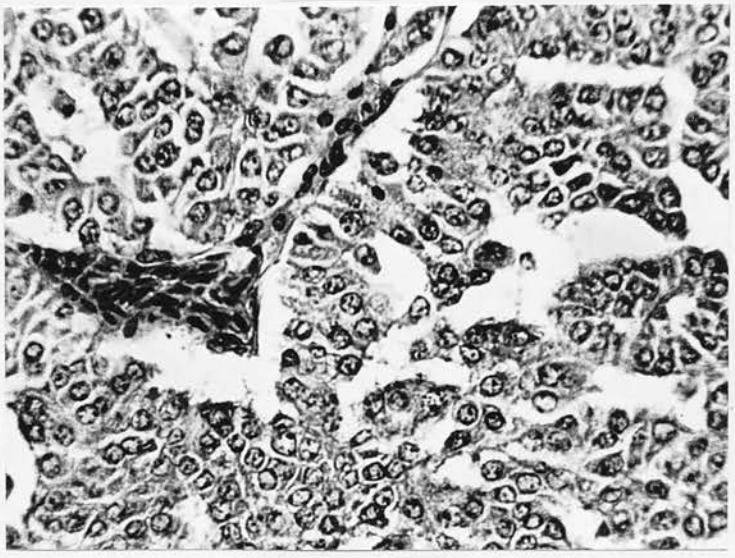


Fig.13

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Recent Investigations on Avian Leucosis and Fowl Paralysis

BY

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AVIAN leucosis, associated as it is with the problem of cancer, offers one of the most challenging problems facing veterinary pathologists to-day. If it became possible at the present time to treat or control this group of diseases, not only would the poultry industry benefit to the extent of some £7,000,000 to £8,000,000 saved annually, as estimated recently by Blaxland (1956), but it is probably true to say that the whole of cancer research would receive a tremendous boost, comparable to that following the discovery of the effects of irradiation on tumours. The leucosis complex, as the term implies, covers a variety of conditions, not all of which, in the speaker's view, should be included. Over the years, workers from the other side of the Atlantic have tended to include neurolymphomatosis, iritis, and osteopetrosis in the complex, not always on the soundest of evidence, and a great deal of confusion has resulted from the general adoption of this scheme. This state of affairs has already been touched upon in previous papers (Campbell, 1954, 1956) and it is not proposed to pursue the matter further at the present meeting. It is sufficient to say that the leucoses proper are considered to be neoplastic conditions arising from haematopoietic tissues, and it is recent studies with these which will be mainly dealt with to-day.

The literature on the leucoses is extremely voluminous, and is widely scattered in a variety of journals, so it is no easy task to keep track with the latest developments. When it is recalled that Hungerford (1951) showed that 69 different synonyms in the English language alone were in popular use when describing these conditions, it is easy to see why this particular field of pathology is inclined to become a morass of confusion in which the worker may become hopelessly bogged down.

As already stated, the leucoses are regarded as being neoplasias of haematopoietic tissue origin, and as such comprise those conditions previously termed leucaemias or sarcomas if they happened to be unassociated with changes in the blood picture. As such, we have the 3 main groups :—

Erythroleucosis,

Myeloid leucosis,

Lymphoid leucosis,

and these may generally be either leucaemic in Virchow's sense, or aleucaemic, or both. In general, however, it may be fairly stated that the erythro form is usually leucaemic, the myeloid form may be both, and the lymphoid form is usually aleucaemic. The rare aleucaemic form of erythroleucosis is termed erythroblastoma, the aleucaemic form of myeloid leucosis may be either diffuse as in the condition affecting the viscera, or it may take a discrete tumorous form known as myelocytoma primarily associated with the bony skeleton of the thorax. The visceral and bony conditions are often present in the same individual. Lastly, the lymphoid form may also be diffuse or discrete, and in the latter case it is called lymphocytoma or visceral lymphomatosis. It is this last term which gives rise to most of the confusion between lymphoid leucosis and fowl paralysis, because the visceral form of the latter is also frequently termed visceral lymphomatosis. The illustrations for a previous article (Campbell, 1956) show certain fundamental differences which in my view justify restricting the term visceral lymphomatosis, well established by usage, to the aleucaemic form of

BURMESTER, B. R., PRICKETT, C. O., & BELDING, T. C. (1946). *Cancer Res.* **6.** 189.
———, GENTRY, R. F., & WATERS, N. F. (1954). *Poult. Sci.* **34.** 609.
———, & WATERS, N. F. (1955). *Ibid.* **34.** 1184.
CAMPBELL, J. G. (1954). 10*th World's Poultry Congress, Edin.* 193.
———. (1954). *Brit. J. Cancer.* **ix.** 163.
———. (1956). *Vet. Rec.* **68.** 527.
CARR, J. G. (1956). *Brit. J. Cancer.* **x.** 379.
COLE, R. K., & HUTT, F. B. (1951). *Poult. Sci.* **30.** 205.
COLES, R. (1954). *Ibid.* **33.** 350.
COTTRAL, G. E. (1954). *Proc.* 89*th Amer. Vet. Med. Assoc.* p. 285.
———, BURMESTER, B. R., & WATERS, N. F. (1954). *Poult. Sci.* **33.** 1174.
DARCEL, C. LE Q. (1950). *Vet. Rec.* **62.** 621.
———. (1953). *Brit. J. Pharmac. & Chemother.* **8.** No. 3. 278.
———, & NEGRONI, G. (1954). Leucaemia Research C.I.B.A. Foundation Symposium.
ELLERMAN, V., & BANG, O. (1908). *Zentralbl. f. Bakt., Parasiten, und. Infektionskr.* **46.** 495.
FOULDS, L. (1934). 11*th Scientific Rep. Imp. Cancer Res. Fund.*
HUNGERFORD, T. (1951). " Diseases of Poultry." Wilkinshaw Co., Sydney.
HUTT, F. B., & COLE, R. K. (1954). 10*th World's Poultry Congress.* 197.
KISSLING, R. E. (1947). *Poult. Sci.* **26.** 74.
LUCAS, A. M., & OAKBERG, E. F. (1950). *Amer. J. Path.* **26.** 75.
MOMMAERTS, E. B., ECKERT, E. A., BEARD, D., SHARP, D. G., & BEARD, J. W. (1952). *Proc. Soc. Exp. Biol. Med.* **79.** 450.
———, BEARD, D., & BEARD, J. W. (1953). *Ibid.* **83.** 479.
OLSON, C. (1941). *Cancer Res.* **1.** 384.
WATERS, N. F. (1954). *Poult. Sci.* **33.** 365.

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AN INFECTIOUS ENTERITIS OF YOUNG TURKEYS ASSOCIATED WITH COCHLOSOMA SP.

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THE relationship of the protozoan flagellate *Hexamita* species to an infectious catarrhal enteritis affecting young turkeys has been the object of an investigation by Hinshaw, McNeil and Kofoid (1938a and 1938b). These workers have reasonably concluded that *Hexamita* species is pathogenic to young turkeys, as it has been found in large numbers constantly associated with bowel lesions, and has proved infective to healthy poults when administered orally. Other flagellates were detected in the bowel contents, notably *Trichomonas* and *Chilomastix*, and amœbæ were also present, but all these were ruled out as causative agents.

No mention of the genus *Cochlosoma* is made in these papers, and as the protozoa and bacteria inhabiting the intestines of the affected turkeys were carefully noted in these American outbreaks, it was presumably not present.

Kimura (1934) and Travis (1938) have both studied *Cochlosoma* species in ducks, magpies and robins. McNeil and Hinshaw (1942), have reported *Cochlosoma* sp. in the intestines of turkeys, but remark that the significance of this parasite is not known.

The purpose of the present paper is to record the discovery of *Cochlosoma* sp. in very large numbers in the intestines of turkeys which were affected with a condition clinically and pathologically indistinguishable from infectious catarrhal enteritis due to *Hexamita* sp. (Hexamitiasis).

The outbreak occurred in early summer, on a large turkey farm in the North-East of Scotland. Young birds were invariably affected, the critical age being from two to ten weeks old. The symptoms reported, and later verified by a visit to the premises, were initially an intense thirst, followed by the development of a watery, frothy diarrhœa, depression, ruffled plumage, drooping head, closed eyes, loss of appetite, weakness, coma and death (Fig. 1). The sick birds congregated about the source of heat and were disinclined to move, but, when forced to, walked with a stilted gait.

The disease appeared to be extremely infectious, spreading rapidly through the pens, so that in a fortnight practically the whole of the young stock, 900 in all, had died. Death usually occurred within 2-3 days of the appearance of symptoms.

Naturally, with such alarming losses, advice was sought, and reports were received from four different sources, two suggesting dietetic errors, one "chilling," and one coccidiosis. Upon visiting the farm, it was obvious that the diet was not to be blamed, and generally the conditions were good, save in two respects —neither the food nor water bowls were protected by a wire cage, so that the young birds were walking in them and soiling the contents with their droppings— and the isolation of young from old stock was by no means perfect.

In all, twenty-eight poults were submitted to post-mortem examination, a number of these being brought alive to the laboratory in order to obtain fresh material.

Examination showed the birds to be in poor condition. The crops were empty; some of the poults had nasal catarrh and slight pulmonary congestion, but the most marked lesions were in the upper digestive tract, the duodenum and jejunum exhibiting a catarrhal enteritis. The bowel contents were watery, and in parts yellow and frothy.

The intestinal tract, as a whole, was atonic, and one of the most characteristic lesions was the presence of dilatations, or bullæ, in the wall (Fig. 2). These bullæ contained yellowish froth, and the mucosa was congested. The cæca appeared to be normal in the majority of birds examined, although two showed a definite congestion of the mucous membrane. The gall-bladders were found constantly to be somewhat distended, and there was an œdematous condition of the connective tissue in this region.

Coccidia were not detected in any of the poults, and no bacteria of any significance were isolated from the intestines, liver or heart blood. Microscopical examination of the heart blood also gave negative results. The possibility of a filterable virus infection was considered, but transmission experiments could not be undertaken because of lack of suitable birds. This, however, is not considered a serious omission, as the nature of the disease does not suggest a virus infection.

Microscopical examination of the intestinal contents revealed little or nothing in birds which had been dead for 12 or more hours. In freshly dead specimens, however, the bowel contents were found to be swarming with a pyriform flagellate, swimming jerkily and with a decided spiral movement. These were especially numerous in the yellow fluid contents of the bullæ, and direct smears stained and examined microscopically showed them to be present almost to the exclusion of any other protozoa.

Attempts were made to cultivate these flagellates in Hartley's broth containing 2½ per cent. glucose over a slope of inspissated ox serum, the whole sealed with paraffin, but in every case bacteria proliferated to such an extent that the protozoa died. In one set of attempts, gentian violet in various dilutions was added to a suspension of intestinal contents until the protozoa were seen to be almost motionless, and then cultures made from this treated suspension, in the hope that the majority of the bacteria would be killed, whilst leaving the flagellates viable, but this, too, was unsuccessful.

These parasites, which appear to belong to the genus *Cochlosoma*, were present in every fresh case examined. A few *Trichomonads* and a few *Hexamita* were also usually detectable, but by far the majority of organisms present were identified as *Cochlosoma* species.

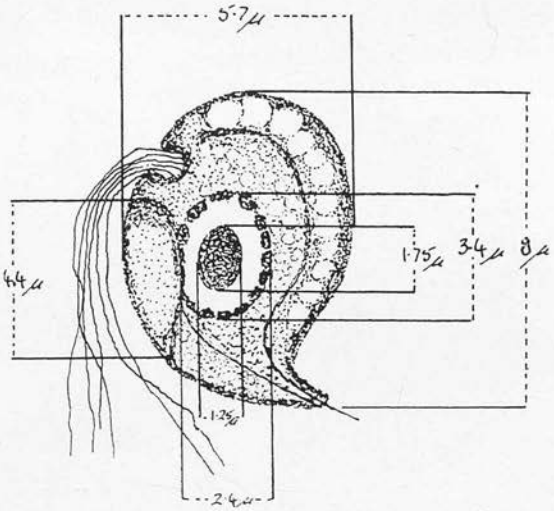
Description of the Flagellate

With dark field illumination, these organisms appear as small periwinkle-shaped flagellates, swimming jerkily with a spiral rotation. A group of flagellæ arises from near the anterior extremity, and trail backwards along the side of the body. The nigrosin dark ground method of outlining the organisms and flagellæ was tried, but in such preparations the flagellæ appeared matted together and it was impossible to estimate the number.

Smears from the bowel contents, stained with May-Grünwald-Giemsa combination, show the main structure of this organism quite well. The drawing (Diag. 1) is based on the study of many individual organisms in several stained

FIG. 1. Typically affected turkey poults, as seen in the field.

FIG. 2. The intestinal tract from a typical case. Note the congested bullæ along the length of the bowel.



DIAG. 1. Drawing of *Cochlosoma* sp. based on the study of stained smears and dark-ground illumination preparations. The dimensions are average figures from fifty individual specimens.

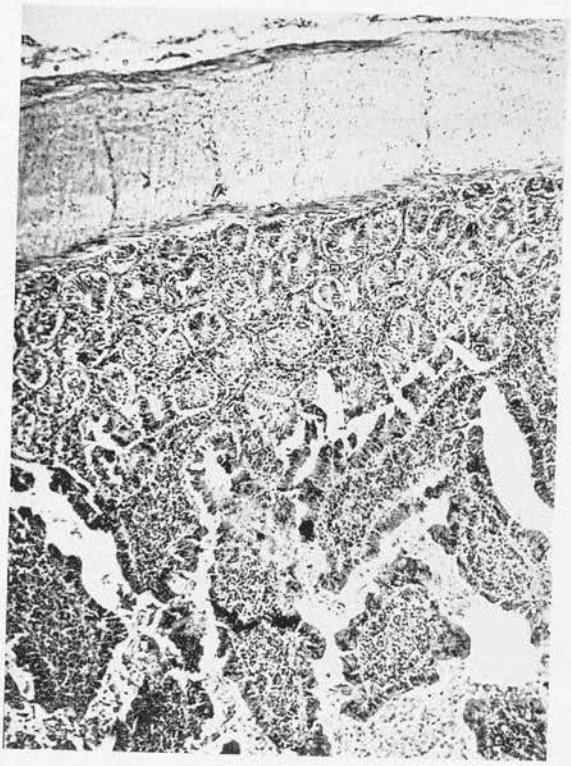


FIG. 3. Tranverse section of affected small intestine. The mucosa is heavily infiltrated, and is sloughing in parts. The débris in the crypts consists of shed epithelial cells and *Cochlosoma* sp. x 80. H. & E.

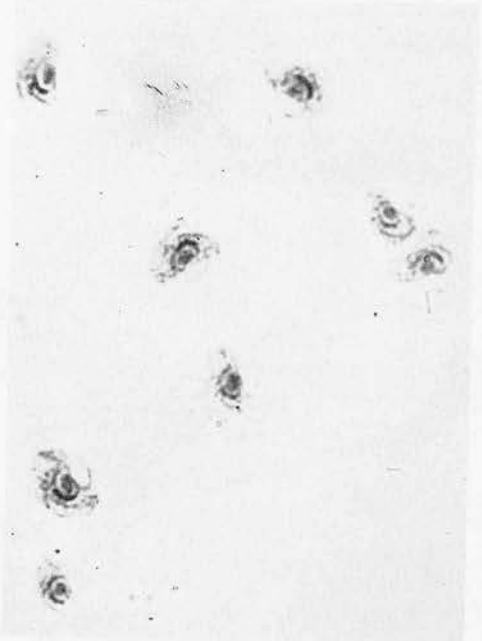


FIG. 4. *Cochlosoma* sp. x 1500. May-Grünwald-Giemsa stain.

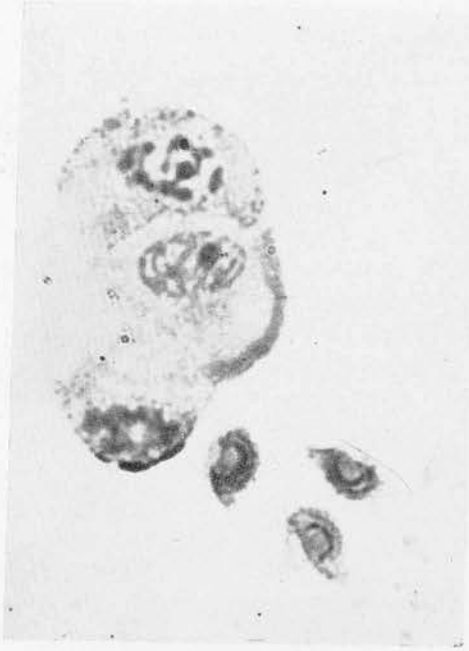


FIG. 5. *Cochlosoma* sp., with desquamated intestinal epithelial cells, for comparison of size. x 1500. May-Grünwald Giemsa.

preparations. With the May-Grünwald-Giemsa method of staining, the cytoplasm is basophilic and vacuolated, the central oval nucleus has a prominent peripheral zone of deeply staining chromatin granules, denser on the dorsal aspect. This thick nuclear membrane encloses a clear hyaline space, in the centre of which is a bright red, oval karyosome. The axostyle is clearly visible as a purple-staining curved structure arising from a primary blepharoplast which is situated antero-ventrally. It curves to the right, i.e., dorsally, following the greater curvature of the cell and passes to the posterior end, where it projects about 2 μ from the cytoplasm. The chromatic basal rod or costa has a similar staining reaction. It arises from a quaternary blepharoplast situated to the left of the nucleus, and curves round to run adjacent to the axostyle. It does not, however, project beyond the curved tail-piece of the organism.

The ventral aspect of the flagellate is provided with a large sucker, appearing in stained preparations as a clear oval area situated in the middle third of the left side. The flagellæ (assumed in the diagram to be six in number, in conformity with all known species of *Cochlosoma*) arise from a small pit at the anterior extremity of the ventral surface. Most of these features, apart from the flagellæ, can be distinguished in the photographs of the organisms.

Histopathology

Fig. 3 shows that the most prominent features of the affected intestine are (a) intense cellular infiltration into the mucosa, and (b) necrosis and desquamation of the epithelium of the villi. Large numbers of the parasite are present in the crypts.

Discussion

The genus *Cochlosoma* is a flagellate belonging to the family Trichomonadidæ and order Protomonadida (Wenyon, 1926). It is of interest to the protozoologist in that it appears to be an intermediate form between the trichomonad flagellates of the Monozoa and the Giardia of the Diplozoa.

For example, the sucker, the two axial fibrils and the centrally disposed nucleus are giardia-like, whilst the flagellæ, arising from a blepharoplast at the anterior end of the body, the presence of a single nucleus, and the axial fibrils, which appear to be homologous with the costa and axostyle, are trichomonad characters.

The only evidence brought forward regarding the pathogenicity of *Cochlosoma* is by Kotlán, who created the generic name in 1923. In one duck he noticed the intestinal wall was swollen and catarrhal at the point where a mass of flagellates were attached, and that the intestinal contents were mixed with blood. Travis (1938) was unable to detect any pathological condition in the infected birds he studied, and he remarks that controlled experiments are needed to demonstrate the effect of these flagellates on their hosts.

Although such experiments were unfortunately not possible in this instance, all the available evidence points to the pathogenicity of *Cochlosoma* to turkey poults under suitable conditions.

Diagnosis can only be made with certainty when freshly dead birds are examined, and in any future outbreak it is to be hoped that transmission experiments will be undertaken.

The diagram shows the main morphology of the flagellate, as seen in prepara-

tions stained with May-Grünwald-Giemsa. It differs mainly from previously published diagrams by Kotlán and Travis in the representation of the nuclear structure (these authors show a simple nucleus, and a very small karyosome) and in the presence of a small, well defined, cup-shaped cavity at the antero-ventral extremity, from which the flagellæ appear to arise. Possibly the primary blepharoplast is associated with this structure.

The diagrams given by these authors also represent the organisms from the ventral aspect. As will be observed from the photo-micrographs appended (Figs. 4 and 5), the organism, being flattened laterally, is always seen from the side in fixed and stained films. This is difficult to reconcile with Travis's description of the organisms as "broadly ovoidal in dorsal or ventral view . . . from a lateral view the body is slender."

It will be seen from Table I. that the dimensions of the *Cochlosoma* sp. found associated with this enteritis of young turkeys agree closely with the figures given by Travis and Kotlán for *C. anatis* and *C. rostratum* respectively. According to Travis, these two are probably synonymous, and so, apart from the slight discrepancy in figures for the nuclear dimensions, which may be due to a difference in interpretation of structure, it appears justifiable to conclude that the *Cochlosoma* species described in this paper is *C. anatis* (Syn. *rostratum*).

TABLE I.

Showing dimensions in microns of *Cochlosoma* sp. from turkeys, as compared with *C. anatis* and *C. rostratum* from North American ducks.

	C. anatis (N. Amer. Ducks). Travis.	*C. rostratum* (N. Amer. Ducks). Kotlán.	*Cochlosoma sp.* (Turkeys, Scotland). —
Length.—			
Mean ...	8.4	8.1	8.0
Range ...	6.5-11.5	6.1-10.0	—
Width.—			
Mean ...	4.9	5.5	5.7
Range ...	4.0-6.0	3.9-6.7	5.5-6.0
Nucleus.—			
Mean ...	2.1	1.4	3.4 x 2.4 (oval).
Range ...	1.2-3.0	1.2-1.7	3.0 x 2.0 - 4.0 x 2.5 (Prominent Karyosome 1.75 x 1.25 μ.)
Length of Sucker.—			
Mean ...	4.8	4.2	4.4
Range ...	4.5-5.0	3.3-4.4	4.0-6.0

Summary

An acute infectious disease of young turkeys indistinguishable from infectious catarrhal enteritis due to *Hexamita* sp. (Hexamitiasis) is recorded, and is believed in this instance to be due to a flagellate protozoan belonging to the family Trichomanadidæ and genus *Cochlosoma* Kotlán. The main features of the morphology of the flagellate are recorded, and its dimensions compared with some other known species.

