

The Effect of Adrenocorticotropin on the Nucleic Acids

and

Histochemistry of the Guinea-Pig Adrenal Cortex

by

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THE EFFECT OF ADRENOCORTICOTROPIN ON THE NUCLEIC ACIDS AND
HISTOCHEMISTRY OF THE GUINEA-PIG ADRENAL CORTEX

Summary of Thesis Presented by
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The effect of adrenocorticotropin (Armour ACTH gel) on the mean amount of deoxyribonucleic acid (DNA) per nucleus, and the scatter about this mean, in adrenal nuclei from mature guinea-pigs was studied, using cytophotometry of Feulgen stained nuclear smears. The results were compared with those from chemical analysis of the same material. The mean adrenal nuclear DNA content was similar to that in a control specimen mounted on the same slide in each instance, except after administration of adrenocorticotropin for 5 and 7 days. Cytophotometric analyses showed a highly statistically significant increase ($P < 0.01$) in mean adrenal nuclear DNA content after treatment of the animals with ACTH for 5 and 7 days. Chemical analyses gave values for adrenal nuclear DNA which were slightly higher than those for pooled kidney nuclei. The significance of these findings is discussed.

Statistical analysis of the results indicates that only specimens on the same slide are to be compared when using cytophotometry of the Feulgen reaction. If specimens on different slides are used for comparison a large error is introduced.

The effect of ACTH was also studied on adrenocortical ribonucleic acid (RNA), plasmalogens, alkaline and acid phosphatase, ascorbic acid and glycogen in mature guinea-pigs after treatment with ACTH for similar periods of time (1,3,5,7,10, 14 and 21 days) and also for 3,6,12 and 18hr and for 28 days.

A gradual increase in adrenocortical ribonucleic acid occurred with ACTH treatment. This increase was found in all zones of the adrenal cortex. Depletion of lipid and of plasmalogens was evident in the zona fasciculata and zona reticularis after ACTH administration for 3,6,12 and 18 hr. In the other experimental groups of animals an increase in lipid and plasmalogens occurred with continued ACTH administration. At 28 days, however, some depletion of these substances was found in the zona reticularis.

ACTH administration caused an increased concentration of alkaline and acid phosphatase in all adrenocortical zones. The increase was most evident after treatment with ACTH for 5 and 7 days, when hyperplasia was maximal.

Ascorbic acid depletion was observed after ACTH treatment for 3,6, 12 and 18 hr and for 1 day. The adrenocortical content and distribution of ascorbic acid was normal in adrenals of guinea-pigs receiving ACTH for longer periods. Glycogen depletion occurred at 12 and 18 hr only.

The findings are discussed and compared with results of other authors who studied the pituitary-adrenal relationship.

It is evident that ribonucleic acid, alkaline and acid phosphatase and ascorbic acid have important roles in adrenocortical Physiology. The results suggest that ribonucleic acid and phosphatases are probably concerned with adrenocortical hyperplasia. It seems more likely, however, that ascorbic acid and glycogen are concerned with secretion of adrenocortical hormones, as depletion of these substances occurred when secretion of ketosteroids was probably at a maximum.

"I come, to pluck your berries harsh and crude;
And, with forced fingers rude,
Shatter your leaves before the mellowing year."

- John Milton.

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ADRENOCORTICOTROPIN

ADRENOCORTICAL DEOXYRIBONUCLEIC ACID

THE EFFECT OF ADRENOCORTICOTROPIN ON GUINEA-PIG

PART I

**THE EFFECT OF ADRENOCORTICOTROPIN ON GUINEA-PIG
ADRENOCORTICAL DEOXYRIBONUCLEIC ACID WITH A
CRITICAL APPRAISAL OF CYTOPHOTOMETRIC METHODS**

ADRENOCORTICOTROPIN (ACTH) is a polypeptide hormone secreted by the anterior pituitary gland. It has been shown to have a variety of effects on the adrenal cortex, including stimulation of the synthesis and secretion of adrenal cortex hormones. In addition, ACTH has been shown to have effects on other tissues, including the brain, heart, and lung. The effects of ACTH on the adrenal cortex are well known, but its effects on other tissues are less well understood. In particular, the effect of ACTH on the synthesis and secretion of adrenal cortex hormones is well known, but its effect on the synthesis and secretion of adrenal cortex hormones is less well understood. In particular, the effect of ACTH on the synthesis and secretion of adrenal cortex hormones is well known, but its effect on the synthesis and secretion of adrenal cortex hormones is less well understood.

C Y T O P H O T O M E T R YINTRODUCTION

Since the pioneer investigations of Friedrich Miescher (1844-1895) much work has been devoted to the chemistry, metabolism and biosynthesis of deoxyribonucleic acid (DNA), and to the role it plays in the life history of the cell nucleus. Concise and convenient methods for the estimation of DNA have, however, become available only comparatively recently. This is especially true of cytophotometric methods whose use was suggested by the work of Caspersson (1936).

Deoxyribonucleic acid is confined to the cell nucleus, forming a stable component of the chromosomes. Biologically it is mainly concerned with transmitting hereditary characteristics, and with tissue growth.

When we consider that much of pathology deals with inflammatory processes and with neoplasia, in each of which tissue growth plays such a fundamental part, we can appreciate the importance of studying the DNA content of cell nuclei, its variation in cell multiplication, and the factors which control these.

It is thought that hormones regulate cellular function by combining with enzymes or their inhibitors. Study of the hypertrophy and hyperplasia caused by anterior pituitary hormones in their target organs is especially interesting and may be expected to yield fundamental information concerning normal growth (Fiala, Sproul & Fiala 1956). The effect of adrenocorticotropin on adrenocortical DNA may now be examined with more confidence, since this hormone is available in relatively pure form, and cytophotometry of the Feulgen reaction has been used by many workers since the pioneer investigation of Stowell (1942), and now rests on a comparatively firm basis as a quantitative technique (Vendrely 1956).

When I decided to study the effects of adrenocorticotropin (ACTH) on the adrenal gland, it seemed logical to lay emphasis initially on the study of the adrenocortical nucleic acids after administration of this hormone. Since deoxyribonucleic acid could be measured quantitatively by a cytophotometric technique, this was considered a logical beginning.

The history of development of cytophotometry, its uses, and my own experience of its use for measuring DNA in isolated Feulgen stained nuclei are first dealt with in Part I of this thesis. The biological importance of this substance, and the cytological techniques generally used for its recognition are considered before I describe experiments which I conducted.

OBJECTS OF THE INVESTIGATION

The objects of the cytophotometric investigation are as follows :-

1. To find out if the administration of ACTH caused any alteration in the mean nuclear DNA of the adrenal cortex.
2. To determine the scatter about this mean.
3. To investigate the possibility of tetraploid and/or octaploid nuclei occurring in the adrenal cortex.
4. To determine whether cytophotometric analyses of Feulgen stained nuclei gave results which agreed with chemical analyses of the same material for mean nuclear DNA content.
5. To investigate the possibility of justifiably comparing results accruing from cytophotometric analyses

of Feulgen stained nuclei mounted on different slides but fixed and stained together.

6. To correlate the results for adrenocortical nuclear DNA content and concentration with changes induced in adrenocortical ribonucleic acid (RNA) and some other histochemically demonstrable substances after ACTH administration.

PHOTOMETRIC PRINCIPLES

The term cytophotometry is preferable to microphotometry or micro-absorption photometry (Drabkin 1947, Moses 1950). In colorimetry the colour of the substance in unknown concentration is brought to colour match with a standard colour representing the substance in known concentration. Photometry implies direct measurement of the colour intensity in terms of the light absorbing powers of the substance at a specific region of the spectrum. It is thus applicable to the (invisible) ultra-violet and infra-red regions of the spectrum also. The substance to be examined must be coloured or be capable of undergoing reactions leading to the production of colour. The colour intensity must be dependent on the concentration of the substance being measured. Many biochemical methods are dependent on photometry. The use of cytophotometry is a much more recent advance, the first application of

cytophotometric methods being introduced by Stowell (1942).

Photometry means the measurement of the light transmitting power of a solution or substance in order to determine the concentration of light absorbing material present....

$$T(\text{Transmittance}) = I/I_0 \text{ (Emitted light/Incident light).}$$

A colourless solvent is used when feasible. Both solutions are examined under equivalent conditions of wavelength, incident light intensity and depth of solution. It is more satisfactory to express T as the negative logarithm or $-\log T$. This value ($-\log T$) is known as the Optical Density (D), or Extinction (E). Where Beer's law is applicable, E is directly proportional to the concentration of coloured substance present.

It is of the utmost importance that substances for comparison by photometry or relative cytophotometry be treated under as carefully controlled conditions as possible. In the case of tissue sections and cell smears it is necessary that the test specimen and a control be mounted on the same slide, so that differences in the conditions of fixation, staining etc., be obviated almost entirely.

LAWS OF ABSORPTION

Photometric and cytophotometric quantitative analyses depend on two laws which relate the ratio of light incident on an absorbing substance to the light transmitted by the substance.

I. Lambert's Law states that the proportion of radiation absorbed by a substance is independent of the intensity of incident radiation.

It is expressed by

$$I = I_0^{-ml}$$

where I_0 and I are the intensities of light entering and transmitted by a thickness l of the substance, and m is a constant, the absorption coefficient of the substance. Generally different amounts of various wavelengths are absorbed, so that Lambert's law applies only where monochromatic light is used.

II. Beer's Law. Beer based his absorption law on experiments made on aqueous solutions of inorganic salts. He measured photometrically the absorption of light by various concentrations of salt solutions. His law states that light absorption by a substance depends on the number

of absorbing molecules through which the radiation passes.

$$T = 10^{-klc}$$

where

T = Transmittance

l = depth of solution

k = constant

and

c = concentration of substance.

alternatively-

$$\begin{aligned} \text{or} \quad \text{Log } T &= -klc, \\ -\text{Log } T &= klc. \end{aligned}$$

It is generally accepted that Beer's law holds, and can be applied to quantitative measurement of substances photometrically.

THE LAMBERT-BEER RELATIONSHIP AND CYTOPHOTOMETRY

When photometry is applied to tissue sections and cell smears, quite different conditions hold from those under which biochemical photometric estimations are made. Technical difficulties prevent cytophotometric analysis of material from a single cell or nucleus in a micro-cuvette measuring a few microns in depth, though micro-chemical analyses on small numbers of cells have been achieved by a photometric technique (Glick & Biskind, 1935).

The question arises whether the Lambert-Beer relationship holds for substances in cell films and tissue sections.

The validity of the method depends primarily on agreement of results obtained with those accruing from chemical estimations. The following points seem pertinent to this problem...

1. Do other methods confirm results from cytophotometric analyses ?
2. Where 'polyploidy' is known to occur, e.g. in rat liver, are values found to be distributed in classes of DNA content which follow a geometrical progression ?
3. Is the constancy which has been found by chemical methods for DNA in diploid nuclei of an individual or species reflected in results obtained for similar tissues by cytophotometric methods ?
4. If sections are cut at various thicknesses, and the amount of material being examined estimated for each, does a linear relationship obtain between the amount of material found and the thickness of the section ?
5. If the amount of DNA per nucleus is assumed to be approximately constant in a given tissue, and the concentration of DNA to vary inversely with nuclear volume, is this inverse ratio shown when extinction is plotted graphically against volume, i.e. is concentration depicted ?

6. Is a scatter of values of DNA from an arbitrary amount X to $2X$ found, as would theoretically be expected due to the accumulation of DNA to twice its resting value during the early stages of mitosis ?

7. Is the substance being estimated homogeneous, or if not, is the resulting error small ?

8. Do cytophotometric methods, using different techniques for a particular substance, give comparable results ?

9. Can the volume of the material being examined be accurately computed ?

10. Is the experimental error of the technique calculable ?

11. Is a linear relationship obtained when extinctions from strips of film which have been exposed for a geometric increase in time are plotted against the logarithm of the time of exposure ?

12. Is the colour produced by the reaction under consideration linear for the concentrations present in the tissue being examined ?

From the following evidence it seems that the Lambert-Beer relationship does definitely hold under the conditions

of cytophotometry as generally practised.

1. The most extensive results available are those comparing cytophotometric results with chemical results obtained from analyses of known numbers of isolated nuclei of the same or similar biological material. Determination of absolute mean amounts of DNA in mouse thymocytes by Kurnick (1950b) gave values which agreed well with those obtained from chemical analyses of similar material by Mirsky & Ris (1949). Good agreement between chemical and cytophotometric analytical results for DNA content of rat liver, and for beef liver, thymus and sperm were obtained by Leuchtenberger, Vendrely & Vendrely (1951). Similar agreement was found for liver and erythrocyte nuclei of various vertebrates by Ris & Mirsky (1949b).

2. Boivin, Vendrely & Vendrely (1948) have shown that spermatids contain half the amount of DNA present in somatic nuclei in general, a finding consistent with the observation of their haploid content of chromosomes.

In a number of organs nuclei occur which have four times (tetraploid) or eight times (octaploid) the haploid number of chromosomes, and these nuclei have been shown by various workers, using cytophotometric techniques, to

contain corresponding amounts of DNA. Swift (1950,a) in an extensive investigation using cytophotometry of Feulgen stained nuclei found that in mouse liver, thymus, pancreas, lymphocytes and Sertoli cells, some nuclei contained tetraploid or octaploid amounts of DNA, and that secondary spermatocytes contained twice and primary spermatocytes four times the spermatid (haploid) value. A similar occurrence of haploid, diploid, tetraploid and octaploid DNA values were found by Swift (1950,a) in kidney cells of the grasshopper *dissosteira carolina*. Studies by Alfert (1950) on the DNA content of mouse oocytes during oogenesis led to results complementary to those of Swift (1950,a) in so far as haploid and diploid nuclei are concerned. Diploid, tetraploid and octaploid values of DNA for rat liver nuclei were found by Thompson (1953) using a cytophotometric method.

3. The constancy in the average amount of DNA per diploid (somatic) nucleus of an individual or species has been demonstrated by various workers using cytophotometric methods. The tissues studied by these authors are tabulated on pages 13 and 14.

Tissues shown to have Constancy in Nuclear DNA Content

by

Various Authors who used Cytophotometric Methods

| Author(s) | Year | Animal or Plant Tissue(s) |
|---|---------|---|
| Swift | 1950a,b | Various tissues of mouse. Plant tradescantia. |
| Alfert & Bern. | 1951 | Rat uterus after Oestrogen injection. |
| Leuchtenberger Vendrely & Vendrely. | 1951 | Various bovine tissues. Rat liver and kidney. |
| Leuchtenberger, Leuchtenberger, Vendrely & Vendrely. | 1952 | Bovine liver and thymus. Rat liver and thymus. |
| Di Stefano, Bass, Diermeier & Tepperman | 1952 | Rat liver after hypophysectomy. |
| Fukuda & Sibatani | 1953b | Liver from scorbutic guinea-pigs. |
| Fukuda & Sibatani | 1953a | Liver of adult rat |

Tissues shown to have Constancy in Nuclear DNA Content

by

Various Authors who used Cytophotometric Methods (ctd).

| Author(s) | Year | Animal or Plant Tissue(s) |
|---------------------------------------|------|---|
| Frazer & Davidson | 1953 | Chick liver and cultured chick heart (fibroblasts). |
| Thompson & Frazer | 1954 | Various rat tissues. |
| Rudkin, Aronson, Hungerford & Schultz | 1955 | Salivary gland of <i>Drosophila melanogaster</i> . |
| Ischitani, Uchida & Ikeda. | 1956 | <i>Aspergillus soja</i> . |
| Naora | 1957 | Various rat tissues |

4. An important contribution was made by Swift (1950a) who investigated the relationship between extinction and section thickness in Feulgen-stained tissue sections of *ambyostoma* liver nuclei. His results showed a linear relationship between extinction and section thickness between 3 and 11 microns. A similar relationship was shown for thyroid colloid stained by the Millon reaction by Pollister & Ris (1947), indicating that Beer's law was followed.

5. The relationships between volumes of individual nuclei and their extinction values were shown by Swift (1950a) to bear an inverse ratio to one another. Similar ratios are evident from the results of Alfert & Bern (1951), Leuchtenberger & Schrader (1951), Schrader & Leuchtenberger (1950). Similar ratios were found by myself (Fig.7). The deviation from a strict linear relationship is due to specimens being mounted on different slides.