It is emphasised that a diagnosis of this disease can only be made with perfectly fresh warm material, as the flagellates die and disappear within a few hours of the death of the host. It was impossible, at the time of the outbreak, to obtain young, healthy poults for transmission experiments, but it is hoped that such experiments will be undertaken at some future date.

REFERENCES.

Hinshaw, W. R., McNeil, E., and Kofoid, C. A. (1938a) : "The Relationship of Hexamita sp. to an Enteritis of Turkey Poults." *Cornell Vet.*, 28 : 281.

Hinshaw, W. R., McNeil, E., and Kofoid, C. A. (1938b) : "The Presence and Distribution of Hexamita sp. in Turkeys in California." *Journ. Amer. Vet. Med. Ass.*, 96, (N.S.46) No. 3 : 160.

Kimura, G. G. (1934) : "Cochlosoma rostratum sp. nov : an Intestinal Flagellate of Domesticated Ducks." *Trans. Amer. Micr. Soc.*, 53 : 102-116.

Kotlán, A. (1923) : Cited by Travis.

McNeil, E., and Hinshaw, W. R. (1942) : "Cochlosoma rostratum from the Turkey." *Journ. Parasit.*, 28 : 349.

Travis, B. V. (1938) : "A synopsis of the Flagellate Genus Cochlosoma. Kotlán, with the Description of Two New Species." *Journ. Parasit.*, 24 : 343.

Wenyon, C. M. (1926) : "Protozoology." Vol. I, p. 681. London : Bailliére, Tindall & Cox.

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BANGKOK HÆMORRHAGIC DISEASE OF CHICKENS: AN UNUSUAL CONDITION ASSOCIATED WITH AN ORGANISM OF UNCERTAIN TAXONOMY

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(Plates LXXII-LXXIV)

In the course of the routine post-mortem examination of poultry in Bangkok, six cases of an unusual disease were observed. There was a common history of several birds in a flock becoming ill and dying within a few hours, and it was unfortunate that in every instance it seemed that only the last bird to die was brought for examination. Previous bodies by that time had been burnt, buried or otherwise disposed of. The outstanding feature in all the birds examined was hæmorrhagic petechiation of the skin, muscles and viscera, and since blood films constantly showed numerous small organisms, it is certain that many previous cases had been reported as fowl cholera or pasteurellosis.

The pathology of all these fowls was similar, and it is unnecessary to record each in detail. Instead, three cases selected for the freshness of the material will be described and a general description of the histopathology will be given, with an account of various attempts to cultivate the organism and to transmit the disease experimentally.

Pathology

Case 1. A four-months-old Light Sussex pullet, the third to die within 2 days in a small flock, collapsed suddenly and died in convulsions in about 10 minutes; it was brought straight to the laboratory for examination. An unusual feature of the still warm bird was the presence of many hæmorrhagic spots shining through the scales investing the featherless part of the legs. Similar hæmorrhages were found in profusion in the subcutis, muscles and practically all the viscera. Mixed with these foci were creamy spots about the size of millet seeds, constituting some 50 per cent. of the total lesions. The brain substance was normal macroscopically, but there was a severe purulent meningitis.

* The material on which this paper is based was collected while the author was serving in an advisory capacity with the Government of Thailand (Siam) in the Department of Livestock Development, Bangkok, during the tenure of an appointment with the Food and Agricultural Organization of the United Nations.

Case 2. The bird was a two-months-old White Leghorn cockerel, examined while moribund. Small hæmorrhagic foci were visible beneath the scales of the legs. Post-mortem examination showed many petechiæ in the subcutis, muscles, liver, lungs, heart, kidneys, proventriculus and intestine. An extensive intraperitoneal hæmorrhage due to rupture of the liver was the immediate cause of death. The spleen was small and pale. Large numbers of *Ascaridia galli* were present in the jejunum-ileum, associated with a mucoid enteritis. A purulent meningitis was present.

Case 3. This bird was a freshly dead Light Sussex cockerel, aged four months, one of several that had died within a few days of each other. Again the most striking external feature was the presence of hæmorrhages beneath the leg scales. Similar lesions with a substantial proportion of creamy spots were present in the subcutis, muscles, liver, spleen, thymus, thyroid, adrenals, testes, proventriculus and intestine. The kidneys showed gross hæmorrhages, while the spleen was swollen and mottled grey, resembling an organ affected with lymphoid leucosis. The lungs were much congested and contained hundreds of small cream-coloured lesions. A mucoid enteritis was associated with a mixed tape- and round-worm infestation (*Raillietina* and *Ascaridia* sp.). The marrow was hypertrophic but pale. The myocardium, cerebrum and cerebellum appeared normal, but meningitis was present, and small lesions about 1 mm. diameter were scattered over the dura. Removal of the purulent exudate revealed patchy meningeal congestion, especially prominent over the dorsal aspect of the cerebellum. All the affected viscera and muscles showed small creamy lesions intermingled with hæmorrhagic foci.

Histopathology

Tissues taken for examination were fixed in 10 per cent. formal-saline and processed in the usual way; paraffin sections were cut at 6-8 μ. Sections were stained routinely with Ehrlich's acid hæmatoxylin and eosin, but other staining methods were used later, such as Gram's method and per-iodic acid-Schiff modified according to Kligman and Mescon (1950). Blood films, marrow and tissue impressions and smears from the meninges were all fixed in methanol and stained with the May-Grünwald-Giemsa combination. In addition, wet blood preparations were examined, but unfortunately the necessary equipment for dark-field and phase-contrast study was not available.

Blood films show large numbers of small bodies resembling toxoplasma. These measured $1{\cdot}5 \times 2{\cdot}0$ μ, but as far as could be ascertained they were always extracellular. In the fresh state they are pyriform, non-motile and refractile. Stained preparations show a deeply basophilic lobed nucleus and pale blue cytoplasm. They occur as either dispersed or aggregated particles, the former being the more common (figs. 1 and 2). When they are clumped, a suggestion of a network of ground substance enmeshing the cells can be made out. Marrow smears, tissue impressions and smears from the meninges all show

similar organisms, but they are particularly plentiful in the meningeal preparations (fig. 3).

Sections of spleen show large numbers of the organisms, mainly concentrated in foci in the white pulp or germinal centres. Fig. 4 shows them lying within the cytoplasm of macrophages, where they probably undergo multiplication to form minute cyst-like structures, with the macrophage nucleus displaced and compressed at the periphery of the cell (fig. 5).

Wandering cells in the red pulp of the spleen are also found to contain the organisms. Groups of large cysts of somewhat variable size (average diameter 0·5 mm.) also occur in the spleen. They have a well-defined wall and are packed with many thousands of organisms. There is little evidence of tissue reaction around these cysts, which are the small cream-coloured bodies visible macroscopically.

The lung is particularly rich in such cysts (fig. 6). Various stages of development may be identified, from immature cysts to mature bodies which rupture, with liberation of great numbers of minute organisms into the surrounding tissue. Discharged cysts usually contain blood, and faintly-staining threads of material which can be stained much more deeply by the per-iodic acid-Schiff method. The final stage is collapse of the cyst wall and absorption by means of foreign-body giant cells (figs. 6, 7, 11 and 14). The lungs show localised pneumonic changes.

Owing to the loose texture of the lung tissue, cysts reach their most perfect state of development in that organ. They contain a uniformly packed mass of small bodies similar to those occurring free in the blood, narrowly demarcated from the cyst wall by a space which may be an artifact due to shrinkage. Occasionally this space consists of a peripheral row of vacuoles. The cyst wall is faintly laminated and externally is closely invested with endothelial cells (fig. 13).

The absence of tissue reaction round intact cysts is remarkable. In one instance only, a peculiar deeply-staining spindle-shaped body of uncertain nature with a clear transverse septum was found lying amongst the contents of a cyst in the lung (fig. 12).

Similar cysts in various stages of development are found in the subcutis, striated muscle (fig. 8) and kidney (fig. 9), which also shows a proliferative glomerulitis with hyaline thickening ; in the adrenal, where discharged cysts are often surrounded by hæmorrhage (fig. 10) ; in the pancreas, which shows a massive histiocytic infiltration into the islets, and in the testis, liver, proventriculus, intestine and heart. The thymus shows cysts and a patchy necrosis with many giant cells. A careful search in the lung and in other infected organs failed to show any microscopic cysts such as are occasionally encountered in the spleen, but this does not rule out their presence, and indeed it seems unlikely that they should be limited to this one organ. A thorough search of representative brain sections failed to reveal any

parasites. The lesions are apparently confined to the meninges. However, a group of cysts was found within the optic nerve in one case.

Cultivation and transmission experiments

Various attempts at cultivation and transmission were made. These included the inoculation of blood rich in parasites into various forms of nutrient broth, inoculation of Sabouraud's medium and Novy, Macneal and Nicolle's medium (Mackie and McCartney, 1948) with blood, marrow and splenic material, and the injection of blood and marrow into the chorio-allantoic membrane of 10-days-old fertile eggs.

Efforts were also made on two occasions to transmit the disease by the intravenous injection of blood from a fresh case into 8-weeks-old chickens. All such attempts gave negative results, although the cultures were subjected to prolonged incubation and the injected chickens were kept for several months before being killed and examined.

Discussion

Diseases of man and animals, including birds, caused by organisms of uncertain taxonomic affinities are well known and in the main have been adequately described in standard works of reference. Examples of such diseases are sarcosporidiosis, occurring in sheep, pigs, cattle, horses, various birds including the duck and chicken, and occasionally in man ; toxoplasmosis, affecting a large number of mammals and birds and only comparatively recently recognised as a disease affecting man ; rhinosporidiosis, a disease of man and horses ; histoplasmosis, affecting man and caused by an organism which Wenyon (1939) has suggested may be related to toxoplasma, though now it seems to be generally accepted as a fungus ; and coccidioidomycosis, due to a known fungus with an obscure life-history (Dickson, 1938).

The organisms associated with these conditions have two main features in common. First, little definite is known about their biology or relationships, and second, they all exhibit morphological characteristics which in the past have caused both protozoologist and mycologist to lay claim to them.

Sarcosporidia, for example, first described by Miescher (Wenyon, 1926, p. 768) in the muscles of a mouse, was regarded by him as a protozoon and named Synchytrium. As this name was already in use for a group of fungus-like organisms, the new organism was renamed Sarcocystis by Lankester (1882). Wenyon, however, stated that sarcosporidia are probably fungi and this seems to be confirmed by the work of Spindler and Zimmerman (1945) and Spindler (1947), who claim to have cultured a fungus belonging to the genus Aspergillus from sarcocysts removed aseptically from muscles. They describe hyphæ-like structures within the Miescher's sacs, with spores or Rainey's corpuscles attached to the septa. In passing, it is worth noting that Wenyon mentions the occasional appearance of sarcosporidial spores free in the blood ; also that mature sarcocysts excite no inflammatory response by the tissues in which they are embedded, though old degenerating ones cause a reaction (Mathews, 1930).

Plate LXXII

Fig. 1.—Blood film showing parasites. Giemsa. ×2500.

Fig. 2.—Groups of parasites in the blood, lying in a matrix containing a single large nucleus. Giemsa. ×2500.

Fig. 3.—Smear from meningeal exudate showing large numbers of parasites. Giemsa. ×2500.

Fig. 4.—Section of spleen (white pulp) showing grouping of parasites in the cytoplasm of macrophages. Hæmatoxylin and eosin. ×800.

Fig. 5.—Small cyst in splenic white pulp. Probably a macrophage whose nucleus has been displaced to the bottom right by proliferation of the contained organisms. H. and E. ×1000.

Fig. 6.—Group of large cysts in various stages of maturation in the lung. The empty (discharged) ones contain red blood cells and faintly staining hypha-like material. H. and E. ×100.

BANGKOK DISEASE OF CHICKENS

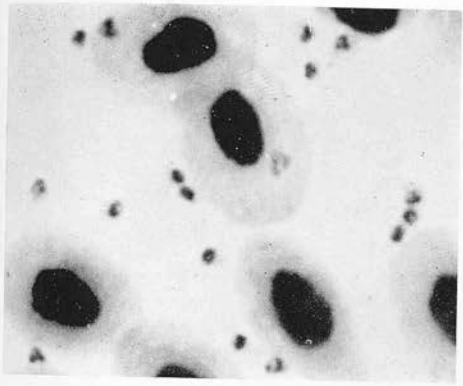
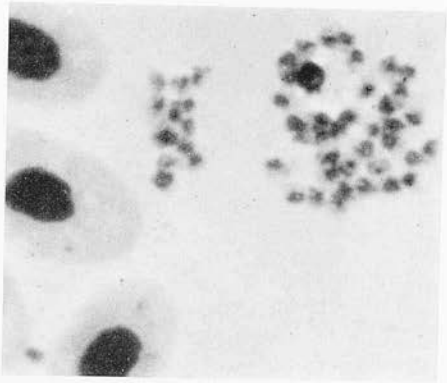


FIG. 1



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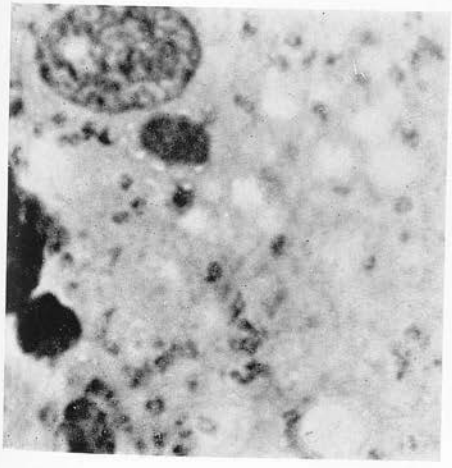

FIG. 3

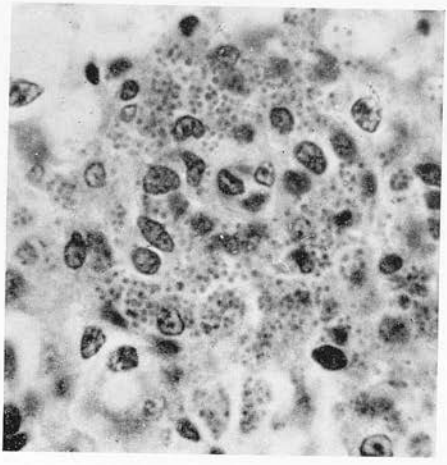

FIG. 4

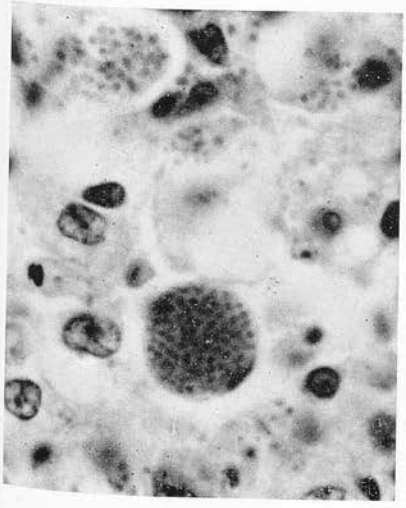

FIG. 5

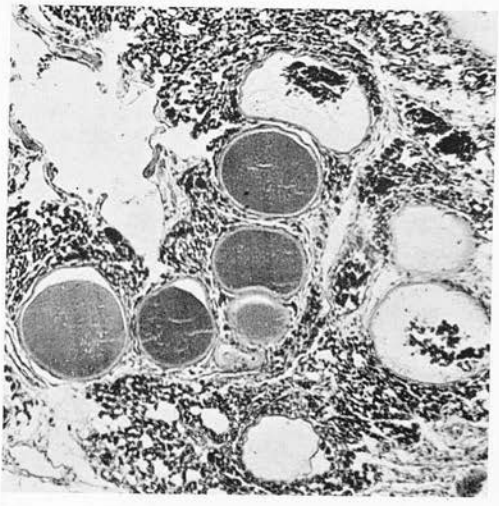

FIG. 6

Plate LXXIII

Fig. 7.—Hypha-like contents of discharged cyst in lung: packed red cells below. Per-iodic acid-Schiff. ×350.

Fig. 8.—Group of discharged cysts undergoing absorption in skeletal muscle. The striations of the cyst walls are microtome artifacts. H. and E. ×80.

Fig. 9.—Large group of cysts in kidney, the darkly staining contents of some of which indicate immaturity. Two partially discharged cysts at top. H. and E. ×80.

Fig. 10.—Remains of cyst in an adrenal gland. Note surrounding extravasated blood. H. and E. ×80.

Fig. 11.—Giant-cell reaction around old partially absorbed cyst in lung. H. and E. ×250.

BANGKOK DISEASE OF CHICKENS

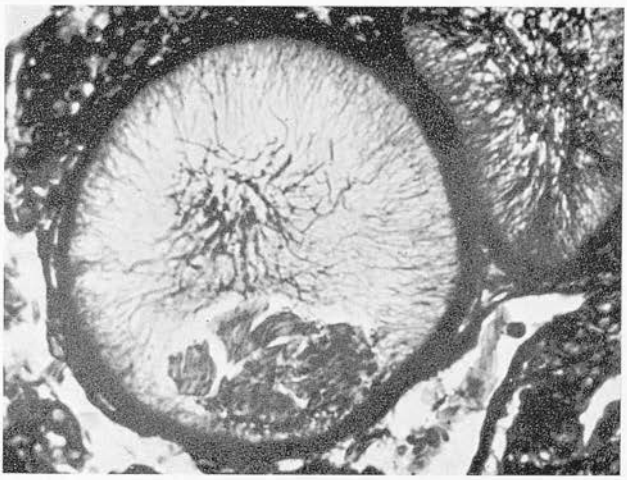

FIG. 7

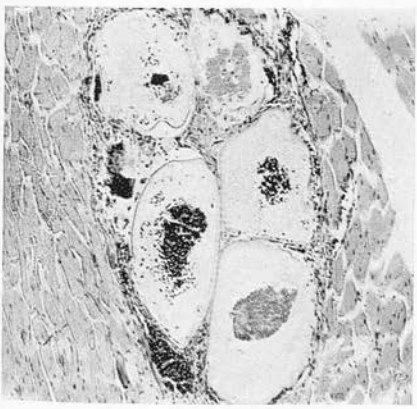

FIG. 8

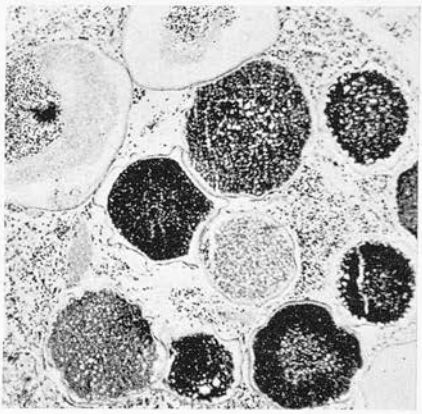

FIG. 9

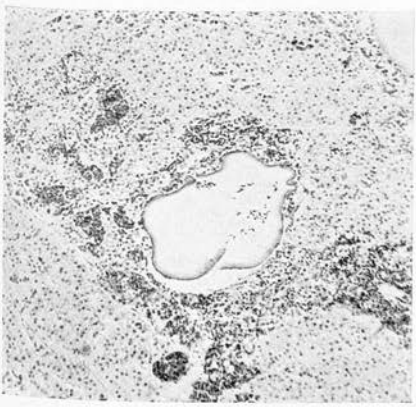

FIG. 10

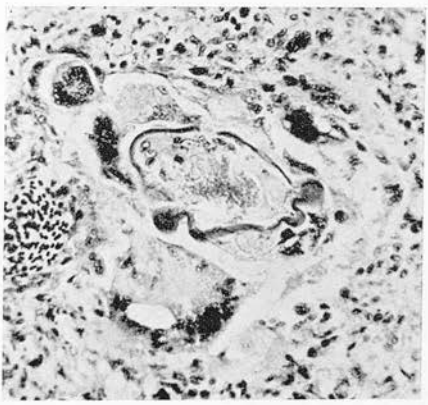

FIG. 11

BANGKOK DISEASE OF CHICKENS

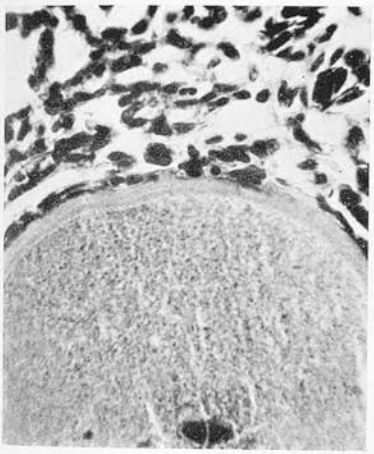

FIG. 12.—Portion of mature lung cyst showing peculiar body of unknown nature at bottom. H. and E. ×600.

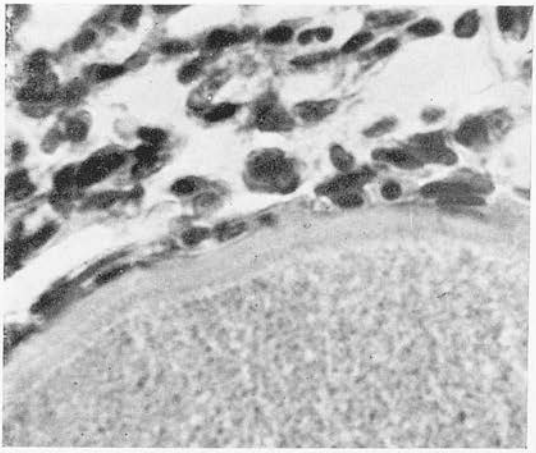

FIG. 13.—Details of part of cyst in the lung, showing structure of the wall and the contained endospores. H. and E. ×1900.