6. A scatter of DNA values in diploid nuclei of an individual or species from an arbitrary amount X to $2X$ is evident in the frequency histograms published by Thompson (1953), and Thompson & Frazer (1953). In these histograms and in those of the author of this thesis and

of Hutchison, Burns & Hale (1957) only a comparatively small number of values lie outside this range. This suggested biological scatter seems logical if we are to accept the inference from the work of Alfert (1950), Price & Laird (1950), Peterman & Schneider (1951) and Walker & Yates (1952). From the results of these authors it seems that synthesis of DNA for a new set of chromosomes takes place, not in prophase, as had been previously supposed, but in interphase - in nuclei which show no morphological changes known to be associated with mitosis. The method of autoradiography was used to confirm this finding (Howard & Pelc, 1951).

7. Inhomogeneity of dye distribution due to changes induced by tissue fixation or staining may increase the error of the method. Treatment of cell suspensions with isolation media containing sucrose is highly desirable (Kurnick, 1951). My own results, from estimation by a cytophotometric method of Feulgen stained DNA in guinea-pig kidney and adrenal nuclei, emphasize the advisability of preserving, as far as possible, the in-vivo homogeneity of nuclear material.

8. The results from the three classical methods for cytophotometric estimation of DNA (ultraviolet microspectrophotometry, Feulgen staining and staining with methyl-green) were stated to be in good agreement, as regards patterns of distribution of DNA values in rat liver and kidney, by Frazer & Davidson (1953). Cytophotometry of methyl-green stained calf thymus nuclei was found by Kurnick (1951) to give results which were stated to agree well with those of Kurnick & Thorell (1951) using densitometry of ultraviolet photographic plates. The amount of methyl-green staining may, however, be an index of the degree of polymerization of DNA rather than an indication of the absolute amount of DNA present (Kurnick, 1950c).

9. Cell crushing as described by Davies, Wilkins & Boddy (1954) may be used to check the accuracy of results of cytophotometric analyses. In their technique a crushing condenser is used to give a flat nuclear preparation, and the product Extinction X Area is said accurately to reflect the amount of stained material. Alternatively, the method of Chalkley (1943) may be used to determine cell volume or

or nucleo-cytoplasmic ratio. Since, however, the amount of stained material is claimed to be accurately reflected in cytophotometric measurements of sections up to eleven microns in thickness by Swift (1950a), the accuracy of the cytophotometry may depend entirely on the accuracy with which area is estimated. It has been found by the author of this thesis that use of the formula πr^2 for area computation, where only two diameters are measured may lead to considerable error.

10. A variation of up to 15% between 'diploid' values for nuclei from the same tissue mounted on different slides was found by Swift (1950a). Where, however, sections or cell smears are mounted on the same slide, and are fixed and stained under identical conditions, it is generally agreed that the error inherent in cytophotometric analyses is somewhat less than 10%.

11. A rotating geometric step-wedge sector was photographed on to each strip of film used by Frazer & Davidson (1952), Thompson & Frazer (1953), Thompson (1953), Burns, Hale & Hutchison (1956), Hale & Burns (1957) and Hutchison, Burns & Hale (1957). It was thus ascertained that the gamma part of the film was being used. (Vide Fig.4).

12. Hillary (1939) made unsuccessful attempts to study the Feulgen reaction by incorporating deoxyribo nucleic acid into agar. Lessler (1951), using solutions of agar containing various concentrations of DNA, found that the Feulgen reaction followed Beer's law for the DNA concentrations normally found in tissues. Using gelatin-DNA preparations, Lessler (1951) found that the intensity of the colour produced by the Feulgen technique was proportional, within limits, to the concentration of DNA present. Photometric measurements indicated a linear increase from 0.2 mg to 1.0 mg per ml in agreement with the Beer-Lambert laws. Above 1.0 mg per ml the Beer-Lambert relationship did not hold and colour increase was less than would be expected.

Howard & Pelc (1951) found that P^{32} enters into organically bound form in the DNA of *vicia folia* root cell nuclei during the resting phase, but not during the actual division of the cell. They effected removal of the incorporated P^{32} by the action of deoxyribonuclease, proving that P^{32} was present in the DNA molecules. Their results indicated that P^{32} was bound in this form

(organic) only in cells which were preparing for division. Further evidence of DNA synthesis during mitosis is given by chemical analyses of the mean amount of DNA per cell nucleus in rapidly growing liver (Price & Laird 1950) and transplanted mouse leukaemia cells (Peterman & Schneider 1951). Increases in mean nuclear DNA content of 83% and 69% respectively were found in these experiments. The investigations by Price & Laird (1950) did not include cytophotometric investigations, and the increase in mean cellular DNA content may well be a reflection of an increased percentage of tetraploid and octaploid nuclei.

CYTOPHOTOMETRIC INSTRUMENTS

Caspersson (1936) is responsible for the pioneer work in this field, which suggested possibilities which have since been extensively explored and applied to many histochemical reactions in various biological fields. An ultra-violet spectro-absorption technique was developed by him which enabled identification of substances in nuclear chromosomes which had the same U V absorption curves as nucleic acids. The apparatus used was extremely complex and expensive, and

did not become generally available. He suggested the possibility of applying similar techniques to quantitation of histochemical reactions. As the biological importance of the nucleic acids became increasingly appreciated simpler cytophotometric methods were developed and many instruments have since been constructed and widely used.

Stowell (1942) describes the first cytophotometer which was developed in the previous few years by the research staff of the Barnard Free Skin and Cancer Hospital, Washington, under the direction of Dr. E. V. Cowdry. It consisted of a lamp, microscope and photocell and amplification and recording apparatus and was used for the quantitative estimation of Feulgen-stained material in individual nuclei of mouse epitheliomata induced by methylcholanthrene.

Pollister & Ris (1947) built an apparatus similar in general structure to that of Stowell (1942) and adapted an electron multiplier tube to the photovolt photometer, thus increasing its sensitivity in measuring 'large' areas such as whole nuclei. They measured the U V absorption by DNA and RNA at 540 m μ and 630 m μ

respectively in calf liver and thymus cells stained by pyronin and methyl-green. They were thus able to find the DNA:RNA ratio for single cells. The lenses, light sources and filters were varied with the spectral region being used; a modified Millon reaction was employed at 364 μ for quantitative determination of nuclear protein.

Pollister & Moses (1949) describe improvements of the apparatus designed by Pollister & Ris (1947). These were concerned mainly with shielding of the light-sensitive element for satisfactory operation with photomultiplier tubes. The new modification enabled photometric estimations to be made at any visible wavelength, and at 253.7 μ in the ultra-violet region.

A built-in camera was used, and means of focussing both the visible and ultra-violet images for microphotography were devised. Construction details and methods of operation for visible and ultra-violet light were described in detail. For U V photomicrography and photometry quartz monochromatic objectives and quartz eyepieces were used, and a condenser with interchangeable top lenses for different numerical apertures. For photometry the object to be measured was centred with the mechanical stage, focussed

with a telescope and the diaphragm closed to circumscribe the area to be examined. With the quartz monochromatic objectives of Kohler the U V image is always somewhat larger than a visible image projected the same distance. If an arc area, therefore, is circumscribed by the scanner diaphragm in visible light, a slightly smaller part of this same region is projected by U V light into the photocell. For photomicrography and photometry with visible light, the spectral region is varied by filters or monochromators as required for the (specific) stage reaction being quantitated. Di Stefano (1948) describes a similar instrument. For measurement of Feulgen staining intensity he used a Wratten 62 filter (peak transmission at 535 m μ); for U V absorption by nucleic acids, a Zeiss double prism monochromator to isolate the 253.7 m μ line, and, for methyl-green absorption at 625 m μ , a Farrand interference filter and tungsten light source. The transmission values of nuclei were measured by means of a standard phototube search unit of a photovolt photometer with an electron multiplier tube. Measurements of DNA in Feulgen stained preparations were made by Swift (1950 a,b). An RCA 931 A electron multiplier phototube was used, output

leads being connected to a Weston microammeter.

Ris & Mirsky (1949b) used the apparatus described by Pollister & Ris (1947) to investigate the possibility of using the Feulgen reaction for cytophotometric estimation of nuclear DNA, and found values which agreed closely with those found with a Beckman spectrophotometer.

Leuchtenberger (1950) used an apparatus similar to that described by Pollister & Moses (1949). A Farrand interference filter, with a peak transmission at 557 m μ , was used for measurements of absorption by Feulgen stained nuclei. A red light, with a peak at 626 m μ , was isolated by a Farrand filter for measurement of methyl-green stained nuclei, and the Millon reaction was measured at 365-366 m μ . Further improvements on the apparatus described by Pollister & Moses (1949) were introduced by Pollister (1952). He states that light-shielding of the phototube was imperfect, and that it was necessary to work with the earlier instrument in subdued light. Movement of the phototube during the cytophotometric routine of examining the image under consideration and allowing the light to pass to the phototube was avoided.

A movable mirror or prism was built into the apparatus to allow visual examination of the image and orientation of the object. Facilitation of measurement of still larger areas was achieved by introducing, close to the photocell, a lens which focussed light on a smaller area of the photosensitive surface. Photomicrography could also be carried out without any disturbance of the instrumentation for photometry. Detailed description of the apparatus is given. Continuous light sources, e.g. tungsten lamp or zirconium arc were used, and a monochromatic light was isolated by means of appropriate filters with line sources, or by a Perkin-Elmer model 83 monochromator with crystal quartz prism. In running absorption curves, the background was first measured throughout the whole visible spectrum and then the object was measured through the same range without moving the cell at each used wavelength. Increased accuracy and speed were thus gained.

The apparatus of Moses (1952) includes some new modifications aimed at improving its general usefulness, and is arranged in the most convenient position for the comfort

of the worker. A special receptacle is provided in the photometer head to contain 9 x 12 cm. plates. Three adjusting screws allow centring of the phototube cathode exactly with respect to the optical axis. Changing of the photocell could easily be arranged for one of different spectral sensitivity, without the need to centre the cathode each time.

The Massachusetts Institute of Technology (M.I.T.) recording microspectrophotometer is described by Wycoff (1952). A D.C. Hydrogen Arc (60 - 110 V) was used which was specially developed for this application. A line free continuum of high intensity is emitted by this source from 200 to 350 μ . Either a prism or a grating instrument is used in a dispersing system of the Van Cittert type. Lithrow's quartz prism monochromator is used. The photomultiplier is enclosed in an insulated box, to exclude stray light. The total reflecting system of the microscope is achromatic and therefore visible light may be used in alignment and field selection.

Sinsheimer (1948) describes a continuous recording visible and U V microspectrophotometer. An RCA I P 28 photo-

multiplier is used in conjunction with an Allen type hydrogen discharge tube as light source. Deeley (1953) employs an RCA 931-A type photomultiplier which is used to stabilize the intensity of light from a Mercury arc lamp. A specially designed crushing condenser (Davies, Wilkins & Boddy (1954), or a Cooke N.A. 1.3 oil immersion, is stopped down to a maximum of N.A. 0.8. A mechanically driven aperture scans the image of the cell being analysed. A logarithm electric circuit with an electric integrator gives ammeter readings which are related directly to the amount of absorbing material present. Results show that neither the error due to random distribution nor that due to glare and other optical effects exceeds 2%. A scanning time for an individual nucleus is 3 secs. and forty (or more) may be analysed in an hour. Appropriate filters are used to isolate spectral regions as required from the 125W mercury vapour lamp (BTH type MB/D stabilized arc). A detailed description of the apparatus is given, with results showing its performance and methods of investigating errors due to distributional effects, de-focussing, glare etc., involved in its use.

CYTOPHOTOOMETRY : SOURCES OF ERROR

The profitable use of any quantitative method requires a knowledge of the error involved in the technique. This is more difficult in cytophotometry than in biochemistry, where calibration with standard solutions of the substance being examined may be accomplished with comparative ease for the photometer used. Increased scatter of values is the usual result of inaccurate cytophotometric analyses, though values consistently too low or too high may occur. **Lack** of experience on the part of the observer, light scatter, uneven distribution of material, improper instrument alignment, use of too large a band width of light, or exposure of too great an area of the photosensitive surface of the photomultiplier, may all lead to a scatter of values due to operative faults rather than to biological variation in the materials being investigated. Further variables may result from inadequate control of the preparation of the sections or of the smears. The exposure and development times must correspond with the gamma part of the curve of the film used in photomicrography. Cytophotometry of out-of-focus preparations or out-of-focus negatives and inaccuracy in the measurement of cell dimensions may also lead to error.

Light Scatter: Where the refractive indices of mounting medium and object differ high extinction values are found due to light scattering from the object into the surrounding medium. The transmitted light is affected by this light increase (Swift 1955). It is important that a medium of similar refractive index to that of the object be used so as to obviate, or at least minimize this reflection and refraction. The method of Shillaber (1944) may be used to ensure that the refractive indices of medium and object are identical, and are appropriate for cytophotometry. Fixed tissues generally have refractive indices of from 1.560 to 1.572 (Swift & Rasch 1956). The refractive indices of resins generally used for mounting sections or smears are lower than this (Greco 1950). Pollister & Ris (1947) emphasize the importance of subtracting a 'blank' from extinction values measured. They used cell nuclei treated with hot trichloroacetic acid (0.3 M at 90°C for 15 min) for blank determinations in their analyses of DNA in *ambyostoma blastula* nuclei by U V Microscopy.

Glass slides and coverslips must be clean, the mounting

medium free of dust particles, and the immersion oil free of bubbles. Background readings should be taken near the object to avoid errors due to variation in thickness of slide, coverslip and mounting medium. Light scatter need not be a problem if these precautions are taken, and the refractive indices matched closely for fixed and stained specimens.

Absorption curve analysis (Swift & Rasch, 1956) allows measurement of the light scatter from all sources. Swift (1950a) found that blanks gave a negligible absorption, and so did not find it necessary to correct for non-specific light loss. By using small diaphragms for the lamp and condenser, as suggested by Uber (1939), and thus controlling the size of the field illuminated and the angle of illumination, Swift found extinctions, at different points of spherical Feulgen-stained nuclei, which would be expected from the thickness. Caspersson (1936, 1950) concluded that an error due to light scatter of less than 5% resulted if the following conditions were adhered to:-

1. The N.A. of the objective lens should not be less than 0.85.

2. The area measured should not be less than 3 lambda in diameter.
3. The ratio between the refractive indices of object and mounting medium should be less than 1.1.
4. The condenser aperture should be as small as is consistent with satisfactory image formation.

Ornstein (1952,b) found that the refractive index nuclei in tissues fixed with Carnoy's fluid was 1.540. If an oil of refractive index 1.530 or 1.550 was used for mounting tissues, the light loss of nuclei at 546 m μ with objective N.A. 1.30 was less than 2%. The "phase contrast" effects were found to be negligible if the difference between the refractive index of medium and object were 0.01 or less. Light scattering of Feulgen-stained nuclei at the absorption peak was less than 2%. Ornstein (1952,a) contends that if approximately parallel illumination is employed the only appreciable residual error in cytophotometric measurements in the visible region of the spectrum where deviations from Beer's law can be discounted, is what has come to be referred to as "Distributional Error".

Use of the Becke line is described by Winchell (1931) and

by Shillaber (1944) to detect refractive index differences between objects and mounting media. When the microscope objective is raised, a white or light ring surrounds the object and moves towards the substance of higher refractive index. On lowering the objective this Becke line moves towards the substance of lower refractive index. When refractive indices of object and mounting media are equal no Becke line is detectable, and the object cannot be seen. Other well known methods of determining relative refringency of object and medium are given by Shillaber (1944).

Schwarzschild & Villiger (1906) found, in micro-densitometry of developed photographic plates, that, if the area surrounding an object is illuminated by the light source used for measuring intensity of transmittance, then serious errors are introduced in measurements of a minute portion of the plate. This Schwarzschild-Villiger (S.V.) effect has according to Naora (1952,1955) been curiously ignored since the beginning of cytophotometry, and leads to error which may be considerable. Naora (1952, 1955) recommends that less than one third of the diameter of the nucleus

being analysed should be illuminated by measuring beams of light. The apparatus described by him provides for this innovation, with the result, it is claimed, that the S.V. effect is eliminated completely.