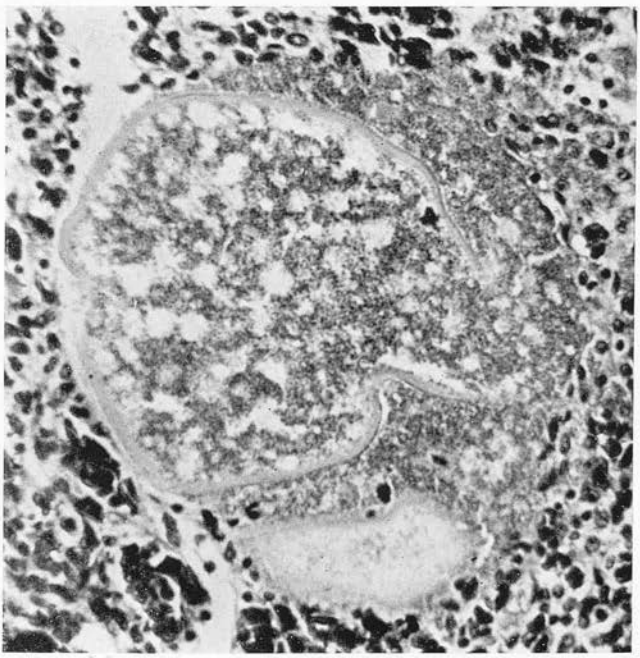

FIG. 14.—A ruptured and discharging cyst in the lung, with liberated endospores. H. and E. ×500.

Again, the organism associated with rhinosporidiosis was considered by Seeber (Wenyon, 1926, p. 777) to be a coccidial parasite. Ashworth (1922-25), however, reported obtaining a slow growth on Sabouraud's medium after seeding with *Rhinosporidium seeberi*, and claimed that it was a yeast or phycomycete. Manson-Bahr (1950), on the other hand, quotes authorities who say that it is probably a protozoon.

The toxoplasmas especially offer great difficulties to the taxonomist. A remarkable feature of these parasites is an almost complete lack of host specificity; even cold-blooded animals have been reported as hosts. Many workers are inclined to consider that all the toxoplasmas reported belong to the same species, and that *Toxoplasma gondii* (Nicolle and Manceaux, 1909) is alone valid. The position of toxoplasmosis of birds is even more obscure. Until recently it was regarded as being due to the same parasite, but serious doubt is now expressed whether many of the recorded cases are really due to toxoplasma, or whether the organisms seen are similar to but not identical with it. Coulston (1942) claims that the so-called toxoplasma of sparrows probably consists of coccidial merozoites which have spread from the intestine via the blood to the liver, spleen, lungs, brain and kidneys, where they are found localised within leucocytes. The coccidial origin of avian toxoplasma also seems to receive support from the observation of Manwell (1941), who has described avian toxoplasma as undergoing schizogony, whereas the mammalian type reproduces by binary fission. This observation is confirmed, with certain reservations, by Manwell *et al.* (1945), who also conclude that what has been called avian toxoplasma, though morphologically similar in some respects to toxoplasma of mammals, is not infective for the latter, appears to differ in certain fundamental characteristics and is probably coccidial in nature. Certainly the photographs illustrating their paper show that the avian " toxoplasma " resembles coccidial merozoites very closely. Yet another confusing factor is the presence in the lungs of birds such as the canary of " x-bodies " (Einschlüsse) which strongly resemble toxoplasma.

Another organism of undetermined position is Globidium. Wenyon (1926, p. 769) considers it to be probably related to the sarcosporidia. Globidium forms quite large cysts (up to 5 mm. in diameter) embedded in the alimentary tract or skin of horses. The wall of the cyst is radially striated and contains a flattened nucleus, and it is considered that it represents the remains of an enormously hypertrophied cell. The spores of Globidium are elongated fusiform structures.

Coccidioides immitis, described by Dickson in man as a thick-walled sporangium 10-80 μ in diameter, is similar in some respects to the organisms under discussion. A generalised infection may lead to involvement of any of the abdominal viscera and chronic meningitis is a frequent complication.

It seems evident that the parasite causing Bangkok disease belongs to this group of poorly understood organisms. The possibility that it is a toxoplasma has always been kept in mind, but in view of its constant extracellular occurrence in the blood, the non-occurrence of binary fission, and the presence in the body of prominent cysts filled with the organism, it obviously does not closely resemble toxoplasma as described in the literature.

Although the few attempts to cultivate the organism resulted in failure, it is felt that indirect evidence points to a mycotic rather than a protozoal nature. In sum, this evidence is as follows :—

1. The cysts closely resemble sporangia and stain well by the per-iodic acid-Schiff method, which has been claimed as a specific

stain for pathogenic fungi in tissue. The writer finds, however, that avian coccidia, especially the merozoites in material from cæcal coccidiosis of the chicken, stain brilliantly by the modified Feulgen technique, *i.e.* these organisms must, on the theory of action of this stain, contain polysaccharides in their make-up.

2. The structure of the cyst wall does not resemble that of protozoal cyst walls, which are homogeneous.

3. The nucleus in the wall seems to indicate that the cyst is derived from an enormously hypertrophied host cell, whose nucleus has been pushed to the periphery by the multiplication of the cellular contents.

4. Hypha-like filaments, forming small septa, are present in the cyst and are particularly evident after the discharge of its contents.

5. The high refractility of the fresh unstained organisms in the blood is not typical of protozoa, but rather indicates a vegetable nature.

It seems possible that this organism is a dimorphic fungus, normally saprophytic, and that a simple unicellular form is developed in animal tissues, adapted for rapid reproduction and invasion. The source of infection may be the vegetating saprophyte, from which air-borne spores may be inhaled. The frequency of lung involvement with its heavy infection and the presence of old healing lesions are points in favour of infection by inhalation.

Until more knowledge regarding this parasite is obtained, it is suggested that " Bangkok hæmorrhagic disease " is primarily a mycotic infection of the lungs which may and frequently does become systemic, leading to a generalised hæmorrhagic mycosis and fatal meningitis. It is also considered that the organism shows many affinities to the particular group which includes sarcosporidia, rhinosporidia and coccidioides, and to a lesser degree the highly debatable avian toxoplasma.

Summary and conclusions

A fatal infection affecting chickens a few months old, characterised by general petechial hæmorrhages, small cysts in the tissues and meningitis, is described. It is associated with the presence of organisms in the blood which bear some resemblance to toxoplasma, but differ from them in that they are always extracellular and non-motile, and in showing little evidence of binary fission. Similar organisms occur in the tissues, where they may be intracellular, especially in the splenic macrophages, where they appear to proliferate. It is thought that parasitised macrophages trapped in various organs develop into large cysts which, upon maturation, discharge their spores into the tissue spaces and the lymph and blood streams.

Infection may be by inhalation, since the oldest lesions are found in the lungs, which are always severely involved.

The organism is considered to be a unicellular phase of a dimorphic fungus which is normally a saprophyte but can become adapted to

a parasitic existence, and that it is possibly related to the debatable group which contains sarcosporidia, rhinosporidia and coccidioides.

I wish to thank Khun Vichitr Pahanakarn, Director General of the Department of Livestock Development, Bangkok, for permission to publish the results of this investigation, which was mainly carried out in his Department.

REFERENCES

Ashworth, J. H.	1922-25.	*Trans. Roy. Soc. Edinb.*, liii, 301.
Coulston, F.	1942.	*J. Parasitol.*, xxviii, suppl., p. 16.
Dickson, E. C.	1938.	*J. Amer. Med. Assoc.*, cxi, 1362.
Kligman, A. M., and Mescon, H.	1950.	*J. Bact.*, lx, 415.
Lankester, E. Ray	1882.	*Quart. J. Micros. Sci.*, xxii, 53.
Mackie, T. J., and McCartney, J. E.	1948.	Practical bacteriology, 8th ed., *Edinburgh*, pp. 186-188.
Manson-Bahr, P. H.	1950.	Manson's Tropical diseases, 13th ed., *London*, p. 712.
Manwell, R. D.	1941.	*J. Parasitol.*, xxvii, 245.
Manwell, R. D., Coulston, F., Binckley, Ellen C., and Jones, Virginia P.	1945.	*J. Inf. Dis.*, lxxvi, 1.
Mathews, F. P.	1930.	*J. Amer. Vet. Med. Assoc.*, lxxvi, 705.
Nicolle, C., and Manceaux, L.	1909.	*Arch. Inst. Pasteur Tunis*, p. 97.
Spindler, L. A.	1947.	*Proc. Helm. Soc. Washington*, xiv, 28.
Spindler, L. A., and Zimmerman, H. E., Jr.	1945.	*J. Parasitol.*, xxxi, suppl., p. 13.
Wenyon, C. M.	1926.	Protozoology, *London*, vol. i, pp. 768, 769 and 777.
„	1939.	*Trop. Dis. Bull.*, xxxvi, 584.

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A POSSIBLE NEW SPECIES OF TREMATODE PARASITE IN THE KIDNEYS OF THE KING PENGUIN (Aptenodytes longirostris).

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and
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THREE years ago, whilst engaged in a post-mortem on the body of a king penguin from Edinburgh Zoological Gardens, it was noticed that the ureters and their main intra-renal branches were rather prominent, and that they contained a whitish muco-purulent material. Microscopical examination of some of this material showed it to consist of granular leucocytes, epithelial cells and mucus. It was whilst examining this that a typical trematode egg was discovered (FIG. 1). Further search revealed numbers of eggs, especially numerous at the proximal ends of the ureter and in its main branches in the kidney. Several small flukes were also found lying free in the lumina, and dissection of a kidney with the aid of a lens, showed many similar parasites lying in cyst-like diverticula in the distal ends of the ducts of Bellini, or in the medullary rays.

Since then, the bodies of five more king penguins have been examined, and in all of these, similar parasites have been found in the kidneys, sometimes in large numbers and all in various stages of development. Blocks of tissue from various organs of all these birds were taken for histological examination, but only the kidneys were found to contain flukes.

A summary of the post-mortem findings in the birds is given below.

King Penguin 31,287 (*No. I*).—*Imported April*, 1936. *Died* 21/8/40.

This bird had pneumomycosis (**Aspergillus* sp. isolated) affecting all the main body air-sacs, the trachea and lungs. There was a mass of granulomatous tissue on the inner aspect of the sternum. Kidneys and ureters contained muco-purulent exudate and flukes.

K.P. 31,289*a* (*No. II*).—*Imported April*, 1936. *Died* 7/11/41.

The usual kidney lesions were present. The liver was enlarged and histological examination showed a certain degree of hæmatopoiesis. *Bacillus coli* was isolated in pure culture from the heart blood.

K.P. 31,611 (*No. III*).—*Hatched July*, 1936. *Died* 14/11/41.

Post-mortem examination showed it to have a greatly dilated left atrium and right side of the heart. There was intense general venous congestion. The mitral valve had several fibrous scars on its free border capable of causing incompetence. Kidneys and ureters as above.

K.P. 32,758 (*No. IV*) *omitted.—See paper.*

K.P. 31,289*b* (*No. V*).—*Imported April*, 1936. *Died* 9/8/42.

This bird had been suffering from severe abscesses involving the pads of both feet. *Staphylococcus aureus* was isolated from the caseous contents of these abscesses. The usual kidney lesions were present.

K.P. 32,767 (*No. VI*).—*Hatched September*, 1937. *Died* 27/8/43.

This bird had definite nephritis. Scores of flukes were found in the kidneys.

There were deposits of urate crystals in the pericardial sac, and an aneurysm of the left cranial vena cava was present.

Histopathology of the Kidney

Histological preparations showed, in every case, that the flukes tended to occur in pairs in cyst-like spaces. The wall of the cyst varied in its structure according to the site of the parasite, which was invariably either in the main ureter branches, in the ducts of Bellini, or in a greatly dilated collecting tubule in a medullary ray. Lesions were confined to the immediate vicinity of the fluke, the rest of the kidney parenchyma remaining normal. The medullary rays are normally demarcated from the rest of the kidney by a thin sheath of connective tissue. When the parasite establishes itself in one of the collecting tubules it gradually compresses all the surrounding tubules in the ray as it grows. Thus the fluke is usually seen lying in a cavity conforming to its own shape, whose walls are composed of greatly compressed non-functional collecting tubules, often appearing as little epithelial-lined vesicles containing a homogenous acidophilic substance. Outside this area of compressed tissue is the connective tissue sheath.

At a later stage, there is an inflammatory infiltration of cells into the cyst wall, due to chronic irritation. These cells are mainly eosinophils, histiocytes and lymphocytes. Scattered about one also sees occasional erythrocytes, and, later, fibroblasts, the whole being enmeshed in a network of fibrin. Briefly, there is set up a fibro-granular reaction (FIG. 3).

Similar changes occur in the walls of the ducts of Bellini and in the branches of the ureters, with the modification that here the epithelial lining sloughs away, and the lumina of these ducts are filled with débris consisting of granular cells—mainly true eosinophils, histiocytes, lymphocytes, epithelial cells and mucus. The fluke appears to ingest this material (FIG. 4). Small nodes of lymphoid cells are often found in the walls of the cysts.

Pathogenicity

Examination of the post-mortem findings in all the penguins in which the fluke was found shows that in nearly every case there was some unassociated condition which could well account for death. Moreover, the histology of the kidneys does not reveal pathological changes sufficient in themselves to cause fatal illness. No doubt, however, the presence of the parasites plays a part in lowering the resistance of the birds to infection, and it seems likely that a very heavy infestation causes nephritis severe enough to produce death, even in the absence of other conditions.

Description of the Fluke and its Egg

A detailed account of the morphology of the parasite will be given elsewhere at a later date by one of us (J.E.N.S.), so only its main features will be described here.

The flukes are cream-coloured, small, and flattened—mature specimens 2.5 to 3 mm. in length by about 1 mm. in breadth at the widest part, which is in the posterior third. Such specimens usually have a brown area near their centre, easily seen with the naked eye.

Stained and mounted specimens (FIG. 2) show well-marked oral and ventral

suckers, though the latter is often half-hidden by a mass of eggs in the uterus. There is a well-developed muscular pharyngeal bulb beyond the oral sucker, and the alimentary tract soon branches into two intestinal cæca. The vitelline glands lie laterally, occupying the middle three-fifths of the body. The rest of the body cavity is filled up with the uterine coils, testes, ovaries, etc., the exact disposition of which will be described later. The epidermis consists of hexagonal scales from the centre of which arise sharp spines.

The egg is operculate, bilaterally symmetrical and light-brown in colour. It has a thick shell, and is embryonated. Its average size is 36.5 x 17.6 μ.

Discussion

In the upper penguin pool at the Zoological Gardens, where most of the deaths took place, Gentoo penguins and King penguins are mixed. A number of the former have also been examined very thoroughly and in one a few flukes have been found in the kidneys. Recently an immature worm has been detected in the kidney of a Blackfooted penguin from another pool lower down. In the former case the parasites have only been examined in sections—no complete specimens were obtained, so it is impossible to say whether they were similar species, whilst in the latter, the immaturity of the single fluke found also made comparison difficult. In both cases, these birds, whose natural habitat is South Georgia, were hatched in the Zoo.

The problem of the mode of infection naturally presented itself very early in the investigation. Obviously there are interesting possibilities. In the first place, if the birds came already infected from the Antarctic, then the flukes must have found a suitable intermediate host in the Zoo pools—almost certainly a snail. Against this theory it must be remembered that the snails in the ponds are fresh-water varieties, whereas in their natural surroundings the penguins live on marine crustacea and cephalopoda. Therefore, if the original infection was brought about by eating a crustacean or cephalopod acting as an intermediate host—then the fluke must have been capable of radically changing its life-cycle to suit the different fauna of a fresh-water pool.

The second possibility is that the infection arises from the ingestion of infected herring. The metacercarial stage of the parasite may be encysted in the tissues of these fish, on which the penguins are fed. If this is the case, it is most likely that the host of the fluke is some fish-eating bird of the northern ocean, and here, again, the problem of adaptation is raised. This theory, if correct, eliminates the possibility of a life-cycle in the penguin pools.

Examination of the fauna of the pools has not, so far, thrown any light on the problem, as they have recently been cleaned out and aquatic snails are difficult to find. The only other forms of fresh-water life occurring in any abundance are water-fleas (Daphnia pulex) and so-called "fresh-water shrimps" (Gammarus pulex). A few sticklebacks are also present.

Summary

The trematode recorded in the foregoing paper belongs to the genus Renicola (Cohn).[1] From a preliminary examination of the material available it appears to be closely allied to *Renicola lari* (Timon-David),[2] but differing in several measurements of diagnostic value, notably in the consistently larger size of

the ventral sucker and in the much greater development of the vitelline glands in the lateral fields. Further study may reveal that the trematode is new, but for the present it must remain *species inquirenda*. It is hoped to publish a full description elsewhere at a later date, when more material is available.

Acknowledgment

We are indebted to Mr. P. G. D. Morris, of the Anatomy Department, Royal (Dick) Veterinary College, Edinburgh, for taking the photo-micrographs.

REFERENCES:

(1) Cohn, L. (1904): Helminthologische Mitteilungen. *Arch. für. Naturgeschichte* 77. Jahrg. Bd. 1. Heft 3. P. 322.

(2) Timon-David, J. (1933): Sur un nouvelle espèce de Renicola trématode parasite du rein des Laridés. *Bull. de l'Institut Océanographique de Monaco*. No. 616.

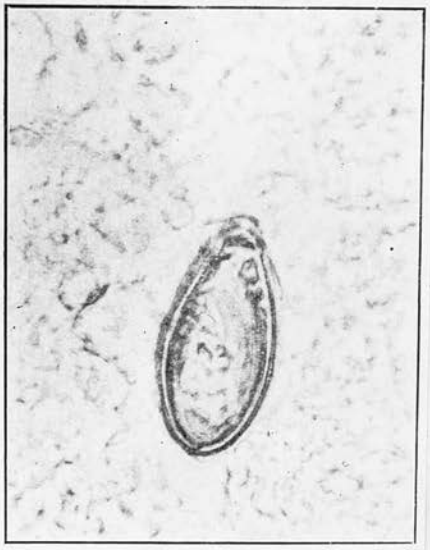

FIG. 1.—Trematode Egg x 876. Actual size 36.5 x 17.6μ.

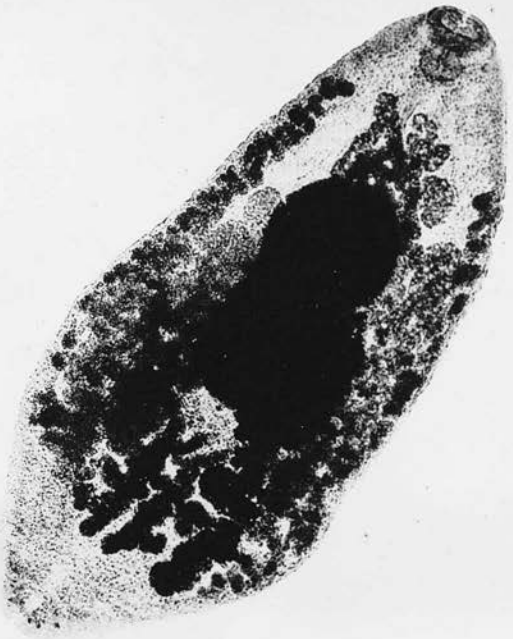

FIG. 2.—Trematode x 54. Kidney, King Penguin. Stained Ehrlich's Acid Hæmatoxylin. Actual length 2.57 mm.

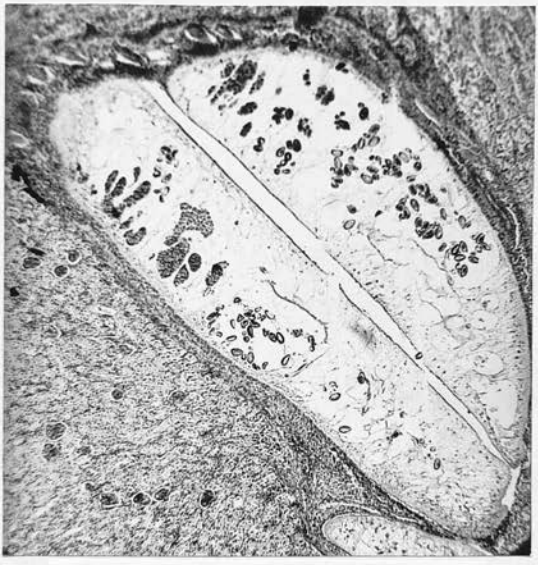

FIG. 3.—L.S. Trematode in Kidney. x 57.

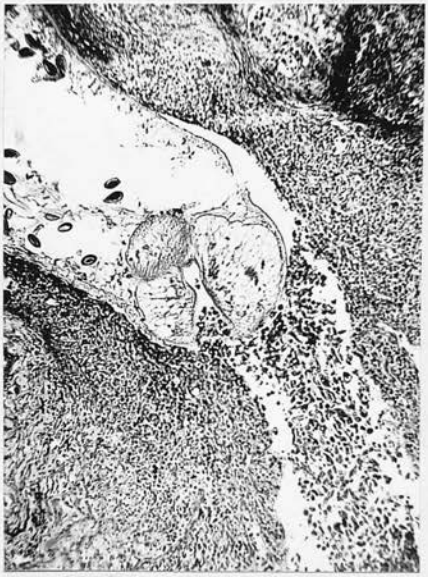

FIG 4.—L.S. Anterior end of parasite lying in a proximal branch of a duct of Bellini. x 65.