Distributional Error: This is the error due to inhomogeneity of dye or material distribution within the object being measured. It is divided by Swift & Rasch (1956) into two types: that due to nonrandom dye distribution, and the error due to variation in the path length of measuring light through the object. The two-wavelength method, as described by these authors, may be used to calculate the error involved. In their experience it was generally found to be negligible. In isolating nuclei a sucrose-calcium chloride medium is preferably used as inhomogeneity in DNA distribution is thus minimized. Nonrandom dye distribution is a marked feature of nuclei isolated in a citric acid medium. My own results (Figs. 17-26) with the citric acid medium showed a greater scatter than my results for DNA values accruing from use of a sucrose-calcium chloride medium (Figs. 27-37). This may be considered

to be due to some degree of inhomogeneity. When the extinction E of a whole sphere is measured by a cytophotometric apparatus, the result differs from that for E which is found if numerous small areas are measured separately and the average extinction of these calculated (Swift & Rasch, 1956). This discrepancy is stated to be large with high values for E , but negligible when "plugs" of less than $2/3r$ are measured through the centre of the sphere.

That errors in cytophotometric analyses of nucleic acids may result from dichroism, where absorbing molecules are so oriented that absorption is greater along one plane of polarization than another is suggested by Commoner (1949 a,b). Schmidt (1937) detected a strong negative birefringence in sperm heads, and concluded that the nucleic acids were oriented in lines parallel to the long axes of the protein fibres. This was confirmed by Caspersson's (1940) observation of dichroism at $257m\mu$ in the sperm bundles of a locust. Commoner (1949 a,b) states that direct evidence of the occurrence of dichroism is in general difficult to obtain from measurements of birefringence, since in most

structures the nucleic acids are present in comparatively small concentrations and the negative birefringence caused by them thus cannot counteract the large opposite effect due to the protein present. Commoner (1949a,b) states that in cytophotometric absorption measurements made with unpolarized light, variations in extinction values may arise from differences in the degree of orientation, rather than from differences in content of any specific substance. He concludes that the entire problem of interpreting intracellular extinction measurements requires to be re-examined with the realization that one is dealing not with true solutions but with oriented aggregates of molecules. Pollister & Swift (1950) criticize Commoner's (1949a,b) conclusions. They suggest that these conclusions are based on inferences from certain physical principles from the meagre information as to the structure of the nucleic acid molecules, and a particular interpretation of selected data of ultra-violet absorption published by Caspersson and his students (1936, 1944). From their own work Pollister & Swift (1950) could find no evidence of dichroism. Commoner (1950) in a reply to the paper by Pollister & Swift (1950) claims that much of their criticism has no bearing on the question at

hand, and that no proof is given that extinctions due to nucleic acids are unaffected by orientation. Frey wyssling (1948) concludes from a quantitative determination of the optical properties of the chromosomes that only a small percentage of the nucleic acid molecules are oriented. This question of the occurrence of dichroism in DNA of Feulgen-stained nuclei cannot be stated to have been adequately investigated, and its elucidation would seem to present many technical problems. Pollister & Swift (1950) state that dyes bound by sperm nucleic acid are not dichroic.

A formula is given by Uber (1939) for correction, where the path-length of light which traverses different parts of the object being analysed varies due to convergence of the beam of radiation. This error is stated to be reasonably constant with respect to thickness. An alternative method of correction is subtraction of a percentage of the absorption read to give the value of extinction with condensers of varying numerical aperture (Swift & Rasch 1956). The two wavelength method, as described by Swift & Rasch (1956) may be used to estimate the magnitude of distributional error.

Dimensions of Specimen : A method for measuring section thickness using a special achromatic lens system is described by Thorell (1947). This method is accurate to approximately 0.3 of a micron. Alternatively, where the mass of the cell is known interferometric microscopy can be used. A method proposed by Chalkley (1943) directly ascertains nucleo-cytoplasmic ratios, or the volume ratios of different cells. It does not, however, deal with direct measurement of nuclear or cell volume. Where stained smears of homogeneous tissue are being used, the image may be projected for tracing, or photographic images of the stained nuclei may be projected to uniform enlargement, the outlines drawn and the area of each nucleus measured in arbitrary units by a planimeter (Frazer & Davidson, 1953). If the formula πr^2 is to be used, it must be ascertained that nuclei are spherical or circular. Where cell or nuclear outlines are irregular, projection and planimetry are definitely preferable. Providing spherical or ellipsoid nuclei are within the depth of focus of the microscope the value extinction X area may be used to compute arbitrary units of stained material. To ensure that this product will give accurate results, as well as to obviate distributional error, results may be checked with those

accruing from use of a crushing condenser as described by Davies, Wilkins & Boddy (1954).

Other Sources of Error: Any stray light (flare) may cause error, unless it is constant. The photometer and monochromator should be light-tight, and there should be no photocell deflection when an opaque object is held in front of the phototube. Flare in the microscope may also result in error (Naora 1952), and condenser apertures should be kept low in cytophotometry. The effect of "noise" is reduced by estimating the average of two or more readings for each object. Srinivasachar (1953) recommends that all values, where duplicate readings differ by more than a certain percentage, should be discarded. The photometer should be tested frequently for proper alignment. Experience on the part of the observer is very necessary, and a good deal of practice is essential before important work is undertaken. Feulgen-stained adult rat liver may be used as a test object, and the values of DNA per nucleus should fall clearly into well-defined groups, the means of which fit a 1 : 2: 4 series. Repeated estimations on the same material should give results which correspond closely.

CYTOPHOTOMETRY: USES (1) OTHER THAN THE FEULGEN REACTION

A modified Millon reaction for nuclear protein, and methyl green-pyronin staining for DNA and RNA were used by Pollister & Ris (1947) for quantitative estimation on maize nuclei. Pollister & Leuchtenberger later (1949) demonstrated the specificity of methyl-green for DNA using cytophotometric methods. From their work they concluded that methyl-green was an exception among basic dyes in that it stained nothing but DNA and no staining of cell components occurred after extraction of DNA. Nuclei from mouse liver and sarcoma were quantitatively analysed by Leuchtenberger (1950). This author studied the DNA and protein content of the nuclei of these tissues, and found that in pyknosis an initial loss of protein occurred before DNA was progressively diminished in amount. From her findings she concluded that DNA occurred in a highly polymerized state in pyknotic cells of these tumours.

Using a cytophotometric apparatus similar to that of Pollister & Moses (1949), Leuchtenberger & Schrader (1950) studied the formation of aerosomal polysaccharide from

dictyosomes in sperms of the insect *arvelius*.

Polysaccharide was also estimated in *arvelius* sperms by Schrader & Leuchtenberger (1951), using staining by the periodic acid-Schiff technique of Hotchkiss (1948). Methyl green staining of deoxyribonucleic acid was estimated by Kurnick (1950) in the nuclei of calf thymus, mouse liver, and chicken erythrocytes. Absolute amounts of DNA were calculated, and the results agreed closely with the chemical findings of Mirsky & Ris (1949).

The amount of DNA per nucleus was estimated in single spermatocytes in different lobes of the testes of the insect *Arvelius Albopunctatus* (De Geer) by Schrader & Leuchtenberger (1951). A true growth occurred in some of these cells with a twofold to an eightfold increase in volume, protein and ribonucleic acid. Nuclear DNA content remained constant. The author concluded that synthesis of DNA and of RNA and protein can proceed independently and that it seemed likely that RNA was closely linked with protein synthesis in these cells.

From ultra violet cytophotometric analyses of macro-and micro-nuclei of *Paramecium Caudatum*, Moses (1950) concluded

that the macro-nucleus is in reality a structure containing a multiple of the genetic elements possessed in the diploid state by the micronucleus. Quantitative estimation of protein by the Millon reaction showed there was twenty times as much protein present as DNA. Ultra-violet spectrophotometry of nuclear nucleic acids demonstrated that RNA composed 10% of the nucleic acids in both types of nucleus.

The effect of X-Radiation on the DNA and on the size of embryonic nuclei of the Grasshopper *Chortophaga Viridifasciata* was studied by Harrington & Koza (1951). The changes induced were cytophotometrically measured, methyl-green staining being used to detect the degree of polymerization. A highly significant loss in staining intensity was found to result from exposure to X-Rays, and it was concluded that a depolymerization of DNA was induced, probably with increased dispersion of DNA in the nuclei.

The physiological pyknosis of erythropoiesis was found by Korson (1951) using methyl-green staining, to be accompanied by a gradual loss of DNA during maturation.

This was comparable to that observed by Leuchtenberger (1950) during pathological pyknosis in mouse sarcoma cells.

The effect of growth hormone administration and of hypophysectomy on the nucleic acid patterns and protein content of rat liver nuclei were studied by Di Stefano, Bass, Diermeier & Tepperman (1952) using U-V spectrophotometry, and cytophotometry with visible light of the Feulgen and methyl-green nuclear reactions. Azure A staining of ribonucleic acid and the intensity of the Millon reaction for protein diminished, but no change in nuclear DNA was found after hypophysectomy. Growth hormone caused an increase in DNA per unit weight of tissue, an increase in the RNA/DNA ratio, but no change in protein content.

Absorption curves of Azure A bound to nucleic acids were determined by Flax & Himes (1952) for a large variety of plant and animal cells using microspectrophotometry. The repeated occurrence of the same three peaks in these was judged to support a proposed extension of the theory of metachromasy based on dye polymerization in solution to explain metachromasy in tissues. The estimation of polyteny in the larval salivary gland of drosophila

melanogaster was the object of DNA estimations by Kurnick & Herskowitz (1952). The amount of DNA stained by methyl green was measured in absolute amounts by the method proposed by Kurnick (1950b). An 140 fold variation was found to occur in the volume of the nuclei of these salivary cells, and in this instance the DNA content was found to be directly related to cell volume.

CYTOPHOTOMETRY : USES (2), THE FEULGEN REACTION

The first investigation involving cytophotometry of the Feulgen reaction was made by Stowell(1942). The DNA was estimated in nuclei from sections of mouse skin epidermoid carcinoma stained by the Feulgen method as modified by de Tomasi (1936) and Coleman (1938). Although large variations were found in the DNA content per unit volume in different specimens of skin papillomas and carcinomas, cytophotometric analysis of Feulgen stained nuclei showed that the mean amount of DNA per nucleus was similar. The possibility of using cytophotometry of the Feulgen reaction for the estimation of the DNA content of nuclei in arbitrary units

was suggested by Pollister & Ris (1947). Their work, however, dealt mainly with ultraviolet absorption spectrophotometry of DNA and RNA stained by methyl-green pyronin, and with cytophotometry of the Millon reaction for proteins in calf thymus and liver cells.

Investigation of cytophotometry of the Feulgen reaction was pursued by Ris & Mirsky (1949b). They studied variables due to fixation, to hydrolysis and to irregular distribution of materials. Comparison was made with results from the use of a Beckman spectrophotometer, and from chemical analyses. Cytophotometry of the Feulgen nuclear reaction was found to give satisfactory results if nuclei of similar structure and DNA concentration were compared. Their analysis were of DNA in liver of rat, chicken, frog and shark.

Ely & Ross (1948) also conducted cytophotometric investigations of nuclei stained by the Feulgen technique. Their work was concerned with the effects of total body X-radiation on the nucleic acid content of cells from the crypts of Lieberkühn in rats. These

authors found that, after 24 hours X-radiation, approximately 50% of these cells were killed and contained a diminished amount of DNA per nucleus.

Schrader & Leuchtenberger (1950) analysed cells of various sizes, from different lobes of the testes of *Ascaris albopunctatus* (De Geer) for DNA content. Whereas nuclear volume and the amount of other cell constituents were found to vary, no change was observed in the nuclear DNA content of different cells. The apparatus used was similar to that constructed by Pollister & Ris (1947), and DNA was estimated in arbitrary units on the basis of computations as described by Swift (1950a).

The question of the significance of the two types of nucleus (macro- and micro-nuclei) in ciliate paramecia was studied by Moses (1950), who showed, using cytophotometry of the Feulgen reaction, that similar concentrations of DNA were present in both types (6 mg/cc). He found that Feulgen values were reproducible from one experiment to the next as is evident also

from the results of Leuchtenberger (1950). My own findings run counter to those of Moses (1950) and of Leuchtenberger (1950).

An extensive investigation was carried out by Swift (1950a) using similar techniques. Swift measured nuclear DNA in various tissues of young and adult mice, the frog, and the grasshopper *Missosteira carolina*. He found a haploid amount of DNA in mouse spermatids, whereas some cells in mouse liver, pancreas, thymus, blood leucocytes and Sertoli cells had tetraploid and octaploid amounts of DNA, although cells with a diploid amount of DNA were predominant in these organs. In mouse embryo liver, where division of cells was occurring, some values ranged between diploid and tetraploid amounts. In the frog Swift found only a few tetraploid values, and these were from liver cells. The grasshopper nuclei fell into four classes in the ratios of 1 : 2 : 4 : 8.

Swift (1950a) experienced a number of variables which influenced Feulgen staining intensity, so that a variation of up to 15% occasionally occurred in mean

diploid values of tissues which were not fixed and stained under identical conditions. He concluded, however, that cytophotometry of the Feulgen reaction gave an accurate relative measure of the DNA in the nuclei of any one preparation.

That variation in volume of mouse primary oocytes was not associated with a change in the absolute amount of DNA per nucleus was demonstrated by Alfert (1950). Pronuclei resulting from two meiotic divisions of primary oocytes had one fourth the amount of DNA found in primary oocytes. The amount of DNA in pronuclei was doubled prior to the onset of division of these nuclei. Pyknotic nuclei, from neoplastic and normal subcutaneously transplanted mouse tissue, were analysed by Leuchtenberger (1950). Though protein loss occurred initially, a later progressive loss of DNA was observed.

"Haematoxylin staining bodies", first described by Gross (1932) in cardiac valvular nodules from a case of Lupus Erythematosus, were the object of study by Klemperer, Gueft, Lee, Leuchtenberger & Pollister (1950). The object of this study was an investigation of the disturbance in

nucleic acid metabolism which occurs in this disease. "Haematoxylin bodies" in lymph nodes, and kidney cells (mesenchymal), were found to consist of DNA as shown by Feulgen and methyl-green staining. Cytophotometry was stated to give their Feulgen : methyl-green ratios and to enable estimation of the degree of depolymerization of the DNA to be made. It was concluded that partially depolymerized DNA was contained in these cytoplasmic structures, and that it was likely that they originated from the nuclei of these mesenchymal cells. The use of the DNA content of the nucleus as a cytotaxonomic character in mantids was explored by Hughes-Shrader (1951). Cytophotometry was conducted by the method of Pollister & Ris (1947) with an apparatus described by Swift (1950a). Eight species of mantids were examined. Those of closely similar species showed similar DNA values. The implications of further findings for the hypothesis that polyploidy has been involved in the evolution of high and low number karyotypes among mantids are considered.

An absolute increase in DNA per cell nucleus, using cytophotometry of the Feulgen reaction was reported by Diermeier,

di Stefano, Tepperman & Bass (1951) in liver nuclei of alloxan diabetic rats. The results are expressed as extinction values per nucleus. Chemical analyses also showed an increase in DNA per unit of wet or dry tissue. The results of these authors might be accounted for by an increase in tetraploid and octaploid nuclei. The percentage of the different classes of nuclei in the livers of these rats does not seem to have been investigated. The possibility of using cytophotometry of Feulgen stained blood films was explored by Korson (1951). A micro-spectrophotometric analysis of red cell nuclei from human bone marrow during physiological pyknosis in the process of maturation was undertaken. A gradual loss of DNA was found during maturation.

A comparison of the accuracy of cytophotometry of the Feulgen reaction and ultra violet microspectrophotometry was made by di Stefano (1948). The base, sugar, phosphoric acid and histone fractions of deoxypentose proteins of *Rana pipiens* tadpole cartilage were followed throughout the standard Feulgen hydrolysis. It was concluded that at an optimal time of hydrolysis one half the total

base content had been removed, presumably the purines. During hydrolyses beyond this time all of the remaining thymic acid molecule and the histone fraction of the protein had been removed from the nuclei. It was claimed by di Stefano that, under properly controlled conditions, this method is comparable to the cytophotometric determination of DNA by the ultra violet absorption method.

The effect of X-rays on the DNA content of embryonic nuclei of the grasshopper, *Chortophaga viridifasciata*, was studied by Harrington & Koza (1951) using the Feulgen reaction and methyl-green staining. In these cells mutations and chromosomal aberrations occurred, and an alteration in DNA content was presumably expected. No loss of DNA content was found to occur, and the authors concluded that it is not safe to make quantitative estimates of DNA stained by either of these methods. Further evidence, from microscopic observations and photographs, that alterations in nuclear size are not necessarily accompanied by changes in DNA content was put forward by Leuchtenberger & Schrader (1951).

Rat liver showed three distinct classes of nuclei whose DNA content showed the ratios 1 : 2 : 4, whereas nuclear volumes varied over a wide range. Examination of cells from the mucosa of the stomach of rats showed that the nuclei had the same DNA content as liver diploid nuclei.

Bryan (1951) studied the relationship between DNA content and protein during the development of male germ cells in the plant *Tradescantia*. A progressive increase in DNA content from the time of tetrad formation until microspore mitosis, and a further increase in DNA prior to anthesis were noted.

The nuclei of normal and neutron irradiated onion bulb roots were examined by Swift & Rasch (1951). During the normal mitotic cycle DNA was found to increase to twice the diploid amount prior to mitosis. No mitoses were found after the roots were irradiated, and intermediate amounts of DNA associated with DNA synthesis were absent. Meristem nuclei were found to contain twice or four times the haploid amount of DNA. Investigation of depolymerization of DNA in buds of *Trillium erectum* using photometry

of the methyl-green : Feulgen ratio was conducted by Moses, Du Bow & Sparrow (1951). No evidence of depolymerization of DNA was found in pollen mother cell nuclei after irradiation. In the discussion following this paper Moses referred to an investigation of the two wavelength method on Feulgen-stained nuclei by these authors. The ratios of extinctions of the Feulgen-DNA complex at 480 and 510 m μ was measured for a standard solution of DNA and for isolated nuclei. The ratio in the former was 2.19, in the latter 2.21, indicating little or no inhomogeneity. They assumed that the absorption curve of the dye coupled in solution is the same as that of the complex in the nucleus, and evidence was found by them that this is so. Observed heterogeneity of the structure of the macronucleus of the protozoan ciliate *Chilodonella uncinatus* was confirmed by Seschachar (1950), using cytophotometry of Feulgen stained nuclei. A progressive increase in size and DNA content of the developing macronuclear anlage was followed by a return of both to normal values. Pathological and normal liver nuclei were examined by Mark & Ris (1949).

These authors found similar values for DNA in spherical nuclei of the same size from normal liver and experimentally induced hepatomas and cholangiomas. From their results they concluded that only sections on the same slide were comparable.

The prolonged effect (8 to 49 days) of a protein-free diet on the DNA content of rat liver nuclei was studied by Ely & Ross (1951), using chemical methods of estimation as well as cytophotometry of the Feulgen nuclear reaction. Although a decrease (average 14%) in nuclear size resulted, increased amounts of DNA per nucleus were found by both methods. The possibility is discussed that the rise in DNA content may be due to the early stage of pyknosis as is stated to have been found by Leuchtenberger (1950) in the early stages of pyknosis in sarcoma. The findings of Ely & Ross (1951) are at variance with those of Davidson (1945), but in the experiments conducted by Ely & Ross rats were fasted for a much longer period of time.

Glick, Engstrom & Malstrom (1951) laid emphasis on the necessity for clear definition of the chemical specificity of any staining reaction before it can be used with confidence for cytophotometry.

These authors infer that errors due to inhomogeneous distribution of material, and chemical effects of fixation and staining processes can gravely interfere with quantitative microscopical work. They state that it must be shown that a stain must have resulted from the reaction of a substance with a particular compound or class of compounds.

The evolution of DNA during the mitotic cycle of embryonic cells from the sea urchin *paracentrotus lividus* was studied by Lison & Pasteels (1949). They concluded that synthesis of DNA was post-telophasic and not prophasic. Contrary to the findings of other authors they concluded that the DNA content of different nuclei of this species do not fall into classes which have values multiple of that found in sperms. Their results show a rise in average cellular amount of DNA during active cell division.

Lison & Pasteels (1951) also found that meiosis, accompanied by the simple separation of four chromatin elements in spermatogenesis in *Talpa*, gave rise to cells having one fourth the mean original value of DNA.

The stage of synthesis of DNA in mitoses during erythropoiesis in human bone marrow was found by Marinone (1951) to be telophase. No cells in mitosis were found to contain double the diploid value of DNA. Leuchtenberger & Schrader (1952) estimated the amount of Feulgen stained DNA in cells from the salivary glands of the snail *Helix aspersa*, and found that the amount of DNA varied with the volume of the nucleus, and with the stage of secretion. The highest values of DNA were found in actively secreting cells, and it was suggested that DNA was used in the formation of secretions.

Studies on various tissues of normal frog embryos (*Rana pipiens*) and of frog embryos in which haploidy had been induced experimentally were conducted by Moore (1952). A wide range of nuclear DNA values was found, and older, more differentiated, tissues were found to have a narrower range of DNA values than younger, less differentiated tissues.

The megaloblasts in normal patients, and in patients with pernicious anaemia who were responding to treatment, were examined for DNA content using cytophotometry of

Feulgen-stained bone marrow smears by Reisner & Korson (1951). No significant deviations from the normal amounts in other human tissues were found, and a gradual progressive loss of nuclear DNA was observed during maturation of megaloblasts. Similar values to normal were found in patients suffering from relapse and during successful treatment.

ISOLATION OF NUCLEI FOR CYTOPHOTOMETRY OF DNA

Aqueous or non-aqueous media may be used for the isolation of nuclei from tissue cells. Aqueous media facilitate the loss of substances of low molecular weight and of protein; non-aqueous media do not permit studies on lipids, and restrict studies on enzymes (Dounce, 1955). If the tissue being examined contains much fibrous material this should be removed prior to homogenization. For homogenization use is made of Waring blender, a Nelco blender or a Potter Elvehjem homogenizer. The tissue is chilled prior to homogenization. Repeated centrifugation and resuspension of the precipitated material may be conducted in a medium containing 0.2 to 5% citric acid (Stoneberg, 1939; Marshak, 1941; Haven & Levy, 1942; Dounce, 1943; Mirsky & Pollister, 1946; Smellie

Humphrey, Kay & Davidson, 1955). Alternatively, a solution containing 30% sucrose may be used (Ris & Mirsky, 1949; Naora, 1951; Frazer & Davidson, 1953). Still other possibilities are a 5 to 10% sodium chloride solution (Dounce, Tishcoff, Barnett & Freer, 1950), or a sucrose-calcium chloride medium (Mizen & Peterman, 1952; Frazer & Davidson, 1953; Alfrey, Mirsky & Osawa, 1955; Naora, 1957). The use of the sucrose-calcium chloride medium gives a homogeneous distribution of DNA, and minimizes cytophotometric distributional error.

Fibre is removed by straining through fine cheese-cloth, muslin or nylon gauze, and the entire procedure is controlled by microscopic examination of tissue smears stained with crystal violet (Bass, McArdle & Grisham, 1956). For studies on nuclear DNA content a pH of 4.0 or lower is generally chosen (Marshak, 1941; Haven, 1942; Dounce, 1943; Mirsky & Pollister, 1946). Little loss of DNA occurs from such preparations (Dounce, 1955).

The advantages of using smears of isolated nuclei rather than tissue sections for cytophotometric estimation of DNA are outlined by Leuchtenberger, Leuchtenberger,

Vendrely & Vendrely (1952), and are as follows:-

- (1) The steps in preparing tissues for sectioning are mostly avoided, and distribution of material is thus more homogeneous.
 - (2) Irregularity of background measurements due to the presence of cytoplasm does not occur.
 - (3) During isolation in aqueous media loss of protein occurs; this is advantageous since there is less "interference" during the Feulgen staining.
 - (4) Standard nuclei e.g. of bull sperm may be added, and control and "test" nuclei in the same preparation analysed.
 - (5) The error due to the need to measure the thickness of sections is avoided.
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THE BIOLOGICAL IMPORTANCE
OF DEOXYRIBONUCLEIC ACID

THE "CONSTANCY" IN THE DEOXYRIBONUCLEIC ACID CONTENT
OF THE NUCLEUS

The stability of DNA was demonstrated in a number of studies on the turnover of radioactive phosphorus, nitrogen and carbon. In DNA the turnover of radioactive phosphorus was found to be extremely slow, when compared with its turnover in most other cell substances (Brues, Tracy & Cohn, 1944). During liver regeneration, however, in the rat the uptake of radioactive phosphorus was much greater, presumably due to synthesis of new DNA prior to mitosis. The phosphorus thus used for DNA metabolism was retained for the most part for at least two weeks, confirming the slowness of DNA metabolism and its stability which is outstanding among biological compounds. Similar findings

were reported by Hammarsten & Hewes (1946), using labelled sodium phosphate. These authors found a specific activity for rat liver RNA 33 times that for DNA. The ratio in the total animal, however, was found to be only 1.5 : 1. Adenine labelled with N¹⁵ was found by Brown, Peterman & Furst (1948) to be much more slowly incorporated (1 : 29) into molecules of DNA than into those of RNA in the resting nucleus. This low ratio, and its variance from data on radioactive phosphorus uptake, were considered to indicate that at least some portion of the phosphate moieties of the nucleic acid might be exchanged independently of purines.

Analyses of mean cellular DNA content for various vertebrates and invertebrates led Mirsky & Ris (1949) to express the view that DNA was a constant component of the chromosomes. The ratio between diploid values found in calf thymus, calf lymph nodes, beef kidney and beef liver, and values for beef sperms varied, however, from 2.5 to 3.1 in contrast to the 2 : 1 ratio found by Boivin, Vendrely & Vendrely (1948), for similar tissues.

Chemical estimation of mean values for DNA per rat liver nucleus under normal conditions, and after experimental induction of precancerous and malignant tumors by Cunningham, Griffin & Luck (1950,a), gave results which indicated that no change in the average liver nuclear content of DNA occurred under these pathological conditions. These values were in good agreement with reported values for mammalian somatic cells. Although evidence of polyploidy was stated to be occasionally encountered, no estimates of the different classes of cells (diploid, tetraploid, etc.) seem to have been made by cytophotometric means. No alteration in nuclear content of DNA was observed in experimental hepatomas or cholangiomas in the rat by Mark & Ris (1949). Only the smallest spherical nuclei of similar size were analysed, however, and it is conceivable that different results might have accrued had nuclei been chosen independent of size. A progressive loss of DNA, after an initial loss of protein, observed by Leuchtenberger (1950) might have seemed evidence counter to the concept proposed by Boivin, Vendrely &

Vendrely (1948), regarding the "constancy" of DNA, were it not observed on pyknotic nuclei of tumor tissue. In the circumstances the variation in DNA values found by Leuchtenberger is not inferred to be abnormal, and is in fact what would be expected during cell death. Similar results to those referred to above were reported by Cunningham, Griffin & Luck (1950,b). No change in the average amount of DNA per liver cell was found by chemical analyses of liver tumors. No cytophotometric studies seem to have been conducted.

An increase in the mean amount of DNA per regenerating liver cell was found after partial hepatectomy, by Price & Laird (1950). The average DNA per cell nucleus was 18.3×10^{-12} g between 12 and 24 hours after partial hepatectomy. In normal liver the value was 10×10^{-12} g. A return to normal, however, was observed after cell division started, and accumulation of DNA prior to mitosis could explain the high value found. Again, in these experiments, no cytophotometric investigation of the percentages of the different classes of cells seems to have been conducted.

Inconstancy of DNA values observed by Schrader & Leuchtenberger (1949), using cytophotometry of the Feulgen reaction for analyses of various tissues of the plant *Tradescantia*, were suggested to be possibly due to different degrees of polyteny.

DNA values of cells from the plant *tradescantia* were found to fall into classes 1 : 2 : 4 : 8 : 16 : 32 by Swift (1950,b). This author concluded that this evidence supported his view that endomitosis occurred in *tradescantia*, contrary to the findings of Schrader & Leuchtenberger (1949), and of Bryan (1951) who found a variation in DNA content presumably associated with different degrees of polyteny. No appreciable alteration in average DNA content of liver nuclei of rats fed with various aminoazo dyes was found by Price, Miller, Miller & Weber (1950) excepting those cases in which 3 - methyl - 4 dimethylaminoazobenzene was administered. In this instance the amount of DNA was doubled in the nuclear fraction. This was attributed to an increase in the number of nuclei per unit weight of tissue. An alternative

explanation might be a decreased percentage of diploid, or an increased percentage of tetraploid and/or octaploid cells. The doubling of nuclear volume observed by Salvatore (1950) in uterine gland cells after injection of oestrogen into ovariectomized rats suggested the investigation of Alfert & Bern (1951). Salvatore's observations were confirmed, and cytophotometry of Feulgen stained nuclei showed no alteration in their DNA content, although the protein content, as demonstrated by the Millon reaction was doubled.

Evidence supporting the "Constancy" of DNA in a particular species or individual was put forward by Davidson, Leslie & White (1951), and its use as a standard for other chemical estimations was discussed. Similar nuclear DNA values for cells in human bone marrow and for leucocytes were found, and these were similar to values for human prostate cells, and for nuclear DNA values in cells of other mammals. The average DNA values for human spermatozoa were rather lower than might be expected from their content of a haploid number of

chromosomes, though this disparity was also observed in bovine tissues. A somewhat higher value for liver nuclei was believed to be accounted for by polyploidy. The average amount of hepatic cellular DNA was found by Di Stefano, Bass, Diermeier & Tepperman (1952) to be unaltered in rats after hypophysectomy or administration of growth hormone, whereas the amounts of RNA and of protein varied appreciably. Using ultra-violet microspectrophotometry for DNA analyses in Ehrlich ascites tumor cells, Leuchtenberger Klein & Klein (1952) found good agreement with mean chemical values. The results indicated that the cells were tetraploid as had been found from chromosome counts. Deviations from the mean value did not differ significantly.

DNA values for leukemic spleen, spontaneous and transplanted, similar to those for normal splenic tissue, were reported by Mizen & Peterman (1952). Variation in the mean amounts of nuclear DNA in bone-marrow cells from different patients with leukemia were found by Menten (1952). It was implied

that these results did not support the views of Boivin, Vendrely & Vendrely (1948) regarding the "constancy" in the diploid value for DNA in a species. The chemical analyses of Menten (1952) were not accompanied by cytophotometric determinations.

Pathological tissue (epidermal cells in senile keratosis), with variation in nuclear size and staining intensity, was examined by Leuchtenberger & Lund (1952), and was found to have considerable variation in cellular DNA content ranging from normal to very high values. The mean values for normal and diseased cells are not reported, but one may presumably conclude that they were raised. Davidson (1947) gives the values for mean nuclear DNA content, calculated by various authors for different tissues of a number of mammals; he is satisfied that the agreement is good. Some of Davidson's experiments show that P^{32} is much more slowly metabolized by liver DNA than by RNA. This slow metabolism further emphasizes stability of the DNA molecule.

Moses (1950) found that macronuclei of *Parmecia* contain forty times the amount of DNA in micronuclei. From the

genetic constitution of these nuclei it seemed that DNA formed an essential component of genetic elements, whose stability is well known. Variation in mean cellular DNA content of mouse tumors was found by Klein (1951) in different neoplasms. Though some cells had values for DNA which corresponded with diploid amounts, other groups of cells had amounts much above normal. No definite parallelism could be found between the increase in DNA and the mitotic index, but a rough parallelism between DNA and cell size did exist.

Increased DNA content per liver nucleus was found by Ely & Ross (1951) in rats starved for a long period (8 to 49 days). Nuclei, however, were assumed to be spherical, and this source of error may have been responsible for results which run counter to those of Davidson (1945) who studied rats starved for one day.