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EXPERIMENTAL ZINC PHOSPHIDE POISONING IN FOWLS

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INTRODUCTION

ALTHOUGH zinc phosphide (Zn_3P_2) has occasionally been sold as rat poison for at least 15 years (A. D. Campbell, personal communication), it was not used extensively in this country until war conditions interfered with the supply of Red Squill. Pest Officers of the Department of Agriculture for Scotland and War Agricultural Executive Committees in England have recently used a bait containing 5 per cent. zinc phosphide and as deaths amongst animals on premises where bait has been laid are frequently attributed to poisoning, this College in the last year or so has received many requests to examine viscera, especially of poultry, for evidence of zinc phosphide poisoning.

No information as to the symptoms and lesions produced by this material was to be found in the literature, though Mr. C. V. Watkins, M.R.C.V.S., kindly gave us an account of his experiences of the condition. Whilst methods for the quantitative determination of zinc were available, nothing was known about the quantities which might be expected in cases of poisoning, or the extent to which small traces found in visceral contents might be derived from the galvanised linings of food and water containers. There was, moreover, no very reliable method for the estimation of very small quantities of phosphide, and further it seemed possible that this substance might be rapidly oxidised in the alimentary tract and so escape detection. Thus, when asked by the Department of Agriculture to pronounce an opinion on fowls suspected to have died from zinc phosphide poisoning, we were rather diffident about cases in which analytical results were negative. Therefore, it was decided to investigate zinc phosphide poisoning in poultry to find the minimum lethal dose of the drug, and the degree of accuracy in diagnosis to be obtained from chemical tests.

Since this investigation began, Hare and Orr (1945) have briefly recorded one case of poisoning in geese and two cases in fowls in which traces of zinc phosphide were found in the crop and gizzard contents, but have given no details as to the total amounts recovered or the methods of estimation used. Later, Ingram (1945) described in considerable detail a case of poisoning in a colt, which was found to have over half a gramme of zinc phosphide in its stomach, but he, too, gave no information as to the analytical methods employed.

METHODS

Small gelatine capsules containing varying doses of a mixture of 10 per cent. zinc phosphide and 90 per cent. starch were given by

mouth to adult fowls. After death the alimentary tract was removed intact and ligatures tied at each end of the crop, gizzard and intestine ; in certain cases other tissues such as liver, kidney, heart, lung and pectoral muscle were also examined.

Phosphide estimations were made as soon as possible on the contents of crop, gizzard and intestine using a modification of the method of Elmore and Roth (1943) which allows of the colorimetric determination of small quantities of volatile phosphorus. A weighed amount of the material to be examined was placed with 50 ml. distilled water in a 150 ml. flask fitted with a stopper carrying a small separating funnel, an air inlet-tube reaching to the bottom of the flask, and an outlet tube connected with a suction pump via three boiling tubes each containing 10 ml. of 1 per cent. $KMnO_4$. The flask was immersed in a water bath at 48 to 50° C. and 10 ml. of 10N H_2SO_4 run in from the separating funnel. Air was gently aspirated through the tubes at about six bubbles per second for one and a half to two hours. The tubes were disconnected and 2 ml. 10N H_2SO_4 added to each ; the contents were warmed to 70° C. and saturated oxalic acid solution was run in till they were almost colourless. They were then cooled and a blue colour developed with 2 ml. of 7·5 per cent. sodium molybdate and 2 ml. freshly prepared 0·2 per cent. stannous chloride in N/20 HCl. Each tube was made up to 20 ml. with distilled water and the contents of the three were mixed, allowed to stand for ten minutes, and compared in a colorimeter with a standard phosphate solution similarly treated. By this means quantities of volatile phosphorus of the order of 0·005 mg. could be estimated with an accuracy of some 5 per cent. either way.

Zinc was also estimated colorimetrically by the method of Schwaibold *et al.* (1938). In this the material is ashed and extracted with N/2 HCl. The acid extract is shaken with 0·01 per cent. dithizone in carbon tetrachloride to remove copper and the carbon tetrachloride layer separated and discarded. After the addition of 3 c.c. of 20 per cent. sodium potassium tartrate to prevent interference by lead, and neutralisation to thymol blue with 6 per cent. ammonia, the zinc is extracted by dithizone solution, and this, after shaking with 0·08 per cent. sodium sulphide, is compared in a daylight colorimeter with a standard zinc solution similarly treated. The method allows of fairly rapid determination of quantities of the order of 0·01 mg. zinc with an accuracy of plus or minus 10 per cent. Recovery experiments from crop contents carried out with both methods proved satisfactory.

RESULTS

Minimum Lethal Dose

To get an approximate idea of the dosage required, two hens, each weighing about 2 kilos, were given respectively 100 mg. and 10 mg. zinc phosphide. The larger dose proved fatal to one hen

in about four hours, whilst the other hen remained unaffected. Three weeks later the surviving hen was given 50 mg. and succumbed in about eight hours. Five fowls were then given doses of 5·25, 10, 15, 20 and 25 mg. per kilo respectively at 5 p.m. and all died overnight except that given the smallest dose. Two fowls were next given what was intended to be 7 and 9 mg. per kilo. respectively, but owing to an error in weighing the bird (which was noted subsequently at *post-mortem* examination) the first actually received 15·4 mg. per kilo, from which it died, whilst the other recovered after a few days' illness. Two further birds were given 9·3 and 9·5 mg. per kilo respectively, only the larger dose proving fatal.

The minimum lethal dose having apparently been determined as lying between 9 and 10 mg. per kilo body weight, attention was turned to the possibility of " chronic " poisoning occurring with repeated sublethal doses. The fowl which had survived 9·3 mg. per kilo was given a similar amount next day and again the day after ; it was seen to be ailing after the second dose, and succumbed within six hours of receiving the third. Another hen, given daily 5 mg. per kilo survived seven doses before succumbing suddenly. We then commenced a similar experiment with 7 mg. per kilo, but the bird died after the first dose, whilst another fowl, given a single dose of 8 mg. per kilo, remained unaffected.

In all the birds so far examined, both zinc and phosphide had been detected easily in the alimentary canal. It seemed possible, however, that in field cases of poisoning, the phosphide might rapidly disappear from the alimentary tract and not be detectable by analytical methods. Five birds were accordingly given 10 mg. per kilo with the object of keeping them for varying periods after death before making a chemical analysis. The dose was chosen as giving the smallest amount of phosphide which was likely to be found under field conditions, and yet be sufficient to kill the fowls, but contrary to our expectations only two died. The remaining three were left a week to recover and were then given 15 mg. per kilo. Again only two died but the survivor succumbed some ten days later to a dose of 17 mg. per kilo.

The results of our experiments with 25 fowls are summarised in Table I, from which it would seem that the lethal amount of zinc phosphide administered as a single dose lies between 7 and 17 mg. per kilo, and is frequently about 10 mg. per kilo. Two fowls withstood amounts totalling some 30 mg. per kilo given in repeated sublethal doses.

Symptoms

Few symptoms were to be seen. The fowls given the largest doses were dull and depressed with ruffled plumage an hour or so after the capsule had been given. Later, they sat very dejectedly in the corners of their cages, where they usually died in a few hours with no signs of struggling. Those given smaller lethal doses survived

TABLE I

Fowl No.	*Breed and Sex*	*Dose of Zn_3P_2 (mg. per kilo)*	*History*	*Post-mortem Findings*
1	L.S., hen	50	Died in 4 hours	Slight yellowish tint of liver and kidneys. Distended gall bladder. Enteritis, severe in duodenum, extending down two-thirds of the S.I. Distinct smell of phosphine in crop and gizzard—most evident in latter.
2	R.I.R. ×, hen	25	Died in 8 hours	Gelatinous exudate in thorax. Slightly mottled liver ; early mucoid enteritis.
3	R.I.R., cock	25	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
4	R.I.R., hen	25	Died in 12 hours	Fatty liver and heart. Pale pancreas containing white spots. No odour of phosphine.
5	W.L., hen	20	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
6	R.I.R., hen	17	Died in 12 hours. Kept at 70–80° F. for 5 days	No evident lesions. Pronounced P.M. changes. No odour of phosphine.
7	Br.L. ×, hen	15·4	Died in under 16 hours	Small quantity serofibrinous exudate on liver capsule. Venous congestion, small quantity of fluid in pericardial sac.
8	R.I.R., hen	15·2	Died in 12–15 hours	Generalised TB., internal haemorrhage from ruptured liver.
9	R.I.R., hen	15·1	Ill but recovered in about 3 days	—
10	W.W., cock	15	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
11	Bl.L. ×, hen	15	Died in 12–15 hrs. Kept at 50–60° F. for 5 days	Sero-fibrinous exudate over surface of liver. Fluid in abdominal cavity.
12	W.W., cock	10	Died in under 16 hours	General venous congestion. Enlarged liver. Small accumulation of serous fluid in pericardial sac.
13	W.L., hen	10	Died in 24–36 hours. Kept for 12 days at 40–50° F.	Early P.M. changes. No detectable odour of phosphine. Small amount of fluid in abdominal cavity. Liver congested.
14	R.I.R., hen	10	Died in under 16 hours	Ascites. No other evident abnormalities apart from some tuberculous lesions. No odour of phosphine.
15	R.I.R., hen	10	Unaffected	—
16	W.W., cock	10	Unaffected	—
17	Br.L., hen	10	Slightly ill but recovered in 2–3 days	—
18	Br.L., hen	9·5	Died in 24–36 hours	Ascites. Sero-fibrinous exudate on surface of liver, small quantity of pericardial fluid.

TABLE I—*continued*

Fowl No.	*Breed and Sex*	*Dose of Zn_3P_2 (mg. per kilo)*	*History*	*Post-mortem Findings*
19	Br.L., hen	9·3	Unaffected — used for repeated doses. Died after 3rd dose	Small quantity of pericardial fluid. Liver congested and enlarged. Contents and lining membrane of gizzard were dark green in colour.
20	W.L., hen	9	Recovered after about 4 days' illness	—
21	R.I.R., hen	8	Slightly ill but recovered	—
22	W.L., cock	7	Died in 18 hours	No evident abnormalities.
23	W.L., cock	5·25	Unaffected	—
24	R.I.R. ×, hen	5	Unaffected	—
25	R.I.R., hen	5	Unaffected. Dose given repeatedly for 7 days before being fatal	Small amount serous fluid around pericardial sac and in abdominal cavity. Duodenal enteritis.

some 12 to 36 hours. Their symptoms were similar but less marked and some diarrhoea, with the passage of greenish faeces, was usually observed. Birds given sublethal doses occasionally appeared quite unaffected though the effective administration of the capsule was checked in nearly every case by feeling its passage down the oesophagus into the crop. Some birds, however, sat or stood dejectedly and ate nothing for two to five days before returning to normal. The faeces in these cases were bright green from bile pigments which were excreted in excessive amounts. Recovery eventually appeared to be complete, some of these birds being kept for several months during which time they were perfectly normal. The two " chronic " cases which were given repeated sublethal doses showed nothing abnormal, apart from the passage of large amounts of bright green faeces, until the administration of the final dose, when they rapidly succumbed with symptoms similar to those discussed above.

Lesions

Macroscopic Findings.—Details of the *post-mortem* examinations of the various fowls are set out in Table I. The most usual finding was a variable degree of venous congestion with a certain amount of serous fluid in the pericardial sac and in the abdominal cavity. In a number of cases there was some sero-fibrinous exudate on the liver capsule. Enteritis in the upper part of the small intestine was noted, especially with the larger doses. The characteristic pungent (carbide-like) odour of phosphine was detected in all cases

except where its absence is specifically noted in the table. It was usually most marked in the crop and was noted only occasionally in the gizzard.

Histopathology. —The microscopical picture in the organs examined varied from mild cloudy swelling to fairly severe fatty degeneration, both constantly associated with venous congestion. Cloudy swelling seemed to be a constant feature of acute poisoning with large doses of zinc phosphide.

The liver cells were swollen, with a fine pale granulation of the cytoplasm, but the nuclei appeared normal. The von Küpffer cells had a more plump appearance than usual. Fatty degeneration was seen in birds given repeated sublethal doses. In paraffin sections the cytoplasm was crowded with minute vacuoles and the nucleus showed signs of definite injury, *i.e.*, it was compact, deeply basophilic, and in many cases disintegrated.

Kidney: The cells mainly affected were those of the convoluted tubules, whilst those of the straight tubules showed little change. The glomerular tufts were swollen, and occasionally free red blood cells were seen lying in an extravascular position.

Heart Muscle: A fine granulation of the cytoplasm with loss of striation was the usual picture.

Small Intestine: Cloudy swelling, desquamation of epithelium and a hyperactivity of the mucosal goblet cells were the only noteworthy features.

Pancreas: In the one case examined (No. 4) white spots were found which were identified as necrosed islets of Langerhans.

ANALYTICAL FINDINGS

In Table II the results of analyses of the contents of the alimentary tract of birds which died following a single dose, are compared with those from four control birds which had received no zinc phosphide. The bulk of the material recovered was found in the crop which contained on an average about 40 per cent. of the administered zinc and about 8 per cent. of the phosphide. Only some 6 per cent. of the administered zinc was recovered from the gizzard contents, whilst the intestines contained even less—about 5 per cent. of the total. (If the apparently abnormally high figures obtained with fowl No. 3 be omitted, these averages drop to about 2·5 per cent. and 3·5 per cent. respectively and are obviously of no significance.) Very small amounts of phosphide (*circa.* 0·5 per cent. of the dose given) were found in the gizzard whilst the intestine seldom contained more than the merest trace. Only traces of zinc were found in the control birds and no detectable amounts of volatile phosphorus were obtained except in the crop of No. 1, where a faint trace of blue developed which was barely readable, but was roughly estimated at 0·0015 mg. The two chronic cases induced by the repeated administration of sublethal doses gave findings roughly similar to those obtained with single lethal doses, except for a much poorer percentage recovery of administered material, especially phosphide. This was, of course, due to the fact that probably only phosphine from the last dose given would be available for recovery.

TABLE II

Fowl No.	Total Zn_3P_2 given (mg.)	Zinc found in Contents of			Volatile P found in Contents of		
		Crop (mg.)	Gizzard (mg.)	Intestine (mg.)	Crop (mg.)	Gizzard (mg.)	Intestine (mg.)
Single Doses							
3	75	1·86	12·7	6·95	—	—	—
2	50	25·2	1·15	1·92	—	—	—
4	61	13·7	0·33	0·43	2·34	0·049	0·006
5	48	19·3	3·74	—	—	—	—
10	36	9·37	0·84	—	—	—	—
8	28·5	11·3	0·65	1·63	0·21	0·007	Nil
12	25	9·1	0·09	—	—	—	—
14	21	16·8	0·13	0·9	0·052	0·037	Nil
7	20·7	9·7	0·28	0·7	0·53	0·083	0·019
18	17	15·8	0·08	0·52	0·226	0·022	0·009
22	16·4	9·45	0·11	0·92	0·106	0·007	Nil
Repeated Doses							
19	3 × 9·3	7·1	0·27	2·2	0·118	0·023	0·033
25	7 × 5	9·64	0·19	0·4	0·023	0·057	Nil
Controls							
IX	Nil	0·17	0·38	1·1	0·0015	Nil	Nil
XIII	Nil	0·27	0·15	0·69	Nil	Nil	Nil
XVIII	Nil	0·11	Nil	0·37	Nil	Nil	Nil
XX	Nil	0·78	0·15	0·09	Nil	Nil	Nil

TABLE III
AVERAGE ZINC CONTENT OF TISSUES

	Liver (mg. per 100g)	*Gall Bladder and Contents (mg.)*	*Kidneys (mg.)*	*Heart (mg.)*	*Lungs (mg.)*	*Breast Muscle (mg. per 100 g.)*	*Intestinal Wall (mg.)*
Fowls given single lethal doses (12) ...	3·04	—	—	—	—	—	0·91
Fowls given repeated doses (2) ...	3·6	0·05	0·3	0·27	0·16	0·38	0·6
Controls (6)	5·98	0·05	0·5	0·13	0·42	0·57	0·75

TABLE IV
EFFECT OF P.M. CHANGES

Fowl No.	*Dose of Zn_3P_2 given (mg.)*	*Time between Death and Autopsy (days)*	*Temp. during Storage °F.*	*Zinc found in Contents of*			*Volatile P found in Contents of*		
				Crop (mg.)	*Gizzard (mg.)*	*Intestine (mg.)*	*Crop (mg.)*	*Gizzard (mg.)*	*Intestine (mg.)*
13	19	12	40–50	7·55	0·11	0·27	0·022	0·01	Nil
11	31·5	5	50–60	2·68	0·44	2·0	0·026	0·034	0·015
6	35·7	5	70–80	24·2	2·24	0·39	0·083	0·02	Nil

We thought originally that some of the zinc might be absorbed and stored in tissues such as the liver, and that if the results were significant, tissue analysis might provide a rapid and reliable means of diagnosis. The results of analysis of the tissues summarised in Table III showed, however, that there was no storage of zinc in either acute or chronic cases—in fact, the livers of birds which had received no zinc phosphide contained significantly larger amounts of zinc than we found in the livers of our experimental fowls. (This might be due to the excessive secretion of bile occurring in dosed birds removing some of the small amounts of zinc normally present.) The apparent differences in zinc content of kidneys and lungs between control and treated birds were not statistically significant. Analysis of faeces passed during the experiments indicated that there is a rapid excretion of the administered zinc, and in cases where repeated sublethal doses were given, faeces collected daily and analysed showed a steady excretion of the bulk of the administered zinc, about 60 per cent. of which was so recovered.

The effect of *post-mortem* changes on the recovery of zinc and volatile phosphorus was determined with three birds which were kept for varying periods after death before being autopsied (Table IV). The recoveries of zinc from crop, gizzard and intestines were of the same order as for birds examined soon after death, being on the average 50 per cent., 3·5 per cent., and 4 per cent. respectively of the doses administered. But as was expected, the recoveries of phosphide were considerably less, that found in the crop being only some 0·6 per cent. of the dose given, whilst the gizzards had only half as much and the intestines little or none. Nevertheless, in every case, in spite of *post-mortem* changes, it was still possible to detect significant amounts in the crop contents though the smell of phosphine was masked by other odours.

DISCUSSION

Our observations confirm field experience that zinc phosphide is a substance highly lethal for poultry, a tablespoonful of the rat bait normally used by pest officers containing sufficient material to poison at least 50 to 60 birds. There was nothing very characteristic about the symptoms which were probably similar to those found in any form of acute poisoning, though the somnolent condition which in some cases preceded death may have been analogous to the narcosis reported by Ingram (1945) in a colt which died from a similar cause. Though symptoms of asphyxia are common in hydrogen phosphide poisoning in man, we found no suggestion of them in our experimental birds. *Post-mortem* changes were also not very characteristic as they were suggestive simply of acute poisoning. We did not see any signs of the liver necrosis recorded by Hare and Orr (1945). At *post-mortem* examination the odour of phosphine is probably the best general guide though it may be missed if the dose is small or if the autopsy is long delayed. There should be no difficulty,

however, in finding phosphine in the crop by analytical methods in the majority of cases even after some delay, provided an unopened crop is received and a sensitive method is used. For diagnostic purposes this need not be quantitative—rapid distillation of the phosphine from boiling acid solution into permanganate and the development of a blue colour by any of the usual methods for the colorimetric determination of phosphorus should suffice. Hydroquinone, amino-naphthol-sulphonic acid or stannous chloride may all be used though stannous chloride is to be preferred as it is much the most sensitive. Even after considerable *post-mortem* change has taken place, a detectable blue can be obtained by such methods. One obstacle to such determinations lies in the fact that free phosphorus gives the same reaction and, while the *post-mortem* changes in well-marked cases of phosphorus poisoning are somewhat different from those found with zinc phosphide, it seems just possible that quantities of free phosphorus too small to give detectable Mitscherlich or Scherer reactions, might be confused with zinc phosphide. There ought to be no difficulty in detecting zinc in the crop contents, as in all probability there would seldom be less than 8 or 9 mg. present and often considerably more. We have used with considerable success a qualitative ammonium mercuric thiocyanate test, suggested by Watkins (personal communication), but it involved a rather tedious separation from other elements and we find the dithizone method described above both simple and effective. For legal purposes, presumably, evidence of both zinc and phosphine would be desirable before stating categorically that zinc phosphide was present, and in such a case a quantitative estimation of zinc would often give a very good idea as to whether sufficient had been consumed to cause death. The routine we have adopted in suspected cases, therefore, is to carry out both a qualitative test for phosphine and a quantitative determination of zinc on the crop contents.

SUMMARY

A method is described for the quantitative estimation of very small quantities of phosphide.

The lethal dose of zinc phosphide for fowls has been determined as lying between 7 and 17 mg. per kilo body weight, being frequently about 10 mg. per kilo.

Chronic effects have been produced by repeated sublethal doses.

Symptoms, lesions and analytical findings in experimental zinc phosphide poisoning are described.

The significance of these findings in toxicological work is discussed.

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REFERENCES

Elmore, J. W., and Roth, F. J. (1943). *J. Ass. off. agr. Chem., Wash.*, **26**, 559.
Hare, T., and Orr, A. B. (1945). *Vet. Rec.*, **57**, 17.
Ingram, P. L. (1945). *Ibid.*, **57**, 103.
Schwaibold, J., Bleyer, B., and Nagel, G. (1938). *Biochem. Z.*, **297**, 324.