Cytophotometric measurements with an improved optical system (Naora, 1951) gave results which did not agree with those of Swift (1950, a). Rat liver nuclei fell into three classes which showed an arithmetical progression of DNA

content, and not a geometrical one. Naora's results, however, seem to indicate that DNA is a constituent of cell chromosomes. Kelly, Payne, White & Jones (1951) found no change in the specific activity of nuclear DNA from kidney, from spleen, from liver or from small intestine of mice with mammary carcinoma or lymphosarcoma. Pregnancy also had no influence. This finding may be interpreted as supporting the "constancy" concept. Further evidence for this hypothesis was put forward by Kurnick (1951). Using chemical estimation of DNA this author concluded that the increase in mass of rat kidney during growth was accompanied by a proportional increase in total DNA content. The average DNA per nucleus remained constant, and this constancy was stated to allow calculation of the total number of cells from the total DNA present.

No cell counts or cytophotometric measurements seem to have been undertaken by Baxi, Samarth & Venkataraman (1951). The initial fall and later rise observed by them in DNA content per unit wet weight of mouse liver, after injection

of a large dose (4 mg/kg) of thyroxine, do not lend evidence for or against the concept suggested by Boivin, Vendrely & Vendrely (1948) since the mean value of DNA per nucleus was not calculated.

Values for tissue DNA were expressed per unit dry weight by Cerecedo, Reddy, Pircio, Lombardo & Travers (1951). In this instance increase in DNA values in mouse kidney, liver and lung were sometimes found to be associated with spread of transplanted or spontaneous tumors, and a correlation with neoplasia seems to have approximately occurred. The unsatisfactory procedure of expressing results as content of DNA per unit weight only is also adopted by Lowe, Williams & Thomas (1951). Their finding of decreased content of DNA in rabbit liver after administration of cortisone would have had more meaning had cell counts and cytophotometric investigations been carried out.

Mean nuclear DNA values for cells in bone marrow and for blood leucocytes in 22 normal males and 17 patients with leukaemia were estimated by Davidson, Leslie & White (1951), using chemical determinations of DNA phosphorus and cell counts. No deviation from the normal values were found in

the haemopoietic disorder investigated.

Evidence that DNA is intimately associated with the chromosomes in embryonic tissues of *Rana pipiens* was found by Moore (1952). Tissues from diploid and haploid embryos were found to have DNA values whose ratio was 2 : 1. The mean amounts of cellular DNA in different tissues of the same embryo showed approximate constancy in spite of a wide variation among individual values. Considerable scatter in nuclear DNA values for salivary gland cells from *Drosophila melanogaster* was found by Kurnick & Herskowitz (1952) using cytophotometry of methyl-green stained cells. This variation was directly correlated with nuclear volume, and presumably associated with variation in the degree of polyteny in these cells.

DNA values for human liver, spleen and lymphosarcoma, analysed by Stevens (1952) were similar to those which had been reported for similar tissues from other mammals. The specific activity of DNA, after incorporation of P^{32} , was comparable to the activity found in rat tissues treated in similar fashion. In this instance tissues from

only one female patient were studied. "Normal values" for P^{32} uptake and DNA content for adult rat liver and spleen were found by Ramback, Moomaw, Alt & Cooper (1952). Amounts of DNA were expressed per gramme of tissue. Whole body X-radiation of rats was stated by Fernstein & Butler (1952) to induce a loss of DNA in the cells of the intestinal mucosa. This was assumed to be accompanied by degradation of the DNA. That DNA "constancy" could not be relied on to give an index of growth in the early stage of tissue culture is evident from the work of Gerarde, Jones & Winwick (1952). Since cell death generally occurred initially in some cells of cultured tissue explants, incorporation of P^{32} was a more reliable guide. After two weeks, however, DNA content was found to be proportional to cell content. Results agreeing with those of Davidson, Davis & Innes (1942) accrued from the work of Menten & Willms (1952). A fall in DNA phosphorus values in human bone marrow during treatment was correlated with percentage distribution of nucleated cells estimated by counts on bone marrow smears. Great variation in nuclear

DNA values for salivary gland cells from *helix pomatia* were found by Leuchtenberger & Schrader (1952). This finding was suggested to be associated with variation in secretory activity.

Similar DNA values for bovine liver and thymus and for rat liver and thymus were found by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952). These values were double the DNA value in sperm heads. The various classes of nuclei in rat liver showed a geometrical progression of DNA values. This is consistent with the general view that DNA forms an inherent and essential part of chromosomes.

A new example supporting the concept of the stability of DNA in the liver of adult animals was put forward by Fukuda & Sibatani (1953). Vitamin C deficiency, induced in guinea-pigs by withholding greens from their diet, was found to cause no change in total liver DNA or mean content of DNA per liver nucleus. These results do not agree with the findings of the Russian workers, Goldstein, Vol'kenzon, Kondrat'era and Ul'yanovska (1950), who say they observed

a decrease in DNA in scorbutic guinea-pigs. These authors suggest that Vitamin C may be involved in DNA synthesis. The intimate relationship between DNA content of mouse liver and growth after partial (65%) hepatectomy was shown by Yokoyama, Wilson, Tsuboi & Stowell (1953). Regeneration was found to be accompanied by a parallel increase in cell counts and DNA content. The mean cellular content of DNA was found to be unaltered. No cytophotometric analyses seem to have been conducted. The constancy in concentration of DNA per unit body weight, and per unit wet weight of liver tissue, from the 4th to the 18th day after partial hepatectomy found by Lombardo, Cerecedo & Reddy (1953) was not inferred to reflect constancy in mean amount per cell type. No cell counts were done nor was the degree of "Ploidy" investigated.

A quicker rate of metabolism of DNA than would be generally expected was found for liver and intestine by Stevens, Dasust & Leblond (1953). Results of incorporation of P^{32} suggested that DNA is formed at a rate approximately double the rate of cell formation in these organs. These findings

were interpreted as indicating that mitosis in these structures is associated not only with formation of additional DNA P, but also with replacement of the original DNA P, both daughter cells having incorporated new DNA P only. Slight variations between DNA values for rat diploid nuclei were found in animals weighing between 12 and 25 g, and a significant difference between some rat liver mean DNA values with variation in weight and "ploidy" were found by Fukuda & Sibatani (1953). No change in these values was found after the animals were subjected to prolonged starvation. Tetraploid nuclei, demonstrated by cytophotometric methods, were said by these authors to predominate in the liver of rats over 100 g. This finding differs markedly from that of Harrison (1953). In cell suspensions of adult rat liver he found about 30% of the cells showed tetraploidy, a figure generally accepted.

Polyteny was suggested as a possible explanation for an increase in the mean cellular amount of DNA found in megaloblastic human bone marrow cells by White, Leslie & Davidson (1953). A constancy was found in this value

for bone marrow specimens which varied over a wide range of cellularity in normal patients. Values for patients with iron-deficiency anaemias were within the normal range. Variations from the constant normal amount of DNA per sperm in presumably infertile male patients were found by Leuchtenberger, Schrader, Weir & Gentile (1953). Subnormal DNA values were correlated with clinically apparent infertility. Values for normal males were constant, however, and equal to one half that found for somatic cells. An approach to the problem of DNA constancy was made by Vincent (1953). Considerable variations between DNA values in different mouse liver nuclei found by this author, using cytophotometry of the Feulgen reaction, led him to conclude that either this reaction was unsuitable for quantitative estimation of DNA in tissue sections, or that DNA constancy was an acceptable hypothesis only if one considered a given population of nuclei in a statistical sense. The latter hypothesis is, of course, the one which is generally inferred when one speaks of DNA "constancy", and the differences in nuclear DNA contents found by this author

using the Feulgen reaction may conceivably be of biological origin rather than due to a fundamental lack of specificity in the Feulgen technique.

Metabolic stability of *Arbacia* egg and sperm DNA is indicated by the results of Vिलlee, Vилlee & La Place (1953). No incorporation of P^{32} into DNA of *Arbacia* sperms, and little uptake of P^{32} by *Arbacia* eggs in vivo was found by these authors. After fertilization, however, a much larger specific activity was found than in the resting state of these cells.

The problem of DNA constancy in a species seems to have been most extensively investigated by Thompson, Heagy, Hutchison & Davidson (1953). Absolute values for nuclear DNA in rat spleen, kidney, pancreas, small intestine, blood leucocytes, bone marrow (nucleated) cells, thymus, heart, lung and salivary gland cells from young and adult rats ranged from 0.65 to 0.70 pg. No variation in values for adult animals was found with sex, strain, weight, or pregnancy. No alteration was found to occur after

administration of a high fat diet, a thiamine deficient diet, or a dose of alloxan which produced diabetes. The value for liver nuclei was higher (0.90 pg in adult animals), a finding consistent with the presence of polyploid nuclei in this organ. The advantages of expressing results of chemical analyses of tissue in relation to the content of DNA are pointed out, and the necessity for confirming DNA "constancy" for a given tissue before such a method of expressing results can be used with confidence is stressed. Further evidence supporting the constancy of DNA in a species is evident from the results of Frazer & Davidson (1953), whose work was concerned primarily, however, with a comparison of the results from various cytophotometric analyses of nuclear DNA content. Chick liver and cultured heart fibroblasts showed similar DNA values; good agreement was also found for values of DNA for diploid liver and kidney nuclei in adult rats.

Goldstein (1954) found that most human monocytes, from a culture of the buffy coat of blood, had amounts of DNA

which were close to the diploid value.

Results of analyses by Leuchtenberger, Vendrely & Vendrely (1951) show similar DNA values for beef liver, kidney and spleen, which were twice the values found for beef sperm. These findings firmly supported the concept of "constancy" of DNA and the probability that DNA formed a fundamental part of the genetic equipment of cells.

Further evidence to support this hypothesis accrued from the work of Thompson & Frazer (1954). Using chemical and cytophotometric methods these authors found similar DNA contents in diploid nuclei from rat liver, kidney, small intestine and pancreas, which were twice that found from sperm heads. No change in mean thymic cellular DNA in normal mice was found by Weymouth, Delfel, Doell, Steinbeck & Kaplan (1955) in various age groups or after irradiation. No significant deviation from normal values was found for human megaloblasts in pernicious anaemia by Reisner & Korson (1951). Nuclei of diploid salivary cells from *Drosophila Melanogaster* were found to contain twice that found in their haploid

homologues by Rudkin, Aronson, Hungerford & Schultz (1955). Similar ratios were found for diploid and haploid nuclei in the plant *Aspergillus soja* by Ischitani, Uchida & Ikeda (1956).

Metabolic inertness of DNA in livers of young rats was demonstrated by Kihara, Amano & Sibatani (1956), who studied the release of incorporated P^{32} throughout the mitotic cycle of the cell. Isotope studies, according to Brown & Roll (1955), had not indicated a significant metabolic lability of the DNA molecular skeleton. Only to a very limited extent did Earle's "L" strain of mouse sarcoma cells in tissue culture assimilate labelled formate or glycine, which seemed to indicate little synthesis of new DNA (Thompson, Paul & Davidson, 1956).

Recent studies by Naora (1957), using an improved optical technique devised by him to minimise light scatter in cytophotometry, indicated a similarity in mean DNA content of somatic nuclei from rat liver, kidney, pancreas, cerebellum and adrenal medulla. He found, however, an initial gradual decrease during early postnatal growth,

presumably associated with increased differentiation of tissues. Solomon (1957) found a constancy in DNA per nucleus during the important embryogenic period between the first and third days in incubated chick embryos. However, there was a rapid increase in cell number and a loss of average cell weight.

BIOLOGICAL ROLES OF DEOXYRIBOSE NUCLEIC ACIDS

The possibility that DNA plays an important part in heredity has been frequently suggested from earlier times, and the parts played by DNA as chromatin in fertilization, mitosis and meiosis make this inference a logical one (Hotchkiss, 1955). Its confinement in almost all cases to the cell nucleus, which seems to play the predominant role in these biological processes, is a further indication that its primary functions are concerned with transmission of hereditary characteristics, and with tissue growth. Precise cytological localization of DNA by the Feulgen reaction (Feulgen & Rossenbeck, 1924) and later by ultra-violet micro-absorption techniques (Caspersson, 1936) and by methyl-green staining (Brachet, 1940) showed the

intimate association of DNA with the chromosomal material of nuclei which undergoes dramatic alterations in mitosis.

The chromosomes were initially shown to be gene carriers by Morgan (1911), and later by Sturtevant (1913). These authors put forward this view from a study of chromosome movements and of genetic determinants conforming to Mendelian laws; they found that a marked parallelism existed. Proteins, the other main constituent of nuclear material, were found by Mirsky (1951) not to have the distribution expected of genetic material.

The finding that DNA is present in constant mean amounts per diploid cell in the various tissues of an individual or species in half this amount in sperms, which contain the haploid number of chromosomes, and in tetraploid and octoploid quantities where the chromosome number is increased to fourfold or eightfold that found in sperm nuclei, further supports the concept that DNA forms an essential constituent of chromosomes. Mutational effects produced by X-rays, by ultra-violet radiation, by sulphur mustard, by caffeine and acriflavine on genetic material,

by indirect, or more generally by direct action on DNA, offer further evidence of the importance of DNA in cell multiplication and heredity.

The deoxyribonucleic acids isolated from individuals of similar species further show a marked uniformity in composition, whereas the deoxyribonucleic acids of different species may contain different amounts and different ratios of the purine and pyrimidine bases (Chargaff, 1955). In fact, new pyrimidine bases were found in deoxyribonucleic acids from certain sources. Thus it is clear that deoxyribonucleic acids from various species may have a wide variety of different structures, and these differences may be connected with transmission of hereditary characteristics common to a species, or individuals of a species.

Variations in the configuration of the DNA molecule are allowed for in the physical structure proposed for DNA by Watson & Crick (1953,a). The structure proposed has two helical chains, each coiled around the same axis, which, it is suggested, may be formed by a polypeptide chain. The two chains are formed from the sugar and

phosphate moieties, and it is suggested that the two chains are bound together by the purine and pyrimidine bases. Only certain pairs of bases fit into this structure ; one member of a pair must be a purine and the other a pyrimidine. If as these authors believe, the bases are present in their most tautomeric forms, then the only pairs of bases possible are adenine with thymine, and guanine with cytosine. This pairing is strongly supported by experimental results which show that adenine and thymine are generally present in similar amounts, as are guanine and cytosine. Moreover X-ray evidence obtained at King's College, London was said to give qualitative support to the proposed physical structure. Such specific pairing suggests a possible copying mechanism for self-propagation of the genetic material (Watson & Crick 1953,b). The considerable untwisting necessary for self duplication may well be reflected, on a much larger scale, by the coiling and uncoiling observed during mitosis in cultured cells.

The metabolic stability of DNA in resting tissues (Brues,

Tracy & Cohn, 1944; Hammarsten & Hevesy, 1946; Davidson, 1947; Brown, Peterman & Furst, 1948; Stevens, 1952; Rambach, Moomaw, Alt & Cooper, 1952; Villee, Villee & La Place, 1953; Brown & Roll, 1955; Kihara, Amano & Sibatani, 1956; Thompson, Paul & Davidson, 1956) and its close relation with tissue growth (Price & Laird, 1950; Alfert & Bern, 1951; Kurnick, 1951; Gerarde, Jones & Winwick, 1952; Lombardo, Cerecedo & Reddy, 1953; Stevens, Dasust & Leblond, 1953; Fiala, Sproul & Fiala, 1956; Solomon, 1957) further emphasize the importance of DNA in heredity.

A direct utilization of cellular DNA for formation of secretory products in the salivary gland of helix is suggested by Leuchtenberger & Schrader (1952). No such suggestion, however, seems to have been made for any vertebrate tissue. The possibility that DNA may function as a co-enzyme for beta-glucuronidase was suggested by Benfield & Fishman (1952). These authors found that DNA restored the activity of diluted preparations of beta-glucuronidase prepared from calf spleen. Since RNA may

act in a similar fashion, though to a lesser extent, this role of DNA is considered to be unlikely by Hotchkiss (1955). Hotchkiss suggests that contamination with metallic ions may have facilitated the enzyme reactivation.