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β-Glucuronidase and Cell Proliferation*

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After repeated feeding of menthol to mice, Fishman (1940) obtained results which, on statistical examination, showed an increase in β-glucuronidase activity in liver, spleen and kidney, as compared with organs from untreated animals. Similar results were obtained in dogs fed with borneol. Glucuronidase in uterus and other sex organs was unaffected by menthol and borneol. In Fishman's own interpretation of these important experiments, β-glucuronidase is assumed to be responsible for glucuronide synthesis in the body. A synthetic role for the enzyme has, however, still to be demonstrated, its physical properties and distribution in the body having been studied solely by means of its hydrolytic action on conjugated glucuronides. Since menthol and borneol have been proved to be excreted as the glucuronides in, e.g. the dog, and may conceivably behave in the same way in the mouse, Fishman suggested that in his experiments he was measuring adaptation by glucuronidase in response to the presence of excess substrate for its hypothetical synthetic action. Later this theory was extended to explain the elevation in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947). Oestrogens did not affect the enzyme in liver, spleen and kidney, and the additional assumption was required, and made, that the enzyme is specific in its synthetic action, according to its source, for different groups of substrate. No such specificity was, however, observed in its hydrolytic action *in vitro*, menthol glucuronide being used throughout in the assay of uterine glucuronidase under conditions found to be optimal for hydrolysis by spleen preparations.

Fishman determined the activity of his enzyme extracts by measuring, by means of its reducing power, glucuronic acid liberated from menthol glucuronide (Fishman, 1939). Sources of error in this procedure, arising largely from its lack of specificity, have been pointed out by other authors (Graham, 1946; Levvy, 1946, 1948), and have led to the development of more satisfactory methods of assay (Talalay, Fishman & Huggins, 1946; Kerr, Graham & Levvy, 1948).

Using phenol glucuronide as substrate in the assay of glucuronidase (Kerr *et al.* 1948), an attempt was made to confirm Fishman's findings (1940) with menthol. Within 24 hr. of a single intraperitoneal injection of L-menthol into mice, there was a marked rise in glucuronidase activity in liver, but not in spleen and kidney. Liver damage was observed and confirmed histologically, and it was subsequently shown that a rise in β-glucuronidase in liver or kidney, depending upon the organ or organs attacked, followed administration of a variety of toxic agents to mice. A more extensive examination of the action of menthol revealed, in addition to the effect on liver, delayed damage to kidney, followed by an increase in glucuronidase activity in this organ also. An increase in the glucuronidase activity of an organ was found, in general, to be associated with active cell proliferation provoked by injury, rather than with the injury itself, and high values were seen in the livers of adult mice after sub-total hepatectomy, and in the liver, spleen and kidneys of infant mice.

* Preliminary accounts of parts of this work have been published elsewhere (Kerr & Levvy, 1947; Kerr, Levvy & Campbell, 1947), and the principal findings were described in a paper read to the Biochemical Society on 27 September 1947 (Levvy, Kerr & Campbell, 1948).

EXPERIMENTAL AND RESULTS

Enzyme assay. In the assay of kidney glucuronidase it was assumed that the conditions for optimum hydrolysis of phenol β-D-glucuronide would be the same as those previously found to hold for spleen and liver preparations (Kerr *et al.* 1948). All preparations of the enzyme were diluted to final volumes giving readings of 20–40 μg. phenol in the assay, after correction for blanks. The results are shown in the tables and figures in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol in 1 hr. from 0·015M-phenol glucuronide at 38° and pH 5·2. The standard error is given wherever possible. Although frequently based upon too small a group of animals to have any statistical value, it shows the variation in the individual figures in a convenient form.

Histology. Portions of organs from animals used for enzyme assay, or whole organs from other animals treated similarly, were fixed immediately in Susa and taken in the usual way through the ethanols to a mixture of chloroform and cedarwood oil, and finally cleared in pure cedarwood oil. After embedding in paraffin wax, sections were cut at 8 μ. and stained with Mayer's haematoxylin and eosin. The distribution of fat was studied in frozen sections, prepared from tissues rapidly fixed by heat in formol saline, and stained with haematoxylin and Sudan III.

Damage, repair and cell division are shown in the tables by an arbitrary system of + signs. In the case of damage, + indicates that while definite it was neither severe nor extensive, and + + + that it was at its greatest for the toxic agent in question. The course of repair is measured likewise, + + + indicating that replacement of damaged tissue by normal cells is practically complete. Under cell division, an estimate is given of the number of mitotic and amitotic figures and hyperchromatic nuclei *in excess of normal.* No histological findings are given for spleen since deviations from normal could never be distinguished in this organ.

Normal mice and vehicle controls. Average values for β-glucuronidase in each organ were the same for normal adult mice (30–40 g.) of both sexes and drawn from three different colonies, and all the results are grouped together in the tables. Spleen showed greater variation in its normal glucuronidase activity than did liver or kidney. Intraperitoneal injection of relatively large volumes of 0·9 % sodium chloride solution, olive oil or nut oil (the vehicles used for administration of toxic agents) had no effect on glucuronidase in any of the three organs examined after an interval of 1–2 days. These results are not shown. The relatively small number of experiments in which nut oil was used as a vehicle, olive oil being unobtainable, are included in the tables with those done with the latter as medium.

Effects produced by a single injection of L-*menthol.* Intraperitoneal injection of L-menthol (Table 1) caused a rapid rise in liver glucuronidase activity, reaching a maximum after 24 hr. and persisting for 7 days. Greatest liver damage was observed after 24 hr., but repair processes were not perceptible at this time. After 14 days, repair was almost complete and the enzyme level had returned to its original value, although cell division still seemed to be slightly in excess of normal. In the first 24–48 hr., kidney was normal in structure and in its enzyme activity, but after 3 days damage was evident and the figure for glucuronidase had risen after 7 days. At the end of 14 days this organ was normal in all respects. No effect of menthol on spleen glucuronidase was observed at any stage. Sex did not influence the results obtained with liver and kidney.

Mills (1947) found beef spleen glucuronidase to consist of two fractions with slightly different pH optima for the hydrolysis of menthol glucuronide, and the pH activity curves for hydrolysis of phenol glucuronide by enzyme from mouse spleen and liver (Kerr *et al.* 1948) had subsidiary peaks at pH 4·5. An experiment was done in which spleen, liver and kidney glucuronidase activities, 24 hr. after injection of menthol, were compared with normal at pH 4·5 instead of 5·2. The change in pH had no appreciable effect on the results compared with those shown in Table 1.

No details of the toxic action of menthol could be found in the literature. A brief description of the changes seen in liver and kidney may be of interest. In the liver, the first deviation from normal was cloudy swelling, followed by fatty change and necrosis surrounding the central vein and extending about a third of the way into the lobule. The nuclei showed hypertrophy and hyperchromatism, many

Table 1. *Changes in β-glucuronidase and histological findings after injection of mice with* L-*menthol*

(333 mg. Menthol/kg. injected intraperitoneally in olive oil. Average enzyme activity and standard error expressed as G.U./g. moist tissue (see text). Number of animals in group shown by figures in brackets.)

Interval (days)	Spleen enzyme	Liver				Kidney			
		Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
Untreated	636±70 (23)	273±13 (23)	—	—	—	363±24 (11)	—	—	—
0·125–0·5	720±63 (9)	467±24 (9)	—	—	—	381±40 (9)	—	—	—
1	690±41 (3)	823±135 (3)	+ + +	0	0	285±46 (3)	0	0	0
2	738±86 (3)	884±74 (6)	—	—	—	344±71 (3)	—	—	—
3	903±208 (3)	953±39 (3)	+ +	+ + +	+	—	+ +	0	0
7	646±86 (6)	775±46 (7)	+	+ +	+ +	603±52 (7)	0	+	+ + +
14	600±14 (3)	318±17 (3)	+	+	+ +	337±23 (3)	0	0	+ + +

binucleate cells appeared (amitotic division), and at a later stage mitotic division became evident. The Küpffer endothelial cells were swollen. In the case of kidney, the damage was not severe, being confined to the distal portions of the convoluted tubules and to some glomeruli, the endothelium of which was swollen and in places necrotic. Intraperitoneal injection of mice with large doses of menthol (about 0·7 g./kg.) caused prolonged depression of the respiration and unconsciousness. No attempt was made to determine the lethal dose.

Repeated administration of L-*menthol and* L-*menthol β-*D-*glucuronide.* Results for β-glucuronidase in spleen, liver and kidney, after intraperitoneal injection of mice with L-menthol or its glucuronide twice or thrice daily for varying periods (Table 2), were similar to those obtained after a single injection of L-menthol. The glucuronide was injected as a neutral solution in 0·9 % sodium chloride solution and menthol itself as a solution in olive oil. (For the preparation of neutral solutions of acid compounds in 0·9 % sodium chloride solution, see Chance, Crawford & Levvy, 1945.) Repeated oral administration (Odell, Skill & Marrian, 1937) of menthol produced an increase in liver glucuronidase activity of the same order as the injections, except in mice receiving a total dose of 9·3 g./kg. in which the rise was barely perceptible. The latter was, however, as great as that obtained by Fishman (1940) and proved to be statistically significant ($P = 0·01$). This experiment was carried out exactly as described by Fishman except that three of the mice were given a solution of menthol in olive oil instead of an emulsion in soap solution. The change in vehicle had no effect on the response of the enzyme, and only solutions in oil were used in the other feeding experiments (total dose 1·2 and 2·0 g./kg.). No histology was done in the experiments listed in Table 2, except in the case of menthol glucuronide, which produced changes similar to those seen after a single injection of menthol.

Changes produced by single injections of a variety of substances. The changes in β-glucuronidase activity and histological findings in liver and kidney after injection of various substances, some of them known liver or kidney poisons, are summarized in Table 3. Spleen was also examined in these experiments. Since any changes in glucuronidase in this organ were relatively small, with wide variation in individual figures, the results are not shown.

Subcutaneous injection of carbon tetrachloride in olive oil caused severe fatty degeneration and early necrosis in liver within 24 hr. After 3 days, damage was extensive, but repair processes had commenced, and after 7 days repair was far advanced. A marked increase in liver glucuronidase activity occurred within 24 hr., and this was maintained for 7 days. There were no marked pathological changes in kidney at any stage, nor was there any rise in glucuronidase in this organ. Intraperitoneal injection of carbon tetrachloride (0·5–2 g./kg.) produced a change in liver glucuronidase similar to that already described for subcutaneous injection.

Mercuric nitrate given subcutaneously in 0·9 % sodium chloride solution had no very marked effect on liver, but produced severe cortical necrosis with hyaline casts in kidney within 24 hr. Kidney glucuronidase activity showed no rise at this stage, but after 3 days, by which time repair was practically complete, it was more than twice its normal value.

The changes in liver after subcutaneous injection of chloroform in olive oil resembled those produced by carbon tetrachloride. Kidney, however, showed an interesting sex specificity in the response of the enzyme to chloroform. In agreement with the observation of Eschenbrenner (1944), this compound was found to cause renal necrosis in male, but not in female mice. The rise in kidney glucuronidase activity, which was confined to male mice, was not seen in the early stages of the damage, but was evident after 8 days, by which time repair was extensive. An increase in liver glucuronidase

Table 2. *Changes in β-glucuronidase after repeated administration of* L-*menthol and* L-*menthol β-*D-*glucuronide*

(Results expressed as in Table 1)

Agent and mode of administration	Total dose (g./kg.)	Interval after 1st administration (days)	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Untreated	—	—	636±70 (23)	273±13 (23)	363±24 (11)
L-Menthol, orally	1·2	3	843±185 (3)	741±146 (3)	—
	2·0	1	—	895±77 (6)	—
	9·3	5	499±48 (6)	369±40 (6)	260±2 (2)
L-Menthol, intraperitoneally	0·8	2	254±38 (3)	1149±136 (3)	—
	1·2	3	599±42 (6)	869±58 (6)	—
L-Menthol β-D-glucuronide, intraperitoneally	2·3	1·5	995±95 (3)	1104±222 (3)	295±13 (3)

Table 3. *Effects of various agents on liver and kidney*

(Results expressed as in Table 1)

Agent and dose	Interval (days)	Sex	Liver				Kidney			
			Enzyme	Damage	Cell division	Repair	Enzyme	Damage	Cell division	Repair
None	—	♀, ♂	273±13 (23)	—	—	—	363±24 (11)	—	—	—
Carbon tetrachloride	1	♂	1138±148 (3)	+++	0	0	139±9 (3)	+ (?)	0	0
(5·3 g./kg.)	7	♂	927±48 (4)	+	+++	++	323±45 (4)	0	0	0
$Hg(NO_3)_2$	1	♂	436±28 (2)	+	0	0	208±42 (3)	+++	0	0
(20 mg./kg.)	3	♂	469±29 (5)	0	+	0	808±61 (5)	0	+	+++
Chloroform	1	♂	939±90 (7)	+++	++	0	194±16 (6)	+++	0	0
(2 g./kg.)	8	♂	711±34 (3)	+	++	++	628±119 (3)	+	++	++
	1	♀	583±27* (3)	—	—	—	251±18 (3)	0	0	0
	8	♀	608±44 (3)	+	++	++	274±15 (3)	0	0	0
Yellow phosphorus	2	♂	91±23 (3)	+++	0	0	338±35 (3)	+ (?)	0	0
	5	♂	744±37 (3)	++	++	+	462±89 (3)	0	0	0
(7·5 mg./kg.)	10	♂	429±80 (3)	0	0	+++	309±32 (3)	0	0	0
Sulphathiazole (43 g./kg.)	3	♂	460±29 (3)	+	0	0	368±13 (3)	0	0	0
Pregnanediol (333 mg./kg.)	1·7	♀	287±65 (3)	0	0	0	321±64 (3)	0	0	0
†Pregnanediol β-D-glucuronide (800 mg./kg.)	1·7	♀	241±36 (3)	—	—	—	264±19 (3)	—	—	—
Ether (40 min. deep anaesthesia)	1·75	♀, ♂	265±30 (4)	0	0	0	309±63 (3)	0	0	0
Sodium sulphapyridine monohydrate (18–36 g./kg.)	2	♀, ♂	327±17 (7)	++	+	+	362±11 (7)	+ (?)	0	0

* Results for glucuronidase in liver obtained after intraperitoneal injection of 0·5 g. chloroform/kg.

† Pregnane-3(α):20(α)-diol glucuronidic acid free from pregnane-3(α)-ol-20-one glucuronidic acid (Sutherland & Marrian, 1947).

activity was observed after injection of as little as 0·2 g. chloroform/kg. subcutaneously.

Yellow phosphorus, injected subcutaneously in olive oil, had no marked effect on kidney, but produced profound and extensive changes in liver (congestion, fatty degeneration and necrosis). From the results of experiments dealt with above it will have been noted that there may be no rise in glucuronidase activity in an organ when damage is at its height. In the case of phosphorus, there was an unmistakable initial drop in the activity of the enzyme, to one-third of its normal value. When, at the end of 5 days, repair was well under way, the enzyme level showed the usual increase, only to fall again when repair was complete.

Of the remaining substances listed in Table 3, ether and pregnanediol produced no pathological changes and had no effects on glucuronidase in either liver or kidney. Pregnanediol glucuronide resembled the parent compound in its effects on the enzyme, but was not examined for histological effects. Ether was given by inhalation, and pregnanediol and its glucuronide were injected intraperitoneally as suspensions in olive oil. Sulphathiazole caused cloudy swelling in liver after subcutaneous injection of a very large dose as a neutral solution in 0·9% sodium chloride solution. There was a small, but significant ($P = 0·05–0·02$) rise in liver glucuronidase activity. This compound had no effect of any kind on kidney. Sodium sulphapyridine, given in the same way as sulphathiazole, caused fatty degeneration and necrosis in liver, accompanied by some cell proliferation, but without appreciable change in the enzyme level. In some animals there was slight damage to the kidneys, again without any rise in glucuronidase activity.

Uranyl acetate. Results for glucuronidase in liver and kidney after subcutaneous injection of mice with varying doses of uranyl acetate in 0·9% sodium chloride solution (Fig. 1) illustrate the point that increasing the dose of a toxic agent may retard the rise in glucuronidase activity in the early stages of poisoning, and may even cause an initial drop in the enzyme level. Each point in the figure is an average for a group of three male mice, killed 2 days after injection. Severe tubular 'nephrosis' was noted at this stage with all four doses of the toxic agent. Cell proliferation could also be seen after all but the

largest dose, becoming more marked as the dose fell. In the case of liver, the histological findings were more difficult to interpret as the damage, which was mainly subcapsular, was transitory and rapidly succeeded by intensely active cell proliferation. Only the latter response was observed after injection of the smallest dose of uranyl acetate. In general, however, damage was greater and repair processes

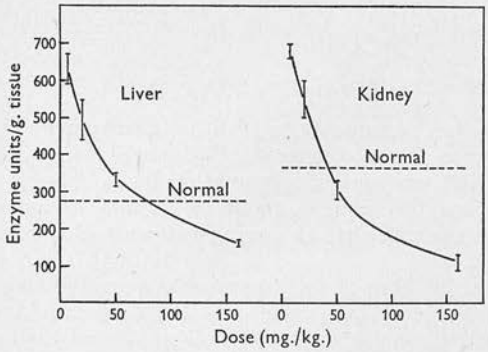

Fig. 1. Liver and kidney glucuronidase activity 2 days after subcutaneous injection of mice with varying doses of uranyl acetate. Mean ± S.E. shown for each point.

slower to appear as the dose was increased. Ten days after injection of the smallest dose of uranyl acetate repair was finished in both liver and kidney, and their enzyme levels had returned to normal. No change in spleen glucuronidase was produced by uranyl acetate.

The possibility that in a severely damaged organ an apparently normal value for glucuronidase may be observed at a certain stage, even though cell proliferation may have commenced, probably explains the fact that only small rises in the liver enzyme were observed after prolonged feeding of menthol (total dose 9·3 g./kg., Table 2) or subcutaneous injection of sodium sulphapyridine (Table 3).

Phenylarsenoxide. Results obtained with this compound (Fig. 2) show the changes in liver and kidney glucuronidase activity at various stages in different degrees of poisoning. Phenylarsenoxide was injected subcutaneously as a neutral solution in 0·9 % sodium chloride solution. Each point in the figure is an average for a group of three male mice, except in the case of the 1 day figures with the larger dose, which are both based on six results. Phenylarsenoxide caused peripheral lobular necrosis and fatty degeneration in liver, and diffuse nephritis in kidney. Damage to both organs was intense 1 day after injection of 1 mg./kg., with no signs of repair. After 3 days, repair processes had become evident, and replacement of damaged tissue was well advanced after 5 days. At the end of 10 days, kidney was entirely normal and liver repair was almost complete. With the smaller dose (0·2 mg./kg.), cell proliferation was marked in both organs after 1 day, and repair was far advanced after 4 days. Phenylarsenoxide had no effect on spleen glucuronidase, and the histological changes produced in liver and kidney were similar for both sexes.

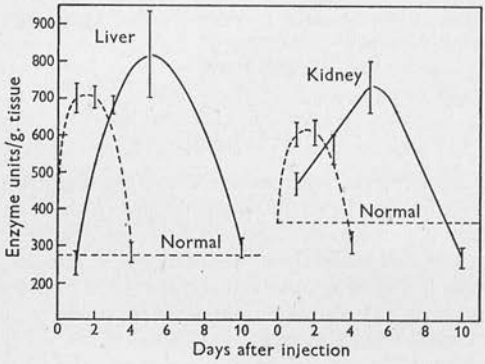

Fig. 2. Liver and kidney glucuronidase activity at varying periods after subcutaneous injection of mice with phenylarsenoxide. ——, 1 mg./kg.; — —, 0·2 mg./kg. Mean ± S.E. shown for each point.

Effects of various substances on the enzyme in vitro. All the substances examined for their effects on β-glucuronidase activity *in vivo*, with the exception of pregnanediol glucuronide, were tested for their effect on the assay *in vitro* in a concentration of 0·1 % (w/v). A solution in the medium used for injection was added to the citrate buffer and shaken vigorously. In no case did the presence of the agent in the incubation mixture affect the activity of β-glucuronidase from normal mice.

Infant mice and partially hepatectomized mice. As shown in Table 4, glucuronidase activity in spleen, liver and kidney was much higher in young mice, ranging in age from 1 to 15 days, than in normal adults. The remaining lobes of liver in adult mice (male and female), 3–8 days after sub-total hepatectomy, were hypertrophied. The glucuronidase level was high and cell proliferation was very active. In preparing the animals, 60 % of the liver was removed by cautery or ligature under ether anaesthesia, the whole operation taking less than 10 min. There was no difference in the final result between the alternative surgical techniques, nor at the various times of examination. One animal (result omitted from Table 4) was comatose and apparently about to expire when killed 3 days after operation. As expected, the remaining fraction of the liver showed no increase in weight, in the glucuronidase activity nor in cell proliferation.

Table 4. *β-Glucuronidase after sub-total hepatectomy and in infant mice*

(Results expressed as in Table 1)

Age (days)	Treatment	Average enzyme activity and S.E. (G.U./g. moist tissue) Spleen	Liver	Kidney
Adult	None	636±70 (23)	273±13 (23)	363±24 (11)
1*	None	5100 (3)	1370 (3)	881 (3)
5*	None	2670 (2)	1294 (2)	702 (2)
5*	None	3820 (2)	1432 (2)	883 (2)
13*	None	1521 (2)	2218 (2)	606 (2)
15	None	5169±2820 (3)	1239±49 (3)	727±108 (3)
Adult	Partial hepatectomy 3–8 days previously	—	1046±88 (10)	—

* Each organ pooled before enzyme assay.

DISCUSSION

Our own results with menthol confirm Fishman's (1940) findings in so far as the enzyme in liver and kidney is concerned. It seems, however, that Fishman suppressed repair processes by overdosage with menthol, and thus obtained a rise in glucuronidase which was only a small fraction of that provoked by the first doses of the compound. No explanation can be offered for our failure to observe the rise in the activity of spleen glucuronidase in menthol-treated mice reported by Fishman, unless the discrepancy has its origin in the wide variation in its glucuronidase level normally shown by spleen. This variation one might expect if glucuronidase activity is a measure of the amount of cell proliferation in progress.