The ability of DNA to induce bacterial transformations and bring about in bacteria an inherited change was considered by Butler (1952) to be evidence strongly in favour of its ability to act as a gene. Butler suggests that DNA is not capable of a sufficient number of variations to act as a template, as has frequently been suggested. From studies on phosphorus metabolism Bonner (1952) concluded that enzymatic syntheses did not occur in genes themselves, but in cytoplasmic particles, presumably microsomes. Results leading to similar conclusions are present in the work of Brachet (1954). This author compared the chemical changes occurring in nucleated and in non-nucleated fragments of unicellular organisms. The nuclei were found by this author to be poor in respiratory enzymes, but to have the enzymes that synthesize nucleotides. Brachet concluded that the nucleus is neither the centre of

cellular oxidations, nor a storehouse or building place for all enzymes. He found that an extensive building up of proteins could go on in the absence of the nucleus. It did seem, however, that enucleation produced a decrease in phosphorylation reactions, possibly due to lack of necessary co-factors. Similar enucleation experiments on *acetabularia mediterranea* led Giardina (1954) to conclude, from protein nitrogen analyses of nucleated and of non-nucleated portions, that, in this species, some proteins are present, the maintenance of which is strongly influenced by the nucleus. The possibility that DNA may be concerned with protein synthesis is discussed also by Todd (1955).

THE FEULGEN REACTION

Specificity:- No staining by the Feulgen (Feulgen & Rossenbeck, 1924) technique of salivary gland chromosomes of *Drosophila* was observed by Mazia & Jaegar (1939), after these were treated with spleen nuclease which removes DNA. This finding supported the specificity of the Feulgen reaction for nuclear DNA. Mazia & Jaegar conclude that

→ DNA must be separately removable from the chromosomes without destruction of the continuous protein structure of the chromosomes. A number of experiments led Brachet (1946) to conclude that the Feulgen reaction enabled precise localization of DNA in cell chromosomes to take place. Di Stefano (1948) used the classical techniques for cytological examination of DNA on cartilage cells from the head region of *Rana pipiens* tadpoles. He obtained values for nuclear DNA in Feulgen-stained specimens which compared accurately with those from ultra-violet absorption methods. He stressed that tissues to be compared must, as far as possible, be treated under identical conditions.

Results in complete contradiction of those of Di Stefano were found by Stedman & Stedman (1950). They found a loss of more than half the nuclear DNA after hydrolysis of smears of isolated nuclei in N hydrochloric acid for 10 min at 60°C. Schiff's reagent was then found to give a strong reaction with the hydrolysate, due, it seemed, to the presence of diffusible fragments of DNA.

The specificity of the Feulgen reaction for DNA has been frequently refuted by Stedman & Stedman (1943, 1944,

1947a,b). These authors claimed to have isolated a protein, which they call chromosomin from nuclei (Stedman & Stedman, 1943). It is neither a histone nor a protamin. This name was chosen, as the authors believe that it forms the principle constituent of chromosomes, the other main constituents being DNA and a histone. Stedman & Stedman succeeded in staining chromosomes with the colour compound formed by the Feulgen reaction with DNA in solution. They conclude that a basic dye complex had initially resulted which stained the acidic protein "chromosomin" of nuclear chromosomes. They concluded that the Feulgen reaction cannot locate the position of DNA, and may act, in fact, as a nonspecific stain for acidic compounds, having initially formed a basic compound by combination with the products of hydrolysis of DNA. Choudhuri (1943) reported results which agreed with those of Stedman & Stedman (1943, 1944) using their "developed nuclear stain", and came to similar conclusions.

An extensive investigation of various tissues from different vertebrates by Stedman & Stedman (1944) led them to conclude that "chromosomin" could readily be distinguished from histone and protamin by its acidic properties. They

found, however, extreme difficulty in removing from it the last traces of nucleic acids, and this removal was attended by considerable loss of material. The Feulgen reaction was admitted to be due to the presence of DNA, but was considered unsuitable for localization of DNA in cell nuclei. The dye formed by interaction of the Feulgen stain with pure nucleic acids in solution was held to be adsorbed on to added chromosomin. Similar deductions were made by Carr (1945), who considered that DNA was not necessarily involved in the reaction. This author found that considerable removal of cytoplasmic constituents by hydrolysis facilitated staining by the Feulgen reaction of the cell nucleus alone. The nuclear staining was believed by the author simply to reflect the adsorbing power of chromosomes.

Ultra-violet absorption bands considered by Caspersson (1936, 1944) to be due to nucleic acids and an overlapping protein band, were thought by Stedman & Stedman (1944) to be due to the tryptophan in "chromosomin", although chromosomin had been identified by an indirect but presumably valid analytical method. Caspersson (1944)

stated that the localization of nucleic acids, using ultra-violet microspectrophotometry, was not affected by "chromosomin". He stated that his results did not contain evidence for or against the presence of this compound, whereas Stedman & Stedman (1943) quoted some of his observations as supporting their finding.

The criticisms of Stedman & Stedman (1943) were answered by Callan (1943) who quoted the experiments of Mazia & Jaegar (1939) to support his view that the Feulgen reaction was specific for aldehyde radicals and the pentose radicals of DNA. Mazia & Jaegar (1939) found that, after treatment with spleen nuclease, salivary chromosomes of *Drosophila* no longer stained with the Feulgen method. These authors concluded that these chromosomes must have a continuous structure to which DNA is so attached that it can be removed separately. The views of Carr (1945) were criticized by Dodson (1946), who found that positive Feulgen staining did not occur after removal of DNA by nuclease treatment. No loss of weight of fixed tissues was observed by Dodson after their hydrolysis with N. HCl. He postulated that the failure of SO₂ water to block the Feulgen reaction in

Carr's (1945) experiments could be explained by an instability of the aldehyde bisulphite product. This proposed lability would allow replacement of the SO_2 by Schiff's reagent until equilibrium was reached.

Dodson concluded that as yet no serious objections to the specificity of the Feulgen reaction for DNA had been sustained.

On the basis of experiments with the Feulgen reaction with colourized Schiff's reagent and with basic fuchsin, and of evidence from the literature, Stowell (1946) concluded that the Feulgen reaction is "relatively specific" for DNA. Danielli (1946,a) stressed the importance of knowing to what extent depolymerization of DNA occurred during the hydrolysis employed in the Feulgen technique as generally applied. He thought that the amount of diffusion of DNA derivatives, within histological material, and into staining solutions etc., and the precise chemical reactions which caused recolorization of Schiff's reagent, still required clarification. The high permeability of cytoplasm referred to by Danielli (1946,b) may easily

allow diffusion of products of hydrolysis if this high permeability persists after fixation. On similar grounds, Danielli (1947) considered untenable the hypothesis of Stacey, Deriaz, Teece & Wiggins (1946) concerning the mechanism of the Feulgen reaction. Danielli also suggested that diffusion of the products of DNA hydrolysis referred to by these authors could explain the negative reaction after excessive hydrolysis. Danielli considered that ultra-violet absorption studies and Feulgen staining of the same chromosomes could well lead to supporting evidence for the specificity of Feulgen staining for DNA. Danielli also asked for further information about the chemical linkages between the sugar and the nitrogenous bases in the DNA molecule.

These criticisms by Danielli (1946, 1947) are answered, possibly satisfactorily, by Baker & Sanders (1946) who found, by ultra-violet absorption methods and by the Feulgen reaction, a similar localization of DNA in bands of salivary gland chromosomes. Baker & Sanders are of the opinion that the Feulgen reaction accurately locates the

sites of DNA. Stedman & Stedman (1947a) repeated their previous criticisms (1943; 1944) . . . Solubility of the effective dye, its avid adsorption by chromosomin, and the possibility of staining chromosomin with "developed nuclear stain" were cited as evidence against the specificity of the Feulgen reaction for DNA and the possibility of localizing DNA by this technique. They gave the findings of Gates (1942) as evidence in this instance. Gates succeeded in staining plant cell walls with the Feulgen reaction after overhydrolysis. No similar finding, however, seems to have been reported for animal material, which differs considerably from the material used by Gates (1942). The avidity of isolated chromosomin for the Feulgen reagent referred to by Stedman & Stedman (1947a) may have been due to the presence of some DNA or its hydrolysis products. No criteria of purity of "chromosomin" are referred to.

No diffusion of hydrolysis products of avian erythrocyte DNA after 5 min hydrolysis of cell suspensions in N. HCl at 60°C was found by Brachet (1947), although these cells were not exposed to the action of fixatives. Brachet could find

no evidence for interaction of SO_2 with partially hydrolysed DNA in vitro, or of staining of chromosomes after these were exposed to thymonucleodepolymerase. He could not reconcile his views with those of Stedman & Stedman (1943, 1944 , 1947a), or with those of Carr (1945).

Increase in colour intensity of the Feulgen "nuclear reaction" was observed by Sibatani (1950) after addition of a histone at any time in the course of the reaction. Other proteins, including casein and egg albumin, were found to have a similar colour enhancing effect. It seemed that adsorption of dye occurred on the proteins. The optimal hydrolysis time for any new material must be investigated carefully. Even then, the Feulgen reaction may be considered at best a compromise between hydrolytic freeing of aldehyde groups by the splitting off of the attached purines, and a progressive loss of derivatives of the DNA molecule (Pearse, 1953). When a technique is devised in which only the first of these two reactions proceeds, methods for localization and quantitation of DNA in histological preparations will stand on a surer footing.

Until then the utmost care must be taken in treating specimens for comparison under identical conditions of fixation, hydrolysis and staining. It seems that results from use of the Feulgen reaction must still be viewed with caution, as advised by Danielli (1946, 1947).

Mechanism of the Feulgen Reaction:- A relatively mild acid hydrolysis of DNA was found by Stacey, Deriaz, Teece & Wiggins (1946) to transform this substance into molecules of *w*-hydroxy-laevulinic aldehyde. These authors considered the restoration of colour to Schiff's reagent in the Feulgen reaction by this labile substance a likelihood. Li & Stacey (1949) claimed to have obtained clear evidence that the purine bases were the first constituents of DNA to be liberated by acid hydrolysis. They believed that highly labile sugar aldehyde linkages in DNA could be broken down readily by hydrolysis and thus react to give colour in the Feulgen technique. They considered it unnecessary to unmask all the "purine-covered" aldehydic groups to obtain positive staining. Overend & Stacey (1949) found somewhat different results after

initial acid hydrolysis of DNA. They claimed that the initial linkages to be broken are those engaged in polymeric binding. Later the linkages binding the purine bases are broken, and then some of the ribose, present in furanose form, becomes converted into the aldehyde-form which reacts with Schiff's reagent to give a coloured compound. The lability of the deoxyfuranose sugar and of the purine sugar aldehyde attachments was thought to account for the variation in staining after different degrees of hydrolysis.

Aldehyde coupling agents were employed by Lessler (1951) to demonstrate the involvement of aldehyde groups in the Feulgen reaction. Lessler postulated the interaction of alternate freed aldehydes with Schiff's reagent. Different results were reported by Lhotka & Davenport (1951). Reactions for aldehydes, other than the Feulgen test for aldehyde groups, did not demonstrate the unmasked aldehyde generally considered to be present after the usual hydrolysis procedure. The possibility was suggested, however, that steric configurations of the hydrolysis product of DNA might fit Schiff's reagent, but not the other aldehyde reagents tested.

Suggested Modifications of the Feulgen Reaction:-

Use of a specially prepared pararosanilin and of $K_2S_2O_5$ instead of $NaHSO_3$ for decolorization is advocated by de Tomasi (1936). This author recommends counterstaining of animal tissues with Orange G. Removal of the colour remaining in Schiff's solution with a proprietary vegetable carbon is advised by Coleman (1938). Thionyl chloride, as a source of sulphurous acid, was found to give satisfactory results for various human tissues by Barger & De Lamater (1948). A method of rapidly preparing Schiff's reagent, by the incorporation of sodium hydrosulphite, was described by Alexander, McCarthy & Alexander-Jackson (1950). The direct use of basic fuchsin instead of leucofuchsin, as proposed by Arzac (1951), is not advised by Pearse (1953).

CYTOCHEMICAL TECHNIQUES FOR DNA, OTHER THAN THE
FEULGEN REACTION

The first observations on the possible chemical basis of staining of nuclear chromatin seem to have been made by Miescher (1874) who found that "nucleinic acid" formed salts with methyl-green. Observations on the staining of chromatin

with basic dyes were made by Paul Ehrlich about the same time. This property of nuclear staining with basic dyes has been generally attributed to the presence in nuclei of nucleic acids. Various authors have used methyl-green, azure B, toluidine blue, crystal violet, methylene blue, and other basic dye stuffs in the study of the cytology of cell nuclei. One of the earliest investigations seems to have been conducted by Mathews (1898), who studied the effect of acid and alkali on the staining of chromosomes with various basic dyes.

The Unna and Pappenheim mixture of methyl-green and pyronin gives differential staining of cells. This was attributed by Brachet (1940) to their nucleic acid content. An extensive investigation by Kurnick (1950 a,b) led him to conclude that the affinity of the nucleic acids for these dyes was dependent on their degree of polymerization. Kurnick concluded, however, that nuclear staining with methyl-green could be used for cytophotometric determination of DNA, if procedures were avoided which led to depolymerization of DNA, and a buffer at PH 4.1 was used in the

staining solution. The deduction that the compound formed by the interaction of methyl-green with the phosphoric acid groups of DNA contained one dye molecule per ten of DNA/P led him to propose a formula for the estimation of absolute amounts of DNA in individual nuclei using a cytophotometric technique (Kurnick, 1950 c).

Results from this analytical procedure agreed with the chemical findings of Mirsky & Ris (1949). It is contended by Swift (1955) that the hypothesis of Kurnick (1947, 1950 a) about polymerization of DNA cannot be accepted without reserve, and he offers other explanations for the decrease in methyl-green binding by DNA. These are at least as tenable.

The concept of a specific interaction of basic dyes with the phosphoric acid group of nucleic acids was supported by the work of Michaelis (1947) who studied the optical properties of basic dyes in solution and in stained preparations. Nucleic acids do not contain all the acidic radicles of tissues, and competition by other substances,

especially protein, for binding with amino groups of basic dyes must certainly occur, especially under neutral or alkaline conditions. In the pH range 3 to 5, staining with basic dyes may be considered, in general, to be due to nucleic acids alone (Swift, 1955). Results of cytophotometry, using methyl-green staining of DNA, have been quite satisfactory (Pollister & Ris, 1947; Pollister & Leuchtenberger, 1949; Harrington & Koza, 1949; Leuchtenberger, 1950; Di Stefano, Bass, Diermeier & Tepperman, 1952; Kurnick, 1950 c, 1952). The popularity of methyl-green for quantitative work is probably due to its relatively greater specificity in comparison with other basic dyes for DNA (Pollister & Leuchtenberger, 1949).

The use of the coupled tetrazonium reaction for DNA is discussed by Danielli (1947). It must be considered an important method for purines and pyrimidines, especially when used in conjunction with other methods which localize DNA. The Feulgen-naphthoic acid hydrazide reaction, as used by Danielli (1947) or by Pearse (1953), gave cytological

results which, in general, confirm those obtained with the Schiff reagent. The resulting dye-substrate complex was stated to be less soluble in water than that resulting from the Feulgen reaction.

Ultra-violet microspectrophotometry was used to localize purines and pyrimidines in chromosomes by Caspersson (1936) whose complex and expensive apparatus did not become generally available. He suggested that quantitative methods could be devised, using similar techniques. The main disadvantages of ultra-violet absorption techniques, as used in cytophotometry, are as follows:-

- (1) There is difficulty in interpreting absorption curves which are a summation of curves due to various compounds.
- (2) The curves undergo variations with variation in pH and in substrate polymerization.
- (3) There is difficulty in controlling non-specific light loss.
- (4) The tissues are damaged.

These drawbacks were, however, insufficient to prevent

fruitful investigations in this field by Pollister & Ris (1947), by Moses (1950), by Di Stefano, Bass, Diermeier & Tepperman (1952), by Flax & Himes (1952), by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952), and by Frazer & Davidson (1953).

Good agreement between cytophotometry with the three classical methods for DNA, the Feulgen reaction, ultra-violet microspectrophotometry, and methyl-green staining, was found by Korson (1951), by Leuchtenberger, Vendrely & Vendrely (1951), by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952), by di Stefano, Bass, Diermeier & Tepperman (1952) and by Frazer & Davidson (1953).