The present work shows that the effects of menthol on β-glucuronidase activity *in vivo* bear no relation to its glucuronidogenic property, but are secondary to its hitherto unsuspected toxic action on liver and kidney. It seems impossible that chloroform, carbon tetrachloride, mercuric nitrate, phosphorus or uranyl acetate should give rise to a glucuronide in the body, and yet all these substances have been found to cause striking changes in glucuronidase activity. Of other substances which caused a rise in glucuronidase, phenylarsenoxide could conceivably form a derivative conjugated with glucuronic acid, and evidence has been obtained that sulphathiazole is partially excreted in rabbits as the glucuronide of a hydroxy derivative (Thorpe & Williams, 1940). In spite of the very large dose injected, sulphathiazole caused only a relatively small rise in glucuronidase activity in mice, and this was confined to liver. No change in glucuronidase was observed after injection of two compounds which are known to be glucuronidogenic, pregnanediol (Venning & Browne, 1936) and sodium sulphapyridine (Scudi, 1944). It should be pointed out that a change in experimental conditions might reveal an effect of sulphapyridine on glucuronidase in liver, since it produced some damage in this organ. The effect of menthol glucuronide on liver glucuronidase was due presumably to menthol liberated by the enzyme initially present. Fishman's theory (see p. 462) provides no explanation for a change in the enzyme brought about by administration of a compound already conjugated with glucuronic acid.

On the basis of the experiments described above it is not possible to decide whether β-glucuronidase is actually concerned in cell proliferation, or whether the increases in activity observed merely reflect an increase in metabolic activity. It is interesting to note, however, that the rise in the enzyme level occasionally slightly preceded the first appearance of cell division which was definitely in excess of normal. Whatever the cause of the parallelism between the glucuronidase activity in an organ and the amount of tissue growth in progress, it provides a straightforward explanation of the changes in the enzyme in liver and kidney which follow administration of menthol and other substances to mice. It seems possible that the same explanation can be applied to the effect of oestrogens on uterine glucuronidase (Fishman & Fishman, 1944; Fishman, 1947), and to a recent observation (Fishman & Anlyan, 1947), which suggests that in some cases of human carcinoma the tumour contained more glucuronidase than the corresponding normal tissue. The possible bearing of our results with carbon tetrachloride on the finding (Pincus & Martin, 1940) that, in liver poisoning produced by this compound, the physiological activity of oestrone is enhanced is of interest.

It is no longer necessary to speculate on the probable role of glucuronidase in the body in order to explain the changes in activity produced by extrinsic agents. The citation by Fishman (1947) of the work of other authors in support of his contention that the enzyme acts synthetically, however, makes it necessary to consider their results from this angle. Florkin, Crismer, Duchateau & Houet (1942) obtained evidence for the condensation of glucuronic acid with borneol in the presence of β-glucuronidase, but the percentage conjugation was very small under extreme conditions, and they concluded: 'Quant à

savoir si cette synthèse enzymatique correspond au mécanisme réalisé *in vivo*, c'est évidemment une autre affaire.' In the work of Lipschitz & Bueding (1939) and Crépy (1946) on the formation of conjugated glucuronides by surviving liver slices, there is no suggestion that the enzyme concerned is β-glucuronidase. De Meio & Arnolt (1944), who studied conjugation of phenol by surviving tissue slices, found that glucuronic acid reversed the inhibition of this process produced by iodoacetate. They also found that feeding phenol and borneol to rats increased phenol conjugation by liver and kidney *in vitro*. Their results are difficult to interpret since it is known that phenol may be conjugated with either sulphuric or glucuronic acid. Even if De Meio & Arnolt are correct in thinking that, contrary to the views of Lipschitz & Bueding (1939), glucuronides are formed by direct condensation of the 'aglucurone' with free glucuronic acid, there is no reason to believe that β-glucuronidase is responsible. With regard to De Meio & Arnolt's second finding, there is, in view of our own work, no need to postulate adaptation by the enzyme or enzymes responsible for conjugation of phenol, since both phenol and borneol may have caused liver and kidney damage in their experiments. Results obtained by Bueding & Ladewig (1939) in studying the effect of chloroform poisoning in guinea pigs on glucuronide synthesis by liver slices are of interest in this connexion. Not only did the liver slices from the poisoned animals show the usual increase in glucuronide synthesis on addition of lactate, but they were apparently more active in forming borneol glucuronide than slices from normal animals. The latter aspect of their results is not touched upon by the authors. While there is thus some evidence to suggest that, following damage to an organ, there may be an increase in its ability to form conjugated glucuronides, it is at present impossible to say whether or not this is due to the rise in β-glucuronidase activity observed when repair is in progress, nor is it certain that the glucuronides are formed directly from free glucuronic acid.

SUMMARY

1. The effect of menthol administration to mice in increasing the β-glucuronidase activity of liver and kidney is due to its toxic action on these organs. The rise in enzyme activity is associated with an increase in cell proliferation following injury. Menthol had no effect on spleen glucuronidase.

2. Among other substances examined, the following caused changes in glucuronidase in liver or kidney in an analogous fashion to menthol: chloroform, carbon tetrachloride, mercuric nitrate, yellow phosphorus, phenylarsenoxide, uranyl acetate, menthol glucuronide and sulphathiazole. The effect of chloroform on kidney glucuronidase was confined to male mice.

3. Livers from adult mice after sub-total hepatectomy, and spleens, livers and kidneys from infant mice showed high glucuronidase activities.

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REFERENCES

Bueding, E. & Ladewig, P. (1939). *Proc. Soc. exp. Biol., N.Y.*, **42**, 464.
Chance, A. C., Crawford, T. B. B. & Levvy, G. A. (1945). *Quart. J. exp. Physiol.* **33**, 137.
Crépy, O. (1946). *C.R. Acad. Sci., Paris*, **223**, 646.
De Meio, R. H. & Arnolt, R. I. (1944). *J. biol. Chem.* **156**, 577.
Eschenbrenner, A. B. (1944). *J. Nat. Cancer Inst.* **5**, 251.
Fishman, W. H. (1939). *J. biol. Chem.* **127**, 367.
Fishman, W. H. (1940). *J. biol. Chem.* **136**, 229.
Fishman, W. H. (1947). *J. biol. Chem.* **159**, 7.
Fishman, W. H. & Anlyan, A. J. (1947). *Science*, **106**, 66.
Fishman, W. H. & Fishman, L. W. (1944). *J. biol. Chem.* **152**, 487.
Florkin, M., Crismer, R., Duchateau, G. & Houet, R. (1942). *Enzymologia*, **10**, 220.
Graham, A. F. (1946). *Biochem. J.* **40**, 603.
Kerr, L. M. H., Graham, A. F. & Levvy, G. A. (1948). *Biochem. J.* **42**, 191.
Kerr, L. M. H. & Levvy, G. A. (1947). *Nature, Lond.*, **160**, 463.
Kerr, L. M. H., Levvy, G. A. & Campbell, J. G. (1947). *Nature, Lond.*, **160**, 572.
Levvy, G. A. (1946). *Biochem. J.* **40**, 396.
Levvy, G. A. (1948). *Biochem. J.* **42**, 2.
Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. (1948). *Biochem. J.* **42**, iv.
Lipschitz, W. L. & Bueding, E. (1939). *J. biol. Chem.* **129**, 333.
Mills, G. T. (1947). *Nature, Lond.*, **160**, 638.
Odell, A. D., Skill, D. I. & Marrian, G. F. (1937). *J. Pharmacol.* **60**, 420.
Pincus, G. & Martin, D. W. (1940). *Endocrinology*, **27**, 838.
Scudi, J. V. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 197.
Sutherland, E. S. & Marrian, G. F. (1947). *Biochem. J.* **41**, 193.
Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
Thorpe, W. V. & Williams, R. T. (1940). *Nature, Lond.*, **146**, 686.
Venning, E. M. & Browne, J. S. L. (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 792.

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β-Glucuronidase as an Index of Growth in the Uterus and other Organs

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The β-glucuronidase activity of mouse liver or kidney has been shown to be related to the degree of cell proliferation in progress (Levvy, Kerr & Campbell, 1948). It was suggested that the rise in uterine glucuronidase observed after administration of oestrogens to ovariectomized mice (Fishman & Fishman, 1944; Fishman, 1947) could also be explained by cell proliferation.

A comparative study has been made of the kinetics of hydrolysis of phenylglucuronide by β-glucuronidase from mouse uterus, liver and kidney, and of the effects on the enzyme activities of various measures designed to produce proliferative changes in one or more of these organs. Mills (1947) showed that ox-spleen glucuronidase could be separated into two fractions, *A* and *B*, with slightly different pH optima for the hydrolysis of menthylglucuronide. Both these fractions have been found in mouse liver and kidney, while uterine glucuronidase appears to be composed entirely of *A*. No evidence has, however, been obtained to suggest that the effect of an extrinsic agent on the glucuronidase activity of an organ is dependent upon which fraction happens to be present. As in liver and kidney, changes in the enzyme level in uterus resulting from a variety of causes appear to be associated with alterations in growth.

In the course of these experiments, some unexpected changes in glucuronidase activity were encountered. In ovariectomized mice, measures

designed to cause a rise in glucuronidase in liver also produced an increase in uterus, whilst an elevated enzyme activity in liver as well as in uterus was seen after administration of oestrone. These effects have been further investigated.

A preliminary account of part of this work has been published elsewhere (Kerr & Levvy, 1948).

EXPERIMENTAL

Enzyme assay. To permit determination of β-glucuronidase activity in a single mouse uterus, the procedure previously described (Kerr, Graham & Levvy, 1948) was adapted for use with the microcells of the Spekker absorptiometer. As before, the tissue homogenate was freed from inactive protein by maintaining it at pH 5·2 and 38° for 30 min., and the enzyme was precipitated by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The enzyme was dissolved in a volume of water such that 0·2 ml. of the resulting solution gave a final reading of 2–4 μg. phenol after correction for blanks. This volume of the enzyme solution was added to 0·1 ml. 0·06 M-phenylglucuronide and 0·1 ml. 0·1 M-citrate buffer at the appropriate pH (see below). After incubation of the hydrolysis mixture for 1 hr. at 38°, 0·5 ml. of a 1 in 5 dilution of Folin-Ciocalteu reagent was added. Protein was removed by centrifuging, and 0·5 ml. of supernatant transferred to a tube containing 0·5 ml. 1·33 N-Na_2CO_3. Colour development was carried out for 20 min. at 38°, and the results were read from a graph constructed with standard phenol solutions put through the same procedure. Assays were done in duplicate, and enzyme and substrate controls were performed as usual. This technique was also adopted for determinations of liver and kidney glucuronidase in the experiments described below, and results are shown in terms of glucuronidase units (G.U.)/g. moist tissue, where 1 G.U. liberates 1 μg. phenol under the standard conditions.

Weight of uterus. Before determining the moist weight of uterus, the tissue was freed from intrauterine fluid by pressing it between pieces of filter paper. The figure then obtained was found to bear a constant relation to the weight after drying at 110° for all conditions of the uterus. No error was introduced into the enzyme assay since the intrauterine fluid contained no detectable amounts of glucuronidase.

RESULTS

Kinetic studies. The pH-activity curve for hydrolysis of phenylglucuronide by mouse-kidney glucuronidase resembled those previously obtained for liver and spleen (Kerr *et al.* 1948) in having two peaks, one at pH 4·5 and the other at pH 5·2. In the case of uterus, however, the activity curve was symmetrical about pH 4·5, and this was still true when the initial purification of the homogenate was omitted. Changes in the enzyme activity in liver, kidney or uterus were not associated with any alteration in the shape of the pH-activity curve (Figs. 1–3). To cover the pH range it was necessary in the case of uterus to pool preparations from two or more mice. For liver and kidney this was only necessary with infant mice. The high figure for uterine glucuronidase in infant mice supports the view that in this organ, as in others, the activity of the enzyme is a measure of growth processes.

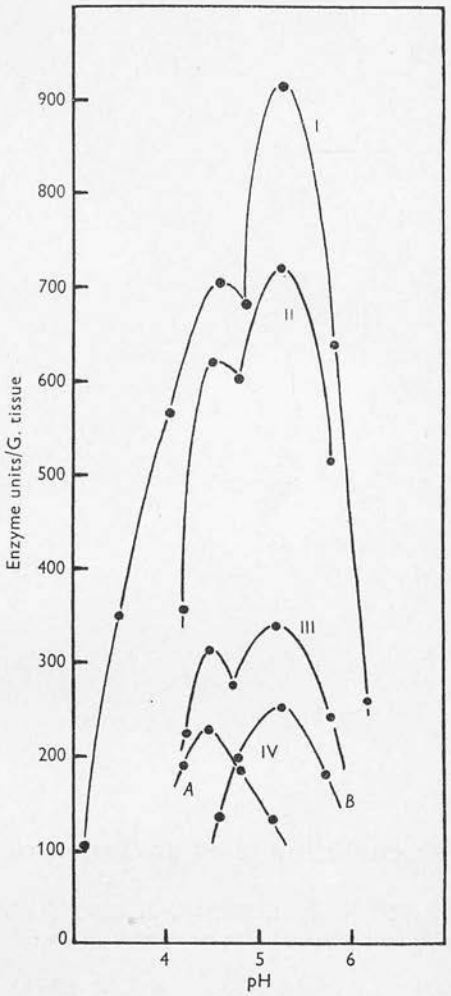

Fig. 1. pH-Activity curves for liver glucuronidase. I, 6-day-old mice; II, adult, 1 day after subcutaneous injection of 5 g. CCl_4/kg.; III, normal adult; IV, the same preparation as III after separation of fractions *A* and *B*.

It was considered that the shapes of the curves for the hydrolysis of phenylglucuronide by mouse liver or kidney indicated the presence of the two glucuronidase fractions found by Mills (1947) in ox spleen, and his technique was applied to their separation. After the preliminary removal of inactive protein by incubation for 30 min. at pH 5·2, the homogenate was made 31·5% saturated with ammonium sulphate. The precipitate thus obtained was devoid of glucuronidase activity. On bringing the preparation to 38·5% saturation with ammonium sulphate, a

large part of the enzyme was precipitated (fraction *A*), whilst all residual activity was removed from solution when the ammonium sulphate concentration was increased to 44·0% saturation (fraction *B*). The separation of the two peaks in the pH-activity curves for liver and kidney achieved in this way is illustrated in Figs. 1 and 2, and it appears that the shapes of the original curves can in fact be explained in terms of Mills's (1947) two fractions. In the fractionation of uterine preparations, all enzyme activity was found in fraction *A*.

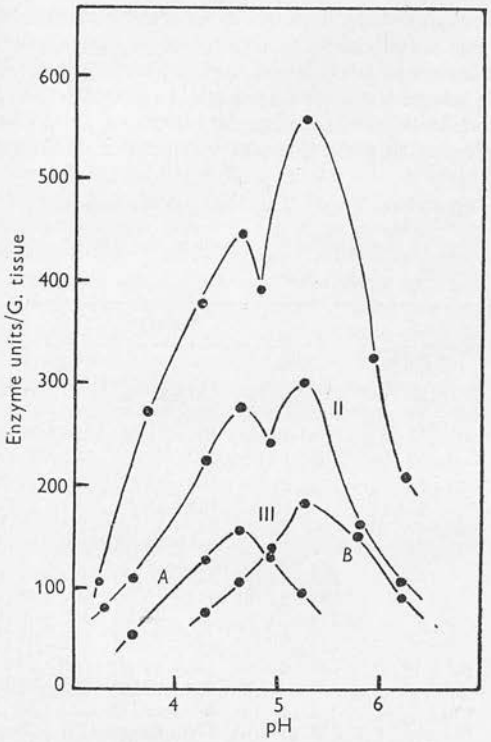

Fig. 2. pH-Activity curves for kidney glucuronidase. I, 6-day-old mice; II, normal adult; III, the same preparation as II after separation of fractions *A* and *B*.

The effect of varying the substrate concentration was studied with uterine enzyme and with fractions *A* and *B* from liver. In every case the activity curve closely resembled that obtained with liver before separation of the two fractions (Kerr *et al.* 1948). K_m, the substrate concentration at which half the observed maximum velocity of hydrolysis was attained, was approximately the same for the two glucuronidase fractions at the figure for the total enzyme in liver (0·0035 M).

Mills (1948) has recently published figures for the pH optima in the hydrolysis of phenylglucuronide by his two glucuronidase fractions from ox spleen, and these correspond exactly with the peaks in the pH-activity curves for mouse liver, kidney and spleen.

The effects of various agents on fractions A and B in liver and kidney

The possibility was considered that differences between glucuronidase fractions *A* and *B* in their distribution and response to extrinsic agents might explain the selective actions of such agents on various organs. The nature of the effect of carbon

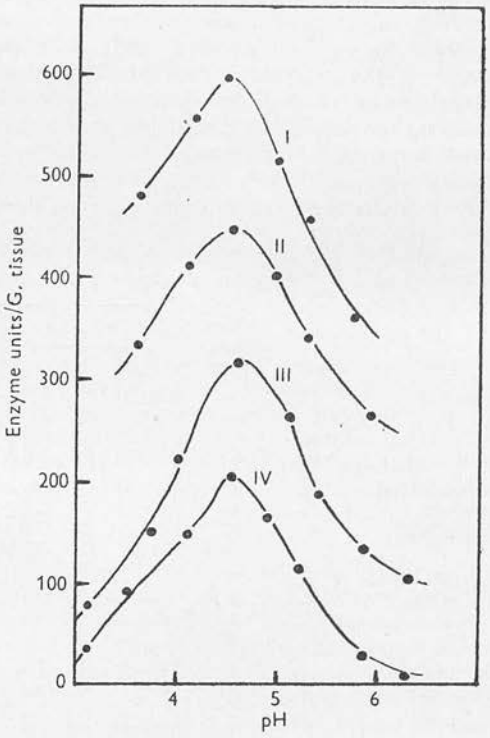

Fig. 3. pH-Activity curves for uterine glucuronidase. I, 10-day-old mice; II, ovariectomized adults, 3 days after subcutaneous injection of 1·7 mg. oestrone/kg.; III, normal adults; IV, ovariectomized adults.

tetrachloride on the pH-activity curve for liver (Fig. 1) renders this possibility unlikely, since both fractions were equally affected in the increase in activity. Carbon tetrachloride is known to be without effect on kidney glucuronidase (Levvy *et al.* 1948), although this organ resembles liver in the composition of the enzyme. In spite of these findings a great many more experiments were done before the possibility just outlined was rejected. In these experiments, the homogenate from each organ was divided into two portions, in one of which *A* and *B* were separated as described above and determined at their respective pH optima, 4·5 and 5·2. The other

portion of the homogenate was brought to 50% saturation with ammonium sulphate and the total enzyme thus precipitated was determined at pH 5·2. Average results for liver and kidney under a variety of conditions are shown in Table 1. Since *A* was determined at a different pH from the total activity, figures for the latter do not agree with the sums of the two fractions. One point not brought out in the table is that individual mice, treated and untreated, showed considerable variation in both liver and kidney in the ratio of the two fractions. Very occasionally, one animal in a group displayed complete lack of *A* or *B* in one of the two organs examined, unaccompanied by any compensatory increase in the activity of the remaining fraction. When this occurred, the size of the group was reduced by one in calculating the average and standard error for the fraction in question, as shown in Table 1.

cutaneous injection of carbon tetrachloride in olive oil were in entire agreement with the conclusions arrived at above, in that this agent showed no discrimination between the two fractions in liver, and was without effect on either in kidney. After subcutaneous injection of mercuric nitrate as an aqueous solution, *A* and *B* rose and fell together in kidney as repair processes became active and were completed. Since mercuric nitrate has little effect on liver (see Table 3), its action on this organ was not studied in the present experiments.

Changes in liver glucuronidase after subcutaneous injection of chloroform in olive oil closely resembled, as one might expect from previous work, those produced by carbon tetrachloride. Taking the results as a whole, there was a suggestion that fraction *A* returned to normal more rapidly than *B*. Before dealing with the effects of chloroform on kidney

Table 1. *Effects of various agents on glucuronidase fractions A and B in liver and kidney*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

			G.U./g. moist tissue					
			Liver			Kidney		
Agent	Sex*	Days after treatment	*A*†	*B*‡	Total‡	*A*†	*B*‡	Total‡
None	M.	—	106±17 (6)	223±12 (6)	281±20 (6)	124±16 (6)	123±38 (6)	266±31 (6)
	F.	—	115±12 (6)	247±38 (6)	334±48 (6)	109±8 (6)	118±16 (6)	266±39 (6)
	cM.	—	127±13 (6)	217±18 (6)	301±18 (6)	130±22 (6)	194±18 (6)	286±20 (6)
	cF.	—	116±12 (6)	167±47 (6)	250±49 (6)	113±10 (6)	130±22 (6)	261±43 (6)
Carbon tetrachloride (5·3 g./kg.)	M.	1	545±18 (3)	654±40 (2)§	830±62 (3)	122±27 (3)	150±61 (3)	206±28 (3)
	M.	4	364±58 (3)	537±27 (3)	763±65 (3)	118±19 (3)	138±18 (3)	257±19 (3)
	cF.	4	499±109 (3)	588±24 (3)	664±57 (3)	126±14 (3)	158±6 (3)	235±23 (3)
	cF.	7	517±27 (3)	568±35 (3)	693±31 (3)	127±14 (3)	186±70 (3)	270±42 (3)
Mercuric nitrate (20 mg./kg.)	M.	3	—	—	—	269±35 (3)	319±55 (3)	463±59 (3)
	M.	6	—	—	—	122±14 (3)	158±15 (2)§	277±19 (3)
	F.	3	—	—	—	224±24 (3)	351±45 (3)	491±62 (3)
	F.	6	—	—	—	122±10 (3)	124±26 (3)	237±61 (3)
Chloroform (2 g./kg.)	M.	1	343±188 (6)	766±147 (6)	881±163 (6)	134±19 (6)	121±17 (6)	206±19 (6)
	M.	4	260±42 (3)	561±44 (3)	766±46 (3)	128±18 (3)	234±22 (3)	302±12 (3)
	M.	7	184±29 (6)	559±38 (6)	650±72 (6)	132±29 (6)	395±54 (6)	524±38 (6)
	F.	1	537±64 (6)	410±194 (6)	812±91 (6)	173±10 (6)	135±9 (6)	227±23 (6)
	F.	4	271±45 (3)	570±52 (3)	696±61 (3)	126±19 (3)	148±35 (3)	238±27 (3)
	F.	7	151±42 (6)	559±106 (6)	705±64 (6)	108±16 (6)	223±78 (6)	310±55 (6)
	cM.	1	453±39 (3)	537±25 (3)	659±174 (3)	119±12 (3)	141±17 (3)	221±38 (3)
	cM.	7	152±20 (3)	549±42 (3)	673±32 (3)	132±20 (3)	124±22 (3)	238±30 (3)
	cF.	1	277±22 (2)§	594±32 (2)§	515±23 (3)	187±21 (6)	256±29 (6)	398±67 (6)
	cF.	4	224±25 (3)	510±20 (3)	620±26 (3)	189±15 (3)	247±18 (3)	352±24 (3)
	cF.	7	359±130 (5)	569±39 (5)	657±78 (5)	175±32 (7)	260±69 (7)	359±67 (7)

* c = castrated.
† One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0·015 M-phenylglucuronide in 1 hr. at 38° and pH 4·5.
‡ One G.U. (glucuronidase unit) liberates 1 μg. phenol from 0·015 M-phenylglucuronide in 1 hr. at 38° and pH 5·2.
§ One animal in group devoid of this fraction.

From Table 1, it appears that in either liver or kidney the distribution of glucuronidase activity between the two fractions was on the average the same for normal male and female mice. Castration 3–4 weeks before sacrifice had no marked effect on the results in either sex.

Results obtained during the prolonged rise in liver glucuronidase activity which follows subglucuronidase, it is necessary to consider the influence of sex on the susceptibility of the mouse kidney to chloroform necrosis. Eschenbrenner (1944) found that chloroform caused renal necrosis in male, but not in female mice, and it was shown later (Eschenbrenner & Miller, 1945) that the effect in males was prevented by castration at an early age. The sex-linked nature of this response was associated with

differences in the structure of Bowman's capsule described by Crabtree (1941), but chloroform necrosis, which involved the convoluted tubules, did not extend to the capsule itself. In previous work on glucuronidase (Levvy *et al.* 1948), the effect of chloroform on the kidney-enzyme level in normal mice was seen to be confined to males. From the figures for total kidney glucuronidase in Table 1, it appears that the response in the male was abolished by castration 3 weeks previously, although this operation was not performed until the animals were adult. There was no necrosis, and Bowman's capsule had become predominantly female in character. In ovariectomized females, chloroform caused a small, but significant rise in the kidney enzyme, associated with necrosis and repair (for results one day after injection, $P = 0{\cdot}02$; grouping results for all three time intervals, $P < 0{\cdot}01$). Extending the period between ovariectomy and injection of chloroform from 3 to 13 weeks did not appreciably affect this response, but in the interval a change towards the male type of kidney became much more pronounced. As already noted, figures for uninjected mice showed no variations in *A* or *B*, corresponding to the changes in kidney morphology. The fact that in the male kidney the rise in glucuronidase appeared to be confined to fraction *B* may reflect an uneven distribution of the two fractions throughout this organ, with predominance of *B* in the convoluted tubules. In a histochemical study, Friedenwald & Becker (1948) found greater glucuronidase activity in rat-kidney tubules than in the glomeruli, when hydrolysis of suitable glucuronides in unfixed, frozen sections was allowed to proceed at pH 5.

The effects of sex hormones on liver and uterine glucuronidase

Fishman (1947) has examined the effects of testosterone propionate and oestradiol benzoate, separately and in combination, on uterine glucuronidase in ovariectomized mice. In the doses used, testosterone did not antagonize the action of the oestrogen in causing a rise in the enzyme level, and Fishman interpreted this as indicating 'a unique type of specificity of action by the oestrogen'. His results, however, show that administration of the androgen along with the oestrogen did not entirely prevent an increase in the wet weight of the uterus. By itself, testosterone produced a rise in glucuronidase activity and an increase in weight. Fishman's results seem entirely compatible with the view that an increase in glucuronidase activity in uterus, as elsewhere, reflects increased growth, and that his failure to observe antagonism between oestrogen and androgen resulted from use of too great an excess of the latter. Figures for uterus shown in Table 2 bear out this argument.

Oestrone and testosterone were given as single subcutaneous injections of the solutions in olive oil, alone or within 3 hr. of each other. Four days after injection of ovariectomized mice with 1·7 mg. oestrone/kg., uterine glucuronidase activity and weight were much greater than in untreated controls. The effects of 0·3 mg. oestrone/kg. and 3·3 mg. testosterone/kg. were alike in that there was a comparatively small rise in uterine weight, with a barely perceptible increase in the enzyme activity ($P = 0{\cdot}1$ and 0·02 respectively at the maximum activity). In a smaller dose (2 mg./kg.), testosterone had no action on the uterus, but this dose completely antagonized the effects of the larger dose of oestrone on the enzyme and the weight.

In the experiments with oestrone and testosterone, glucuronidase was determined in liver and kidney as well as in uterus. In ovariectomized mice a marked rise in liver glucuronidase, preceding that in uterus, was observed after injection of oestrone in a dose of 1·7 mg./kg. (Table 2). This effect was also seen in normal and castrate males, but was absent in intact females, even after 4·3 mg. oestrone/kg. Reducing the dose of oestrone to 0·3 mg./kg. abolished the action on liver in ovariectomized mice. Histological examination revealed intense mitotic activity, with little evidence of damage in the livers of oestrone-treated castrate males and females. In normal males, the effect on mitosis was slight, but there was a marked increase in binucleate cells. Testosterone, itself without any action on the liver, antagonized the stimulant effect of oestrone on the enzyme and on cell division. Bullough (1946) has studied the effects of oestrone on mitotic activity throughout the body of the adult female mouse, and concluded 'that those substances which have come to be called oestrogenic or female sex hormones are in fact general mitosis stimulators'. Oestrone produced no effect on liver in his experiments, which were, however, confined to the normal female.

Fractionation of the glucuronidase preparations was carried out in many of the experiments listed in Table 2. Fractions *A* and *B* were both involved in the liver response to oestrone, while all uterine activity was invariably found in fraction *A*.

The effects of liver regeneration on uterine enzyme and weight

Administration of carbon tetrachloride to rats has been shown to produce an increase in the weight of the uterus in immature animals (Talbot, 1939), and to enhance the effectiveness of administered oestrone in ovariectomized animals (Pincus & Martin, 1940). Partial hepatectomy causes a similar increase in the potency of administered oestrogen (Segaloff, 1946). From the work of Roberts & Szego (1947) it appears that increased sensitivity to oestrogens occurs during active liver regeneration rather than in the initial stages of injury. In all these studies the animals were treated with an oestrogen or alternatively the ovaries were still present.

It was, therefore, with surprise that increases in the β-glucuronidase activity and the weight of

Table 2. *Effects of oestrone and testosterone on liver, kidney and uterine glucuronidase*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

Treatment	Sex*	Days after treatment	Total G.U./g. tissue: Liver‡	Total G.U./g. tissue: Kidney‡	Total G.U./g. tissue: Uterus†	Uterine weight (mg.)
None	F.	—	334±48 (6)	266±39 (6)	333±53 (6)	234±56 (6)
	cF.	—	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
	M.	—	281±20 (6)	266±31 (6)	—	—
	cM.	—	301±18 (6)	286±20 (6)	—	—
Oestrone (4·3 mg./kg.)	F.	1	258±39 (3)	370±33 (3)	463±41 (3)	167±66 (3)
	F.	4	244±32 (3)	357±44 (3)	439±63 (3)	192±76 (3)
Oestrone (1·7 mg./kg.)	F.	1	365±60 (3)	223±47 (3)	388±45 (3)	320±24 (3)
	F.	4	267±26 (3)	253±41 (3)	441±87 (3)	253±31 (3)
	cF.	1	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	cF.	2	481±34 (3)	275±67 (3)	343±47 (3)	47±8 (3)
	cF.	4	569±73 (6)	281±33 (6)	548±106 (6)	211±20 (6)
	cF.	6	399±67 (3)	356±52 (3)	346±41 (3)	52±10 (3)
	cF.	8	315±18 (3)	299±23 (3)	223±16 (3)	46±5 (3)
	M.	1	879±98 (9)	283±95 (9)	—	—
	M.	4	303±12 (6)	221±50 (6)	—	—
	cM.	1	359±43 (3)	324±22 (3)	—	—
	cM.	4	562±58 (3)	333±52 (3)	—	—
Oestrone (0·3 mg./kg.)	cF.	1	271±51 (3)	359±72 (3)	181±13 (3)	51±10 (3)
	cF.	2	274±34 (3)	321±30 (3)	247±38 (3)	47±2 (3)
	cF.	4	269±34 (3)	327±61 (3)	226±34 (3)	102±15 (3)
	cF.	6	256±38 (3)	305±22 (3)	181±36 (3)	63±9 (3)
Testosterone (3·3 mg./kg.)	cM.	1	271±50 (3)	172±14 (3)	—	—
	cM.	4	311±36 (3)	201±23 (3)	—	—
	F.	1	254±32 (3)	297±20 (3)	289±27 (3)	172±54 (3)
	F.	4	272±40 (3)	343±48 (3)	326±19 (3)	193±35 (3)
	cF.	0·5	236±62 (3)	339±38 (3)	199±20 (3)	57±12 (3)
	cF.	1	289±34 (3)	319±56 (3)	260±14 (3)	101±19 (3)
	cF.	2	284±25 (3)	363±46 (3)	183±35 (3)	44±6 (3)
	cF.	4	251±48 (3)	326±46 (3)	189±39 (3)	35±11 (3)
Testosterone (2 mg./kg.)	cF.	1	253±54 (3)	345±16 (3)	156±31 (3)	32±13 (3)
	cF.	4	259±18 (3)	345±25 (3)	154±31 (3)	39±9 (3)
Testosterone (2 mg./kg.) + oestrone (1·7 mg./kg.)	cF.	1	266±51 (6)	379±28 (6)	179±34 (6)	46±12 (6)
	cF.	4	279±20 (5)	340±56 (5)	205±31 (5)	47±7 (5)
	M.	1	261±20 (3)	314±31 (3)	—	—
	M.	4	295±78 (3)	323±64 (3)	—	—

*, †, ‡, see Table 1.

uterus were obtained 7 days after injection of ovariectomized mice with chloroform or carbon tetrachloride (Table 3). That these changes were not due to a direct action of the toxic agent on the uterus, but were secondary to the effect on liver, was shown by further experiments in which mice were submitted to partial hepatectomy 3 weeks after ovariectomy, with similar results. With all three methods of treatment, the rise in uterine weight at its greatest was statistically significant ($P < 0.001$). Liver repair is far advanced after this period (Levvy *et al.* 1948). In a separate group of six mice, the uteri were examined histologically 9 days after partial hepatectomy. Metoestrus, pro-oestrus and, in one case, full oestrus were observed, as compared with dioestrus in ovariectomized controls. It should be noted that not more than 40 % of the liver was removed in the partial hepatectomies in the present experiments. Results obtained with mercuric nitrate suggest that changes in kidney are without effect on uterine weight and glucuronidase activity.

Fractionation of uterine-glucuronidase preparations from ovariectomized mice treated with chloroform and carbon tetrachloride showed all activity to be present in fraction *A*.

DISCUSSION

The original purpose behind the experiments described above was to decide whether the sites of action of various agents on glucuronidase activity were determined by differences in the properties of the enzyme. No evidence of this was obtained. An increase in β-glucuronidase activity appeared to be governed solely by the ability of the agent to stimulate growth processes in the organ in question. Results for uterus did, however, emphasize the need for a preliminary kinetic study with each new organ

Table 3. *Effect of liver regeneration on uterine enzyme and weight*

(All values are given as mean ±S.E., followed (in parentheses) by the number of animals in the group.)

Treatment	Sex*	Days after treatment	Total G.U./g. tissue			Uterine weight (mg.)
			Liver‡	Kidney‡	Uterus†	
None	F.	—	334±48 (6)	266±39 (6)	333±53 (6)	234±56 (6)
	cF.	—	250±49 (6)	261±43 (6)	174±45 (6)	34±18 (6)
Chloroform (2 g./kg.)	F.	1	812±91 (6)	227±23 (6)	265±45 (6)	277±44 (6)
	F.	4	696±61 (3)	238±27 (3)	342±34 (3)	372±36 (3)
	F.	7	705±64 (6)	310±55 (6)	321±51 (6)	306±29 (6)
	cF.	1	515±23 (3)	398±67 (6)	181±33 (3)	28±10 (3)
	cF.	4	620±26 (3)	352±24 (3)	162±31 (3)	33±7 (3)
	cF.	7	657±78 (5)	359±67 (7)	469±51 (7)	103±20 (7)
Chloroform§ (6 g./kg.)	cF.	8	605±75 (3)	435±56 (3)	386±61 (3)	99±18 (3)
	cF.	10	501±30 (3)	335±52 (3)	306±22 (3)	65±12 (3)
Carbon tetrachloride (5·3 g./kg.)	cF.	1	712±55 (3)	301±42 (3)	203±48 (3)	25±6 (3)
	cF.	4	664±57 (3)	235±23 (3)	285±52 (3)	18±9 (3)
	cF.	7	715±60 (6)	290±59 (6)	496±64 (6)	77±23 (6)
	cF.	10	379±56 (3)	292±12 (3)	205±20 (3)	42±5 (3)
Partial hepatectomy	cF.	2	572±53 (3)	284±22 (3)	225±67 (3)	75±14 (3)
	cF.	4	532±83 (3)	305±29 (3)	240±42 (3)	64±8 (3)
	cF.	6	625±89 (3)	342±34 (3)	412±31 (3)	123±14 (3)
	cF.	8	535±61 (6)	328±54 (6)	395±43 (6)	115±19 (6)
	cF.	12	424±41 (3)	329±32 (3)	277±29 (3)	53±6 (3)
Mercuric nitrate (20 mg./kg.)	cF.	3	241±32 (3)	523±27 (3)	179±18 (3)	30±4 (3)
	cF.	6	238±26 (3)	323±24 (3)	168±26 (3)	39±6 (3)

*, †, ‡, see Table 1.

§ Divided into three daily doses of 2 g./kg.; timed from first injection.

examined for glucuronidase activity. The choice of pH 5·2 for determining activities in liver or kidney with phenylglucuronide would seem to be justified for most purposes.

Changes in the susceptibility of the mouse kidney to chloroform necrosis were faithfully reflected in the figures for glucuronidase activity. The value of such figures as a biochemical index of growth is illustrated by the discovery of new facts relating to liver and uterus. The action of oestrone on liver in normal and castrate males and in ovariectomized females, and the antagonizing of this action by testosterone are in accordance with the view of Bullough (1946) that the effects of such hormones are more widespread throughout the body than is generally appreciated. The absence of any action by oestrone on liver in normal females suggests some form of control of this organ by the ovary.

The changes observed in uterus during liver regeneration in the ovariectomized mouse can only be explained on the assumption that the body is capable of producing an extra-ovarian growth hormone for uterus in significant amounts. In this case, there is obvious need for care in interpreting certain experiments (Talbot, 1939; Pincus & Martin, 1940; Segaloff, 1946; Roberts & Szego, 1947) in which the action of liver damage and regeneration in enhancing the effectiveness of administered or ovarian oestrogens is claimed to be due to 'depressed inactivation' or 'accelerated activation' of the hormones. In view of the effect of oestrone on the liver, a hitherto unsuspected complication must be looked for in the action of this compound on the uterus of the ovariectomized mouse.

SUMMARY

1. Both β-glucuronidase fractions found by Mills (1947) in ox spleen are present in mouse liver and kidney, whilst the uterus contains only one of these.

2. The two glucuronidase fractions in liver and kidney respond identically to agents causing changes in the enzyme activity.

3. In the uterus, as in other organs, changes in glucuronidase activity reflect changes in growth, and the action of oestrone on the enzyme is antagonized by testosterone.

4. Oestrone produces marked increases in glucuronidase activity and cell division in the liver in ovariectomized mice. This action, which is also seen in normal and castrate males, but not in normal females, is antagonized by testosterone.

5. During liver regeneration following chloroform or carbon tetrachloride poisoning or partial hepatectomy, uterine weight and glucuronidase activity increase in ovariectomized mice in absence of administered oestrogen.

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REFERENCES

Bullough, W. S. (1946). *Philos. Trans.* B, **231**, 453.
Crabtree, C. (1941). *Endocrinology*, **29**, 197.
Eschenbrenner, A. B. (1944). *J. nat. Cancer Inst.* **5**, 251.
Eschenbrenner, A. B. & Miller, E. (1945). *Science*, **102**, 302.
Fishman, W. H. (1947). *J. biol. Chem.* **159**, 7.
Fishman, W. H. & Fishman, L. W. (1944). *J. biol. Chem.* **152**, 487.
Friedenwald, J. S. & Becker, B. (1948). *J. cell. comp. Physiol.* **31**, 303.
Kerr, L. M. H., Graham, A. F. & Levvy, G. A. (1948). *Biochem. J.* **42**, 191.
Kerr, L. M. H. & Levvy, G. A. (1948). *Nature, Lond.*, **162**, 219.
Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. (1948). *Biochem. J.* **42**, 462.
Mills, G. T. (1947). *Nature, Lond.*, **160**, 638.
Mills, G. T. (1948). *Biochem. J.* **43**, 125.
Pincus, G. & Martin, D. W. (1940). *Endocrinology*, **27**, 838.
Roberts, S. & Szego, C. M. (1947). *Endocrinology*, **40**, 73.
Segaloff, A. (1946). *Endocrinology*, **38**, 212.
Talbot, N. (1939). *Endocrinology*, **25**, 601.

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Further Observations on Changes in β-Glucuronidase Activity in the Mouse

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Increased β-glucuronidase activity in mouse liver or kidney was shown to reflect an increase in the proliferative activity of the tissue (Levvy, Kerr & Campbell, 1948). A similar relationship in uterus has been found to explain changes in the glucuronidase activity of this organ produced by certain sex hormones (Fishman & Fishman, 1944; Fishman, 1947; Kerr, Campbell & Levvy, 1949). Investigation of unexpected changes in glucuronidase activity in mouse tissues led to the discovery of new factors controlling growth in liver and uterus (Kerr *et al.* 1949). Further studies of these factors are described in the present communication. Additional examples of parallelism between the β-glucuronidase activity

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of a tissue and its state of proliferation, with particular reference to the actions of growth inhibitors, are also presented.

EXPERIMENTAL AND RESULTS

Methods

The assay of β-glucuronidase activity in tissue preparations and the measurement of uterine weight were carried out as described by Kerr *et al.* (1949).

The effects of certain steroid hormones on liver and uterine glucuronidase

In a previous communication (Kerr *et al.* 1949) it was shown that oestrone causes a marked rise in glucuronidase activity and in cell division in the livers of ovariectomized mice. This action was also seen in normal and castrated males, but not in intact females. In similar experiments with oestriol and oestradiol (Table 1), no change in liver glucuronidase activity was observed in male or ovariectomized female mice. Both compounds appeared to stimulate cell division in liver, but this effect was seen only with the larger doses studied, and at its greatest was slight compared with that produced by oestrone. Oestriol and oestradiol had the expected stimulant actions on uterine weight and glucuronidase activity (Fishman & Fishman, 1944; Fishman, 1947). It is not impossible that under different experimental conditions these steroids might show an effect on liver enzyme activity.

It has already been noted (Kerr *et al.* 1949) that testosterone antagonizes the effects of oestrone on liver and uterine glucuronidase. As can be seen from Table 1, progesterone (1·8 mg./kg.) antagonized the action of oestrone (1·7 mg./kg.). Given alone, a single injection of progesterone had no effect on the enzyme in liver or in uterus, even when the dose was increased threefold.

No significant changes in kidney glucuronidase activity were observed in the experiments listed in Table 1.

Table 1. *The glucuronidase activity of mouse liver and uterus after injection of certain steroid hormones*

(In this and all subsequent tables values are given as mean ±S.E. followed (in parentheses) by the number of animals in the group. M. = male, F. = female, O.F. = ovariectomized female.

Glucuronidase activity is expressed in units (G.U.). One G.U. liberates 1 μg. phenol from 0·015M-phenylglucuronide in 1 hr. at 38° at pH 5·2 (for liver, kidney and breast tissue) or at pH 4·5 (for uterus).

In the experiments of Table 1 hormones were administered by single subcutaneous injection of solutions in olive oil.)