MATERIALS AND METHODS USED IN THE WORK REPORTEDIN THIS THESIS

Animals:- Female guinea-pigs of body weights ranging from 350 to 500g were used. The animals were divided into 12 groups of 6 guinea-pigs with approximately the same body weight. All groups were maintained and handled in an identical manner. Animals were fed a regular commercial diet with additional greens. Each of the 6 animals in each of the test groups received intramuscular injections of 5 mg of ACTHar gel (long acting adrenocorticotropin manufactured by Armour laboratories) per 500 g body weight. The injections were given morning and evening for varying periods of time, according to the table on page 104 which also denotes the isolation medium employed. One control group of guinea-pigs (C1) was used for comparison with groups 1,3,5 and 7. A second control group of animals (C2) was similarly employed for comparison with groups 3A, 10, 14 and 21. A third control group of guinea-pigs (C3) was used for comparison with group 3B. From the control groups of animals normal kidney nuclei were isolated for comparison with adrenal and kidney nuclei from the ACTH treated animals. They were also used for comparison with similarly isolated adrenal or kidney nuclei from a group of control animals.

| Group No. | Control Group No. | Daily Dosage of ACTH. | Duration of ACTH Administration. | Isolation Medium. |
|-----------|-------------------|---------------------------|----------------------------------|--------------------------|
| 1 | C1 | 10mg per 500g body weight | 1 day | Citric Acid |
| 3 | C1 | " | 3 days | " " |
| 5 | C1 | " | 5 " | " " |
| 7 | C1 | " | 7 " | " " |
| 3A | C2 | " | 3 " | Sucrose-Calcium Chloride |
| 10 | C2 | " | 10 " | " |
| 14 | C2 | " | 14 " | " |
| 21 | C2 | " | 21 " | " |
| 3B | C3 | 50mg per 500g body weight | 3 " | Citric Acid |

Control animals received no ACTH. All animals in each test group were killed on the morning of the day following their last injection. The cavies were killed by stunning and bleeding from the cervical blood vessels. The adrenals and kidneys were placed in ice, and freed from surrounding connective tissue. In each instance the adrenals were removed in less than four minutes following death of the animal.

Isolation of Nuclei:- Nuclei were isolated from the pooled adrenals and from the pooled kidneys of the six animals in each group. Nuclei from the animals in the control groups, C1 and C3, and in groups 1, 3, 5, 7 and 3B were isolated by the citric acid method described by Smellie, Humphrey, Kay & Davidson (1955). This procedure involves disintegration of the tissues in a Potter-Elvehjem homogenizer containing 5 ml. 0.05 M citric acid followed by straining through nylon gauze, centrifugation, resuspension of nuclei in 0.01 M citric acid, and recentrifugation etc. until clean nuclei free of debris are obtained. The progress in isolation is checked by

microscopic examination of smears stained with crystal violet. Nuclei from control group C2, and from groups 3A, 10, 14 and 21 were isolated by the sucrose-calcium chloride method of Ailfrey, Mirsky & Osawa (1955). Samples of the nuclear suspensions were used by Dr. Hutchison (Dept. of Biochemistry, Glasgow University) to estimate the DNA content chemically using the method described by Ceriotti (1952); the number of nuclei per unit volume of the suspension was estimated by a haemocytometer.

Fixation and Staining:- Nuclei from the pooled kidneys of the control animals were smeared on one end of all glass slides used for the corresponding experiment. Nuclei of kidney or adrenal cells from the control or test series were smeared on the other end of these slides for comparison. The smears were dried in air at room temperature, fixed in acetic acid-alcohol (1 : 3 mixture) for 18 hours, and again dried in a desiccator at room temperature. A number of slides were stained by the Feulgen method for DNA, the leucofuchsin solution being prepared by Coleman's (1938) modification of de Tomasi's

(1936) method. The hydrolysis took place in N. HCl at 60°C, the time varying from five to thirty minutes. The optimum hydrolysis time, determined by visual examination of the depth of staining, was found to be 20 min. All specimens of the sucrose-calcium chloride group were taken through all stages of fixation and staining on the same slide rack. The citric-acid specimens were also treated together. All nuclear smears were hydrolysed in N.HCl at 60°C for 20 min, washed in distilled water for a few sec and placed in the leucofuchsin solution for 30 min. The slides were then washed in three changes of the acid metabisulphite solution described by de Tomasi (1936), dehydrated in graded alcohols, cleared in xylene and mounted in De Pex (Gurr: refractive index 1.524).

Photomicrography:- The stained nuclei were photographed under conditions of Kohler illumination with light from a mercury arc, using a Wratten 62 filter (Kodak), a 1.3 N.A. apochromatic oil-immersion condenser and a 1.3 N.A. apochromatic oil-immersion objective. The optimum exposure

time was used, i.e. that which resulted in the maximum deflection of the microdensitometer recording pen within the range of the straight part of the gamma (time-density) curve of the 35 mm film being used (Kodak microfile). A separate strip of 35 mm film was used for each slide. Ten fields of the control nuclei, and twelve to fifteen fields of the corresponding control or "test" nuclei, were photographed on to the same strip of film as a rotating step wedge sector which was exposed for the same length of time. The optimum exposure time was found in all cases to be one second. The step-wedge sector was constructed so that adjacent strips of film were exposed respectively for 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 0 seconds. Fig.3 shows a positive print of such a step wedge. Fig.4 shows microdensitometer readings plotted against the logarithms of the times of exposure in arbitrary units. A copy was drawn of each field of photographed nuclei and the individual nuclei numbered on each copy. Nuclei which were spindle shaped, which were near the edge of the field or which were overlapping were rejected.

Cytophotometry - The Recording Microdensitometer

The apparatus used is a prototype of the one described by Dr. Walker, Biophysics Research Unit, King's College, London (1955). The instrument supplied to the Institute of Physiology, University of Glasgow, was the first of its type made commercially by Joyce, Loebel & Co. Ltd., Newcastle-upon-Tyne, England. This instrument makes use of a double-beam system in which two separate light beams fall alternately on a photosensitive surface. Difference in the intensity of these light beams causes movement of an optical attenuator, the result of the movement being to abolish this difference in light intensity. The position of the optical attenuator is utilized to give a direct record on graph paper of the optical density of a portion of a film or of a photographic plate. As the film or plate is moved mechanically across the light beam the pen traces out the varying density of the film. The essential features are described by Walker (1955). Densities up to 3.0 from circular areas as small as 0.2 mm diameter may be measured. This corresponds to 0.0015 mm diameter on the specimen.

Accuracy is independent of light intensity, and deviation of less than 0.5% from a straight line was found by Walker (1955) for an Ilford dyed gelatine wedge. Reproducibility was excellent over a large range in density.

The alignment of the instrument was checked at the beginning of each separate series, and the objective and condenser focussed on the film. The same sensitivity setting was used for each strip of film. Generally, forty to fifty nuclei from each of the 42 separate microscopic specimens were analysed. Three separate traces were recorded across each nuclear image, the adjacent centimeter or so on each side of the traced portions being scanned also.

Calculation of Arbitrary Units DNA per Nucleus:-

The area enclosed by the projected baseline, corresponding to background film densitometer reading, and the densitometer trace of the nuclear image was measured by planimetry for each of the three nuclear cross-sections. The area of each was divided by the width in millimeters of the same trace at the baseline. These calculations gave three densitometer deflection values for each nucleus. The average densitometer

deflection for the three traces was estimated, this being the nuclear extinction value.

The photographic nuclear image of each nucleus was projected on to the sheet of graph paper which was used for its cytophotometry. The outline was drawn, and its area measured with a planimeter. All nuclear images were projected to uniform enlargement, using a microscope with 2" objective and 6X eyepiece. The product Extinction (E) X Area (A) gave the amount of DNA present in each nucleus in Arbitrary units.

Each control specimen was compared only with the corresponding control or test specimen which was mounted on the same slide. The extinctions, the areas, and the number of arbitrary units of DNA were totalled for each of the 42 specimens, and the averages for each estimated.

RESULTS

Methodology:- Preliminary investigation of the effect of Armour ACTH gel on the adrenal weights of mature guinea-pigs gave results which indicated that increase in weight and lipid depletion of the adrenal glands occurred more readily in female guinea-pigs, after the administration of physiological doses of ACTH, than in males. An increase in adrenal weight and a loss of adrenocortical lipid are generally accepted as criteria of adrenocorticotropin activity. As limitation of the dosage of ACTH to physiological levels was considered desirable, mature female guinea-pigs were chosen for these experiments. Comparatively smaller doses of ACTH were found necessary with guinea-pigs than with albino rats (Hutchison, 1957).

Control animals received no injections. The avoidance of injections in the controls was deliberate. I feared that injections, even of an inert substance, might evoke a discharge of endogenous ACTH. The adrenals from my control animals reflect conditions in normal glands unaffected by

exposure to the stress of injections. Rats receiving saline injections showed no adrenal lipid depletion, and no appreciable difference was found between their adrenals and those of normal animals (Bergner & Deane, 1948). In retrospect, however, I feel that perhaps I ought to have injected a 16% gelatine solution into my control animals.

It was possible to remove both adrenals from each experimental animal within four minutes of death. Post-mortem changes were thus kept at a minimum. Rapid freezing in ice ought to have minimized autolytic enzymatic tissue destruction.

General macroscopic post mortem examination revealed no abnormality in any of the animals used.

An increase in the weight of the pooled pairs of adrenals from each test group was found. This increase was almost linear up to 7 days (Fig. 8), and was interpreted as indicating that an adequate dosage of the hormone had been given.

Purity of the nuclear suspensions was invariably found by examination of smears stained with crystal violet solution.

The absence of nuclear particles was also evident from microscopic examination of specimens stained by the Feulgen technique, and from examination of the photographic negatives.

Fixation with the simple acetic acid - alcohol mixture gave good preservation of nuclear DNA. Loss of protein, which probably occurred, may well have been an advantage.

Fixed, unstained nuclear smears mounted in DePex were almost invisible by microscopy using visible light. This must mean that the refractive index of the isolated nuclei was very close to that of the mounting medium, DePex (Gurr) with a refractive index of 1.524. Ornstein (1952**b**) believes that the refractive index of isolated nuclei is 1.540. Error due to light scatter was therefore in the region of 2 - 3% (Ornstein, 1952**b**).

Subjective assessment, by microscopic examination, of the optimum time for hydrolysis of fixed smears, prior to staining with Schiff's reagent, was found to be feasible. The optimum time for hydrolysis was similar for kidney and adrenal nuclei in the guinea-pig, and with each of the two

methods used for isolation of nuclei; I did not consider it necessary to use methods, such as cytophotometric measurements of stain intensity, to find the optimum hydrolysis time with these nuclei.

There was consistent homogeneity of distribution of DNA when the nuclei were isolated by the sucrose-calcium chloride method. This is evident from a photograph of adrenal nuclei isolated by this method (Fig.2). A certain degree of non-random distribution of material, however, almost invariably resulted from isolation of nuclei by the citric acid method.

Planimetry of outlined projected areas of photographic nuclear images gave results reproducible within 2 - 3%. Where the edges of images were vague or definable with difficulty the nuclei were not examined.

I found that the use of the formula πr^2 for nuclear area led to error which was frequently considerable, although in general, nuclei seemed from microscopical examination to be approximately circular. I am convinced that, if results

are to be presented with confidence, planimetric measurements are essential as in the present series.

Practice in the use of the cytophotometric technique is necessary before results from analysis of the same nucleus, or of nuclei from the same or similar material, are accurately reproducible. Beginners tend to choose too short an exposure time, or microscopic fields which are too crowded. Analysis of nuclear images, which are overlapping or near the edge of the microscopic field, gave a traced baseline representing the film background; This background is frequently irregular and therefore not acceptable. Beginners, too, sometimes find difficulty in aligning the instrument.

Difficulty in localization of photographic nuclear images was overcome by the incorporation, beneath the specimen table, of a light which could be switched on when required. This light facilitated centring of the nuclear image under analysis in the light path which passed through the film negative.

The use of point to point densitometer traces, where each individual densitometer measurement represents 0.0015 mm

of the specimen, to a large extent, except at the periphery of the specimen, avoids error due to light scatter. The precautions advised by Naora (1952) i.e. that photometry should be restricted to an area bounded by less than $2/3$ the nuclear diameter, were thus taken.

With this method I determined the DNA content of very nearly two thousand individual nuclei. The procedure was time consuming and laborious. The estimation of DNA in an individual nucleus entailed planimetry of three densitometer traces, measurement of the diameter of each at its baseline and estimation of the average densitometer deflection for each trace. Planimetry of the outlined projected area of each nucleus was also necessary before the content of DNA could be estimated.

DNA Content of Control Kidney Nuclei:- Mean values obtained by cytophotometry for the relative DNA content in arbitrary units per nucleus of control kidney specimens isolated by the citric acid method are shown in Table I. Histograms showing the distribution patterns of DNA values for these are shown in Figs. 17 to 26.

Mean values of DNA in arbitrary units found for control kidney specimens isolated by the sucrose-calcium chloride method are shown in Table 2. Histograms showing the patterns of distribution of these values are shown in Figs. 27 to 37.

Mean values for the absolute DNA content obtained by chemical analyses of nuclei from the same pooled control kidneys are shown in Table 3.

Values in Tables 1 and 2 for each control kidney specimen are to be compared only with those for the corresponding control or test specimen which was mounted on the same slide. The results obtained from the different pairs of specimens on different slides cannot be legitimately compared. A statistical analysis of variance (Tables 4, 5) confirmed this generally held view.

The mean content of nuclear DNA found for 52 nuclei from 6 pooled control kidneys (1) was 14.9 arbitrary units. The corresponding figure for 52 nuclei from a second group of normal kidneys was 15.4. These values show good agreement. The difference (0.5 arbitrary units) is not statistically

significant.

It is evident from the histograms (Figs. 17 to 37) that values for each control kidney specimen show a scatter of values from an arbitrary amount x to $2x$. The nuclei from the control kidneys show the following scatter:-

from 10 to 20 and 11 to 21 in Fig.17,
from 10 to 20 in Figs.18 and 19,
from 12 to 24 in Figs. 20 and 21,
from 11 to 22 in Fig. 22,
from 10 to 20 in Fig. 23,
from 9 to 17 in Fig. 24,
from 9 to 19 in Fig. 25,
and from 11 to 22 in Fig.26.

A similar scatter, which varies less from slide to slide, is shown for almost all control kidney nuclei isolated by the sucrose-calcium chloride method.

This extends:-

from 6 to 12 in Figs. 27,28 and 29,
from 5 to 10 in Fig.30,

from 6 to 12 in Fig.31,
from 5 to 10 in Fig.32,
and from 6 to 12 in Figs.33, 34, 35, 36 and 37.

Tetraploid values in arbitrary units for DNA in control kidney nuclei isolated by the citric acid method are as follows:-

36 in Fig.17,
26 and 31 in Fig.18,
40 in Fig.20,
25 and 26 in Fig.24,
and 25 in Fig.25.

Tetraploid values for control kidney nuclei isolated by the sucrose-calcium chloride technique are the following:-

16 in Fig.28
and 20 in Fig.34.

Thus, out of 1,029 control kidney nuclei examined, only nine were found to have a tetraploid amount of DNA.

DNA Content of Control Adrenal nuclei:- The mean DNA content in arbitrary units of 40 nuclei isolated from six

pooled control adrenals by the citric acid method was similar to that for 46 nuclei from the corresponding control kidney specimen, (Table I). These values are 14.6 and 15.5 respectively. The difference (0.9) is not statistically significant. Fig.18 shows the scatter of DNA values for these adrenal nuclei. The DNA values extend from an arbitrary amount x to $2x$, except for one value of 11 units. No tetraploid value was found. Mean values obtained by chemical analysis for the absolute DNA content of these adrenal nuclei is 7.7 pg. and for the corresponding control kidney 6.5 pg. (Table 3).

A comparison of mean DNA values for control kidney and adrenal nuclei isolated by the sucrose-calcium chloride method shows values which are similar. The mean amount of DNA for 51 normal kidney nuclei was found to be 7.0; that for 48 normal adrenal nuclei 7.3 (Table 2). The difference between mean values for these kidney and adrenal nuclei (0.3 A.U.) is not statistically significant. Mean values obtained by chemical analysis were 5.9 pg. for control kidney nuclei, and 7.7 pg. for control adrenal nuclei (Table 3).