Treatment	Sex	Days after treatment	Glucuronidase activity (G.U./g. tissue) Liver	Glucuronidase activity (G.U./g. tissue) Uterus	Uterine weight (mg.)
None	O.F.	—	270±18 (18)	166±21 (18)	36±9 (18)
	M.	—	273±14 (12)	—	—
Oestrone (1·7 mg./kg.)	O.F.	1	431±42 (3)	300±54 (3)	26±5 (3)
	O.F.	4	562±33 (9)	524±56 (9)	210±11 (9)
	M.	1	879±98 (9)	—	—
	M.	4	303±12 (6)	—	—
Oestradiol (2 mg./kg.)	O.F.	1	289±13 (3)	231±12 (3)	62±2 (3)
	O.F.	4	301±15 (3)	357±15 (3)	291±4 (3)
Oestradiol (6 mg./kg.)	O.F.	1	277±16 (3)	257±36 (3)	116±7 (3)
	O.F.	4	287±20 (3)	398±23 (3)	273±10 (3)
	M.	1	292±19 (3)	—	—
	M.	4	279±13 (3)	—	—
Oestriol (2·5 mg./kg.)	O.F.	1	281±17 (3)	209±11 (3)	57±6 (3)
	O.F.	4	306±22 (3)	305±15 (3)	257±11 (3)
Oestriol (5 mg./kg.)	O.F.	1	289±22 (3)	451±38 (3)	275±16 (3)
	O.F.	4	279±26 (3)	500±41 (3)	298±21 (3)
	M.	1	281±17 (3)	—	—
	M.	4	295±18 (3)	—	—
Progesterone (1·8 mg./kg.)	O.F.	1	265±29 (3)	156±8 (3)	38±3 (3)
	O.F.	4	284±15 (3)	167±10 (3)	41±4 (3)
Progesterone (5·4 mg./kg.)	O.F.	1	277±19 (3)	172±12 (3)	33±5 (3)
	O.F.	4	272±19 (3)	189±7 (3)	36±4 (3)
Progesterone (1·8 mg./kg.) + oestrone (1·7 mg./kg.)	O.F.	1	266±24 (3)	188±14 (3)	34±4 (3)
	O.F.	4	289±22 (6)	174±13 (6)	32±3 (6)

Changes in uterus during liver regeneration

In absence of administered oestrogen, small but definite increases in uterine weight and glucuronidase activity occur in ovariectomized mice during liver regeneration (Kerr *et al.* 1949). Further experiments (Table 2) show that administration of testosterone or progesterone (by subcutaneous injection in olive oil) prevented the changes in uterus, without influencing the rise in liver glucuronidase activity, after partial hepatectomy. It should be noted that the rise in uterine weight during liver regeneration was never more than about half that caused by an effective dose of oestrogen (Table 1). Vaginal smears were not unlike those obtained with a dose of oestrone, 0·3 mg./kg. (Kerr *et al.* 1949), which caused a similar increase in uterine weight. Vaginal smears taken from ovariectomized mice during liver regeneration were sent to Prof. G. F. Marrian, F.R.S., who gave us the following opinion: 'The smears were not positive on the basis of the Marrian & Parkes (1929) criteria. In many smears there was a lack of leucocytes and considerable mucification. In some there was a lack of leucocytes and some nucleated epithelial cells were present, while in others cornified cells plus leucocytes were observed. The action appeared to be similar to that observed with a dose of oestrogen just below that required to produce a positive smear on the basis of the Marrian & Parkes (1929) criteria.'

Table 2. *The effects of testosterone and of progesterone on uterus in ovariectomized mice after partial hepatectomy*

(Dose divided between two injections, one 6 hr. after partial hepatectomy, the other 3 days later. Measurements made 8 days after partial hepatectomy. G.U. defined in Table 1.)

Treatment	Glucuronidase activity (G.U./g. tissue) Liver	Glucuronidase activity (G.U./g. tissue) Uterus	Uterine weight (mg.)
None	270±18 (18)	166±21 (18)	36±9 (18)
Partial hepatectomy	559±49 (12)	351±30 (12)	111±13 (12)
Partial hepatectomy + testosterone (4·0 mg./kg.)	572±48 (6)	247±27 (6)	43±7 (6)
Testosterone (4·0 mg./kg.)	283±17 (6)	195±15 (6)	32±4 (6)
Partial hepatectomy + progesterone (3·6 mg./kg.)	509±36 (3)	186±16 (3)	38±5 (3)
Progesterone (3·6 mg./kg.)	282±25 (3)	174±21 (3)	35±2 (3)

The glucuronidase activity of proliferating breast tissue

Fishman & Anlyan (1947) found that the glucuronidase activity of human malignant growths was higher than that of most normal tissues. This observation has been confirmed in mice (Karunairatnam, Kerr & Levvy, 1949). Fishman & Anlyan (1947) also noted that the lactating breast in a human subject had a higher glucuronidase activity than normal breast tissue. Table 3 shows the glucuronidase activity of three spontaneous breast tumours in mice, and of healthy breast tissue at various stages of pregnancy and lactation. Histological examination showed the tumours to be adenocarcinomata. In the absence of any data for the kinetics of hydrolysis of phenylglucuronide by breast or tumour glucuronidase, the enzyme assays were done as recommended for liver and kidney (Kerr *et al.* 1949). In each animal, the mammary glands were pooled before homogenizing and taken through the rest of the procedure in the usual way.

Table 3. *Glucuronidase activities of healthy and malignant breast tissue*

(G.U. defined in Table 1.)

Description	Glucuronidase activity (G.U./g. tissue)
Normal adults (virgin)	92±7 (3)
7–14 days pregnant	173±9 (3)
14–21 days pregnant	165±16 (3)
1 day post-partum	151±19 (4)
11 days post-partum	97±4 (3)
Spontaneous adenocarcinomata (individual results)	197, 423, 454

If the figures in Table 3 may be taken as a guide, proliferation of the breast commenced in the mouse in the early stages of pregnancy and had ceased at 11 days post-partum, although the animals were still lactating at that time. At no stage was the glucuronidase activity of healthy breast tissue as high as in the tumours.

The glucuronidase activity of rat liver

It was considered desirable to examine the relationship between the glucuronidase activity and the state of proliferation of a tissue in another animal than the mouse. Table 4 shows figures obtained for the glucuronidase activity of infant, adult, and regenerating rat liver. Assays were done as described for mouse liver (Kerr *et al.* 1949). Liver glucuronidase activity in 4-day-old rats was more than twice the value for normal adults, and enzyme

activity in liver regenerating after partial hepatectomy was even higher. In individual operated animals, the activity of the portion of liver removed was never more than half the figure for the remaining hypertrophied lobe.

Table 4. *Glucuronidase activity of rat liver*

(G.U. defined in Table 1.)

Description	Glucuronidase activity (G.U./g. tissue)
Normal adults	1382±264 (10)
Normal infants (4-day-old)	2977±301 (4)
Adults, 4 days after partial hepatectomy	3177±541 (6)

Effect of growth inhibitors

Colchicine. In view of the inhibitory action of colchicine on growth processes in general (Lits, Kirschbaum & Strong, 1938), a study was made of its action on the glucuronidase activities of various organs in the mouse. The drug was dissolved in 0·9% sodium chloride solution and injected subcutaneously. As can be seen from Table 5, it had no marked effect on the enzyme levels in normal adults, even in a dose of 6 mg./kg., a dose which is usually rapidly fatal.

Given 6 hr. after partial hepatectomy, colchicine (1·5 mg./kg.) prevented hypertrophy of the remaining fragment of liver and the associated rise in glucuronidase activity (Table 5). When the interval between operation and colchicine injection was increased to 24 hr., regeneration took its normal course. Scheifley & Higgins (1940) found colchicine to be effective in partially hepatectomized rats only if given in the initial stages of recovery. Given within a few minutes of oestrone (1·7 mg./kg.), colchicine (1·5 mg./kg.) antagonized the effects of the oestrogen in liver and uterus in ovariectomized mice.

Table 6 shows results obtained in two experiments with infant mice. In each experiment, a litter was

Table 5. *Action of colchicine in combination with measures leading to changes in glucuronidase activity in adult mice*

(G.U. defined in Table 1.)

Treatment	Sex	Days after initial treatment	Glucuronidase activity (G.U./g. tissue) Liver	Kidney	Uterus	Uterine wt. (mg.)
None	M.	—	273±14 (12)	288±22 (12)	—	—
	F.	—	304±29 (12)	306±25 (12)	318±38 (12)	229±27 (12)
	O.F.	—	270±18 (18)	316±22 (18)	166±21 (18)	36±9 (18)
Colchicine (1·5 mg./kg.)	M.	2	273±22 (3)	277±42 (3)	—	—
	F.	2	243±23 (3)	350±45 (3)	213±14 (3)	194±5 (3)
	F.	4	281±18 (3)	379±19 (3)	205±10 (3)	183±6 (3)
	O.F.	2	282±16 (3)	379±31 (3)	187±22 (3)	39±8 (3)
	O.F.	4	269±22 (3)	357±25 (3)	168±24 (3)	29±3 (3)
Colchicine (3 mg./kg.)	M.	1	233±27 (3)	291±22 (3)	—	—
Colchicine (6 mg./kg.)	M.	1	260±15 (2)	252±18 (2)	—	—
Partial hepatectomy	M.	4	780±50 (6)	360±24 (6)	—	—
Partial hepatectomy + colchicine (1·5 mg./kg.) 6 hr. later	M.	4	243±26 (8)	285±17 (6)	—	—
Partial hepatectomy + colchicine (1·5 mg./kg.) 24 hr. later	M.	4	718±68 (3)	280±38 (3)	—	—
Oestrone (1·7 mg./kg.)	O.F.	1	431±42 (3)	313±17 (3)	300±54 (3)	26±5 (3)
	O.F.	4	562±33 (9)	309±20 (9)	524±56 (9)	210±11 (9)
Oestrone (1·7 mg./kg.) + colchicine (1·5 mg./kg.) simultaneously	O.F.	1	307±9 (3)	370±23 (3)	246±32 (3)	32±2 (3)
	O.F.	4	320±20 (6)	362±19 (6)	251±27 (6)	40±8 (6)

Table 6. *Effect of colchicine in young mice*

(Subcutaneous injection of 1 mg. colchicine/kg. in 0·9% NaCl solution every second day. Litter-mate controls injected with pure NaCl solution. G.U. defined in Table 1.)

Description	Age (days) At start	At end	Average body wt. (g.) At start	At end	Increase in wt. (%)	Glucuronidase activity (G.U./g. liver)
Treated	9	18	6·88	6·70	−2·6	347±31 (4)
Controls	9	18	6·73	11·10	64·9	673±49 (4)
Treated	10	15	7·90	8·00	1·3	310±17 (4)
Controls	10	15	8·10	9·21	13·7	1043±62 (4)

removed from the mother and divided into two groups. One group received repeated injections of colchicine and the other was kept as control. Both groups received the same diet. The control animals had gained in weight and their liver glucuronidase activities were normal for their age (Karunairatnam *et al.* 1949) at the end of each experiment. The colchicine-treated mice, on the other hand, did not gain in weight and their liver enzyme levels fell to within the range for normal adults.

Sorbic acid. 'Parasorbic acid' (D-4-hydroxypent-1-ene-1-carboxylic acid lactone) inhibits the growth of connective tissue (Haynes, 1948). It was considered of interest to investigate the possibility that the related compound, sorbic acid (penta-1:3-diene-1-carboxylic acid), which is readily available, might have a depressant action on tissue growth and glucuronidase activity *in vivo*. It has no effect on the enzyme *in vitro* (Karunairatnam & Levvy, 1949). In the following experiments, sorbic acid (Light & Co. Ltd.) was given to mice by subcutaneous injection of a neutralized solution in 0·9% sodium chloride solution.

Single injections of 160 mg. sorbic acid/kg. had no effect on the glucuronidase activities of liver, kidney and uterus in adult mice (male and ovariectomized female), nor was there any striking change in the histological appearance of the organs. The general picture was unchanged when this dose was repeated on several consecutive days (Table 7).

Table 7. *Effect of sorbic acid on liver, kidney and uterine glucuronidase in normal mice*

(G.U. defined in Table 1.)

Dosage	Sex	Days after injection	Glucuronidase activity (G.U./g. moist tissue) Liver	Kidney	Uterus	Uterine wt. (mg.)
None	M	—	273±14 (12)	288±22 (12)	—	—
	F.	—	304±29 (12)	306±25 (12)	318±28 (12)	229±27 (12)
	O.F.	—	270±18 (18)	316±22 (18)	166±21 (18)	36± 9 (18)
160 mg./kg. injected daily throughout experiment	O.F.	6	258±26 (3)	382±36 (3)	175±20 (3)	41± 8 (3)
	O.F.	10	263±24 (3)	359±25 (3)	168±16 (3)	35± 7 (3)
240 mg./kg. (single injection)	M.	2	284±37 (3)	361±27 (3)	—	—
	M.	4	27±30 (12)	31±25 (12)	—	—
	M.	5	133±21 (3)	234±19 (3)	—	—
	M.	6	240±29 (3)	345±26 (3)	—	—
	M.	9	287±27 (3)	389±24 (3)	—	—
	M.	11	327±18 (3)	394±31 (3)	—	—
	F.	2	275±21 (6)	360±27 (6)	275±19 (6)	247±17 (6)
	F.	4	17± 9 (12)	34±28 (12)	301±20 (12)	202±14 (12)
360 mg./kg. (single injection)	M.	1	259±26 (3)	370±36 (3)	—	—
	F.	2	245±31 (3)	345±28 (3)	265±19 (3)	209±17 (3)

After a single injection of 240 mg. sorbic acid/kg., a profound depression was seen after 4 days in the glucuronidase activities of liver and kidney, but not of uterus (Table 7). Individual animals sometimes showed zero enzyme activities in liver or kidney. With this dose of sorbic acid, necrosis was seen in the liver, but the kidney was apparently normal. A fall in glucuronidase activity in a severely damaged organ has been noted with other poisons (Levvy *et al.* 1948). The histological findings thus provide some explanation for the action of sorbic acid on the enzyme in liver, but not in kidney.

When the dose of sorbic acid was increased to 360 mg./kg., the mice never survived single injections for more than 2 days, by which time any action on the enzyme was not apparent (Table 7). Histological examination revealed severe necrosis in the liver, slight damage in the kidney, and an arrest of mitosis throughout the body. Repeated daily injections of 240 mg./kg. were also rapidly fatal.

Experiments in which infant mice received 160 mg. sorbic acid/kg. (Table 8) yielded results resembling those obtained with colchicine, in that growth was checked and the liver enzyme activity reduced to the adult value.

Many experiments were carried out in which the action of sorbic acid was studied following partial hepatectomy or injection of oestrone or carbon tetrachloride. Results of representative experiments are shown in Table 9. Sorbic acid did not prevent the rise in liver glucuronidase activity which follows injection of carbon tetrachloride (Levvy *et al.* 1948), nor did it influence the changes produced in liver and uterus by oestrone (Table 1). It did, however, prevent the increase in liver enzyme activity and the associated changes in uterus which follow partial hepatectomy (Table 2). The action of sorbic acid in a dose of 240 mg./kg. in depressing kidney glucuronidase activity, observed after 4 days in normal animals (Table 7), was still seen after partial hepatectomy or injection of carbon tetrachloride, but not after oestrone administration. In the livers of partially hepatectomized mice injected with sorbic acid, cell division was frequently seen to be arrested

Table 8. *Effect of sorbic acid in young mice*

(Subcutaneous injection of 160 mg. sorbic acid/kg. as a neutral solution in 0·9% NaCl solution daily. Litter-mate controls injected with pure NaCl solution. G.U. defined in Table 1.)

Description	Age (days)		Average body wt. (g.)		Increase in wt. (%)	Glucuronidase activity (G.U./g. liver)
	At start	At end	At start	At end		
Treated	10	22	8·10	8·95	10·5	290±22 (4)
Controls	10	22	8·10	13·50	66·6	636±29 (4)
Treated	11	22	6·20	6·66	7·4	283±15 (4)
Controls	11	22	6·15	10·15	65·1	574±32 (4)

Table 9. *Action of sorbic acid in combination with measures leading to changes in glucuronidase activity in adult mice*

(G.U. defined in Table 1.)

Other treatment	Sex	Days after other treatment	Glucuronidase activity (G.U./g. tissue)			Uterine wt. (mg.)
			Liver	Kidney	Uterus	
Single injection of 240 mg. sorbic acid/kg. within 1 hr. of other treatment						
CCl_4 (5·3 g./kg.)	M.	4	576±49 (3)	62±28 (3)	—	—
Partial hepatectomy	M	4	298±32 (6)	78±23 (6)	—	—
Oestrone (1·7 mg./kg.)	M.	4	428±32 (3)	367±35 (3)	—	—
Daily injection of 160 mg. sorbic acid/kg. commencing 3 days before other treatment						
CCl_4 (5·3 g./kg.)	M.	7	858±48 (3)	378±25 (3)	—	—
Partial hepatectomy	O.F.	8	325±36 (3)	347±26 (3)	142±18 (3)	46±9 (3)
	M.	8	307±29 (3)	307±32 (3)	—	—
Oestrone (1·7 mg./kg.)	O.F.	4	468±25 (3)	362±29 (3)	377±25 (3)	197±8 (3)

in the metaphase. Displacement of the chromosomes was occasionally observed in cells in late anaphase.

DISCUSSION

In the variety of experiments described above in which a change was observed in glucuronidase activity, the only other common factor would appear to be a change in the state of proliferation of the tissue studied. The results are thus in accordance with the views put forward concerning this enzyme in previous communications (Levvy *et al.* 1948; Kerr *et al.* 1949; Odell & Burt, 1949). In common with the practice of most other workers in this field, our determinations of glucuronidase activity were made with aqueous extracts of freshly homogenized tissue, after a brief period of incubation to coagulate inactive protein. In recent work, carried out at the Rowett Research Institute, we have found that normal mouse liver contains relatively large amounts of an insoluble glucuronidase precursor. Preliminary results suggest that this precursor disappears from liver when the activity of extracts rises after partial hepatectomy. Incubation at acid pH leads to transformation of the precursor into the soluble enzyme, and this occurs to some extent under the conditions used for the final assay of the enzyme. It is evident that varying the mode of preparation of the enzyme for assay may profoundly alter the type of result obtained during changes in the state of proliferation of a tissue. For example, extracts prepared from tissue homogenates which have first been submitted to prolonged incubation may be expected to contain glucuronidase arising from the precursor as well as the enzyme originally present in an active state.

Colchicine had no effect on the glucuronidase activities of normal adult tissues as measured by our own procedure. It did, however, prevent increases in activity following on measures which normally stimulate cell division. It seems probable from these findings that the action of the drug on the activity of the enzyme was secondary to its effect on mitosis, and that the small number of dividing cells in an adult liver or kidney makes a negligible contribution to the total glucuronidase activity.

Sorbic acid arrested growth in infant mice and liver regeneration in partially hepatectomized mice. In these experiments it kept the liver glucuronidase activity at the normal adult level in the same way as colchicine. The failure of sorbic acid to prevent rises in liver and uterine glucuronidase activity in other experiments may have been due to inadequate dosage. The toxicity of the compound, however, precluded increases in dosage. It is possible that the falls in liver and kidney glucuronidase activity produced in normal mice by 240 mg. sorbic acid/kg. may

have been due, not to the compound itself, but to some metabolite, such as parasorbic acid, since the effects were not seen until 4 days after injection. More information is obviously required regarding the action and fate of sorbic acid in the body before its effects on glucuronidase can be fully understood.

It seems probable that the changes seen in uterus during liver regeneration in ovariectomized mice were due to an extra-ovarian oestrogen since they were antagonized by testosterone and by progesterone. The bearing of this finding on certain experiments which claim to show a change in the metabolism of oestrogens during liver damage and regeneration has already been discussed (Kerr *et al.* 1949). Toxicity for liver may explain the actions of some compounds with feebly oestrogenic properties. It seems quite possible that such compounds could cause urinary excretion of a 'true oestrogen', as defined by Emmens (1943).

The action of oestrone on liver was not shared by oestriol and oestradiol under the conditions of our experiments, but it did resemble an oestrogenic action in that it was antagonized by testosterone (Kerr *et al.* 1949) and by progesterone.

SUMMARY

1. Under similar experimental conditions, oestriol and oestradiol do not show the same action as oestrone (Kerr, Campbell & Levvy, 1949) in increasing liver glucuronidase activity in male and ovariectomized female mice. The action of oestrone on liver is antagonized by progesterone.

2. Testosterone and progesterone antagonize the increases in uterine weight and glucuronidase activity seen in ovariectomized mice during liver regeneration (Kerr *et al.* 1949), without affecting the enhanced enzyme activity in liver itself.

3. The glucuronidase activity of mouse breast tissue rose in the early stages of pregnancy and had returned to normal at 11 days post-partum. At no time was the activity in healthy breast tissue as high as in the mammary tumours studied.

4. Colchicine, itself without action on the glucuronidase activities of normal adult mouse tissues, antagonized the effects of measures which cause an increase in activity. Liver glucuronidase activity in infant mice was reduced to the adult value by colchicine.

5. Sorbic acid inhibits cell division to some extent. This does not, however, explain all the effects of the compound on glucuronidase.

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REFERENCES

Emmens, C. W. (1943). *J. Endocrinol.* **3**, 316.
Fishman, W. H. (1947). *J. biol. Chem.* **169**, 7.
Fishman, W. H. & Anlyan, M. D. (1947). *Cancer Res.* **7**, 808.
Fishman, W. H. & Fishman, L. W. (1944). *J. biol. Chem.* **152**, 487.
Haynes, L. J. (1948). *Quart. Rev. Chem. Soc.* **2**, 46.
Karunairatnam, M. C., Kerr, L. M. H. & Levvy, G. A. (1949). *Biochem. J.* **45**, 496.
Karunairatnam, M. C. & Levvy, G. A. (1949). *Biochem. J.* **44**, 599.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1949). *Biochem. J.* **44**, 487.
Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. (1948). *Biochem. J.* **42**, 462.
Lits, F. J., Kirschbaum, A. & Strong, L. C. (1938). *Amer. J. Cancer*, **34**, 196.
Marrian, G. F. & Parkes, A. S. (1929). *J. Physiol.* **67**, 389.
Odell, L. D. & Burt, J. C. (1949). *Cancer Res.* **9**, 362.
Scheifley, C. & Higgins, G. (1940). *Proc. Mayo Clin.* **15**, 536.