A histogram of the distribution of DNA values for these normal adrenal nuclei examined shows a scatter of values from 5 to 10 units (Fig.35). Two control adrenal nuclei out of 88 gave tetraploid values of DNA (Figs.18, 35).

DNA Content of Test Kidney Nuclei:- Mean amounts of DNA (in arbitrary units) in nuclei from the pooled kidneys of each group of six guinea-pigs are shown in Tables 1 and 2. Each value is to be compared with that for the corresponding control kidney nuclei. It is evident that the values are similar. In two cases, however, mean DNA values for test kidney specimens showed differences which are statistically significant. These are the results for three day (Table 1) and 10 day experimental animals (Table 2); ($0.01 < P < 0.5$). The differences, however, are small. In the other seven specimens analyses gave results which did not show a statistically significant variation from that for the corresponding control kidney specimen. Histograms of the distribution of nuclear DNA values for the "test" kidney specimens are shown in Figs. 19, 21, 23, 25, 27, 29, 33, 34 and 36. A scatter of almost all DNA values from an arbitrary

amount x to $2x$ is evident, as for the control kidneys. Few values lie outside this range. Tetraploid amounts of DNA were found in only two nuclei out of a total of 430 analysed (Fig.23, 19 A.U. and Fig.33, 17 A.U.).

DNA Content of Test Adrenal Nuclei:-

Mean DNA values for adrenal nuclei from groups of guinea-pigs which received injections of ACTH are shown in Tables 1 and 2. Adrenal from a group of control animals and from those which received ACTH for 1,3,5 and 7 days were isolated by the citric acid method. Adrenals for a second group of control animals, and from test groups of guinea-pigs which received injections of ACTH for 3, 10, 14 and 21 days were isolated by the sucrose-calcium chloride method. The distribution of DNA values for 1, 3, 5, 7 day experimental animals are shown in Figs. 20, 22, 24 and 26 respectively. The scatter of values for 3, 10, 14 and 21 day test animals is shown in Figs. 28, 30, 32 and 34 respectively.

The mean nuclear DNA values for adrenal nuclei from a group of six animals which received five times the usual dose of ACTH (i.e. 50 mg ACTH per 500 g body weight per day) are shown in Table 2. The distribution pattern of

individual nuclear DNA values for these and for the corresponding control kidney nuclei are shown in Fig.37.

The mean DNA value for the one day experimental animals (Group 1) is similar to the value for the corresponding control kidney nuclei (Table 1). The difference is not statistically significant.

Adrenal and control kidney mean nuclear DNA values for 3 day test animals (Group 3) also agreed well (Table 1). The difference between these kidney and adrenal DNA values is not statistically significant.

The mean adrenal nuclear DNA values for the five day experimental animals was higher than that for the corresponding control kidney nuclei (17.9 and 14.6 A.U. respectively). The mean DNA values for adrenal nuclei from animals which received ACTH for 7 days was also higher than that for the corresponding control kidney (21.4 and 16.8 A.U. respectively). Statistical analyses of the results in both of these instances in which adrenal nuclear DNA values were

found to be elevated showed that the difference in DNA values was highly significant ($P < 0.01$).

The mean DNA content of 55 adrenal nuclei from the ten day test group of animals was found to be the same as that in 52 corresponding control kidney nuclei.

Values for mean DNA content of adrenal nuclei from the 14 day and 21 day test groups of animals did not show a significant difference from the values for the corresponding kidney nuclei in each case (Table 2).

The distribution of individual DNA values for the adrenal test specimens in Figs. 20, 22, 24, 26, 28, 30, 32 and 34 shows a scatter of almost all DNA values from an arbitrary amount x to $2x$, this being similar to the scatter in the corresponding control kidney in each instance.

Tetraploid amounts of DNA were found in nuclei from some "test" adrenals:-

| | |
|-------------------------|----------------------|
| Fig.20; 28, 39, 48 A.U. | Fig.22 ; 27, 29 A.U. |
| Fig.24; 31 A.U. | Fig.26 ; 40 A.U. |
| Fig.32; 14 A.U. | and Fig.37 ; 14 A.U. |

Tetraploid amounts of DNA were found in only nine nuclei from 421 analysed.

The difference between the range of DNA values in arbitrary units between nuclei isolated by the citric acid method (Table 1) and those isolated by the sucrose-calcium chloride technique is presumably due to the difference in the isolation technique. The materials homogenized by these two techniques were treated under different laboratory conditions at different times, and the separate results are not directly comparable.

Mean values obtained by chemical analysis for the absolute DNA content per nucleus of the same pooled test adrenals are shown in Table 3. The fact that the arbitrary units in which the cytophotometric results of analyses of material isolated by the sucrose-calcium chloride technique are expressed, are of the same order as those given in picograms ($g \times 10^{-12}$) in table 3 is purely coincidental. In general, it is apparent that the mean absolute amounts of DNA per adrenal nucleus are slightly higher than those for the corr-

esponding control kidney specimens.

Relationship between Extinctions and Projected Areas of

"Test" Adrenal Nuclei:- The ratios -

$$\frac{\text{average "test" adrenal extinction}}{\text{average corresponding control kidney extinction}}$$
 and

$$\frac{\text{average test adrenal projected area}}{\text{average corresponding control kidney projected area}}$$
 are shown in Figs. 5 and 6 respectively. The values of these ratios are shown in Table 6. These ratios are plotted rather than the absolute extinction and projected area values for the test adrenal nuclei, as specimens on the same slide only are comparable.

It is evident that there is, in general, a reciprocal relationship between adrenal nuclear extinction and projected area. This is not strictly true, however, for the five day and seven day test specimens, in which a highly statistically increase in the mean amount of adrenal nuclear DNA content was found. Apart from these two values, the reciprocal relationship is striking.

The initial relative increase in adrenal nuclear projected

area and the fall in extinction values is slight. These values have almost returned to normal after three days ACTH administration. There is, however, a marked relative increase in adrenal nuclear projected areas at seven and ten days accompanied by a corresponding fall in mean extinction values. After ten days ACTH administration there is a tendency for these values to return slowly towards normal.

The adrenal nuclear concentration of DNA expressed as mean extinction value/mean projected area is shown in Fig.8 for nuclei isolated by the citric acid method. The ratio is divided by 10 for convenience of plotting against pooled adrenal weights.

It will be seen that there is an almost linear decrease in the concentration of adrenal nuclear DNA up to 7 days ACTH administration. This is accompanied by a progressive rise in the pooled adrenal weights. Though the values for nuclei isolated by the sucrose-calcium chloride technique are not comparable to these, it may be stated that the concentration of DNA in these had not returned to normal after ACTH

administration for 21 days. Concentration values are plotted for nuclei isolated by the citric acid method as the periods of ACTH administration for these were less varied than those during which animals from the second groups of experiments were given ACTH (Table 2).

Statistical Treatment of Results:- Results of an analysis of variance of nuclear DNA values for nuclei isolated by the citric acid method are shown in Table 4, and those for nuclei isolated by the sucrose-calcium chloride method in Table 5. Mean adrenal and kidney DNA values - the standard error are plotted in Figs. 9 to 12. The difference between principal and control mean DNA values - the standard error are plotted in Figs. 13 to 16.

As the data are not orthogonal all readings after the first thirty were rejected for the purpose of analyses of variance. The average difference between principals and controls is not significant ($0.1 < P < 0.05$). The difference between the slides is very highly significant and interaction is even more striking, indicating a variation in response to the different laboratory

procedures for different slides. It is inferred that comparison of mean DNA values from specimens on different slides is not justifiable. Principals and controls on the same slide only may therefore be compared, and it is concluded that application of a simple t test to results for principal and control specimens on the same slide is indicated and is adequate.

A simple t test was therefore applied to the results from each of the 21 pairs of principal and control specimens. The difference between the 5 and 7 day test adrenal mean nuclear DNA values and those for the corresponding control kidneys was found to be highly statistically significant ($P < 0.01$). The difference between a 3 day and the 10 day test kidneys and their corresponding controls was found to be significant ($0.01 < P < 0.05$). No statistically significant difference was found between the other principal specimens and their corresponding controls.

DISCUSSION

The results in general support the concept of constancy in the mean amount of nuclear DNA per resting diploid nucleus in a species. This is the attitude of Boivin, Vendrely & Vendrely (1948). The findings are in general in accord with those found for other tissues by Stowell (1942), by Mark & Ris (1949), by Swift (1950 a, b), by Alfert & Bern (1951), by Leuchtenberger, Vendrely & Vendrely (1951), by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952), by di Stefano, Bass, Diermeier & Tepperman (1952), by Fukuda & Sibatani (1953 a,b), by Frazer & Davidson (1953), by Thompson & Frazer (1954), by Rudkin, Aronson, Hungerford & Schultz (1955), by Ischitani, Uchida & Ikeda (1956) and by Naora (1957). The above all used cytophotometric methods. There is also agreement with the results of Davidson (1947), of Cunningham, Griffin & Luck (1950 a,b), of Price, Miller, Miller & Weber (1950), of Price & Laird (1950), of Davidson, Leslie & White (1951 a,b), of di Stefano, Baas, Diermeier & Tepperman (1952), of Mizen & Peterman (1952), of Moore (1952), of Stevens (1952), of Rambach, Moomaw, Alt & Cooper (1952), of Menten & Williams (1952), of Thompson, Heagy,

Hutchison & Davidson (1953), of Frazer & Davidson (1953), of Thompson & Frazer (1954), of Weymouth, Delfel, Doell, Teinbeck & Kaplan (1955), of Naora (1957) and of Solomon (1957) who all used chemical methods.

The findings run counter to those of Schrader & Leuchtenberger (1949), of Moses (1950), of Goldstein, Vol'kenzon, Kondrat'era & Ul'yanovska (1950), of Klein (1951), of Lison & Pasteels (1951, 1953), of Menten (1952), of Leuchtenberger & Schrader (1952) and of Leuchtenberger, Schrader, Weir & Gentile (1953).

The mean nuclear DNA content found in two test kidneys (Tables 1 and 2) is greater than that in the corresponding control kidney specimens, and in these instances the difference is statistically significant ($0.01 < P < 0.05$). This difference is, however, small and similar findings have been reported for other tissues where a comparatively large number of nuclei have been analysed for their DNA content by cytophotometric methods (Vendrely, 1956). Until more is known about the specificity of the Feulgen reaction, it would be unwise to assume that these differences are

biological rather than technical. Use of the improved apparatus described by Deeley (1953) and re-investigation of the Feulgen reaction should facilitate the assessment of the significance of apparent small differences in mean nuclear DNA content of similar tissues in a species.

The highly statistically significant differences between the mean nuclear DNA content in test adrenals and in nuclei from corresponding control kidneys in the five and seven day groups of experimental animals is consistent with the hyperplasia of adrenal cells found in guinea-pig adrenals after ACTH administration for this period of time. The increase found is explainable by a synthesis of new DNA prior to cell division. These results are in accord with those found for other actively growing tissues by Price & Laird (1950), by Leuchtenberger & Lund (1952), by Walker & Yates (1952), by Leslie & Davidson (1953), by Thompson, Heagy, Hutchison & Davidson (1953), by Alfert & Swift (1953), by Davies & Walker (1953), by Deeley, Richards, Walker & Davies (1954), by Walker (1955) and by Naora (1957).

The findings for actively growing tissue differ from those of some authors for other proliferating tissues, (Klein, 1951; Kurnick, 1951; Gerarde, Jones & Winnick, 1952; Yokoyama, Wilson, Tsuboi & Stowell, 1953; Solomon, 1957).

The results support the view that DNA plays a major part in formation of chromosomes, at least in so far as guinea-pig adrenal and kidney are concerned. They agree in general with the early findings of Morgan (1911) and of Sturtevant (1913), and indirectly support their proposition that chromosomes are gene carriers.

The increase in adrenal nuclear size, unaccompanied by an increase in mean nuclear DNA, found for most "test" adrenal nuclei is comparable to a similar finding for uterine nuclei following administration of oestrogen to ovariectomized rats (Alfert & Bern, 1951).

A direct relationship between mean nuclear DNA content and nuclear size has been reported for the macro and micronuclei of paramecia by Moses (1950); the macro-

nucleus has much more DNA. A direct relationship between size of nucleus and DNA content has also been found for salivary gland nuclei of *Drosophila* by Kurnick & Herskowitz (1952), and for salivary gland nuclei of *Helix pomatia* by Leuchtenberger & Schrader (1952). These results are, in general, similar to my own for 5 and 7 day test adrenal nuclei. No instance of increase in mean nuclear DNA content of a tissue accompanied by proliferation and increase in the mean nuclear projected area seems, however, to have been reported.

From the results of the chemical analysis it is evident that control and test adrenal nuclei gave a slightly higher value for mean nuclear DNA than did the corresponding kidney nuclei in each instance. We (Hutchison, Burns & Hale, 1957) have found that, with the guinea-pig adrenal gland, homogenization beyond 15 to 20 sec leads to a considerable reduction in the number of nuclei in the suspension. The adrenal nuclei appeared to be more fragile, in the isolating media used, than those of the kidneys. Thus one possible explanation of the higher values obtained for adrenal nuclei

throughout the experiments may be that they were slightly contaminated with nuclear fragments which had not been removed during the differential centrifugation. This would have the effect of raising slightly the value for the mean DNA content per nucleus. The mean value obtained for kidney nuclei is close to the mean value for liver nuclei in guinea-pigs reported by Vendrely (1952).

Apart from the 5 and 7 day test adrenals there is general agreement between the results obtained by cytophotometric and by chemical estimation. The arbitrary units in which the cytophotometric analytical results are expressed are only comparable between each principal specimen and the corresponding control. The results, shown in the different tables are, therefore, not to be compared.

The general agreement between chemical and cytophotometric results was also found for vertebrate liver and erythrocyte nuclei by Ris & Mirsky (1949**b**) for mouse thymocytes by Kurnick (1950**b**) and for beef liver, thymus and sperm by Leuchtenberger, Leuchtenberger, Vendrely & Vendrely (1952). The same agreement was found in liver following hepatectomy (Leuchtenberger, 1950) in liver after administration of growth

hormone to hypophysectomized rats (di Stefano, Bass, Diermeier & Tepperman, 1952), in cells concerned with microsporogenesis in *tradescantia*, (Bryan, 1951) and in rat kidney, liver, small intestine, pancreas and spermatozoa (Thompson, 1953; Thompson & Frazer, 1954).

The results from the cytophotometric analyses of the 5 and 7 day test adrenal specimens are somewhat different from those obtained by chemical analyses. The cytophotometric results show an increase in the mean adrenal nuclear DNA content which is highly significant statistically ($P < 0.01$). The increase found is consistent with the increase in mitoses observed in the zona fasciculata. Synthesis of new DNA prior to mitosis would account for a real increase in mean adrenal nuclear DNA observed after administration of ACTH for these periods of time. The ratios:-

$$\frac{\text{Mean adrenal nuclear DNA}}{\text{Mean control kidney nuclear DNA}}$$

found for the 5 and 7 day test adrenal test specimens by cytophotometric analysis are 1.22 and 1.27 and by chemical analysis 1.11 and 1.17 respectively. Both methods may be at

fault; the chemical method, since contamination by nuclear fragments due to the marked fragility of guinea-pig adrenal nuclei may occur, and the cytophotometric method due to the error generally inherent in the technique. Nuclei in the early stages of mitosis may be disrupted more easily than resting nuclei; cytophotometry may give more reliable results in these instances. Absolute proof, however, is lacking, and improvements in techniques for isolation of fragile nuclei and for cytophotometric analysis of nuclear DNA are required before it can be decided whether the variation found is biological rather than technical.

I found individual values for kidney or adrenal nuclear DNA which correspond to the tetraploid amount. Those found were approximately twice the mean diploid amount in each instance (Figs. 17 to 37). This is in agreement with similar findings by Swift (1950a) for some nuclei from mouse liver, pancreas, thymus, blood lymphocytes and Sertoli cells, and from frog liver. Naora's (1951, 1957) experience was the same with some rat liver nuclei. The nuclei of Ehrlich

ascites tumor cells show the same phenomenon (Leuchtenberger, Klein & Klein, 1952); so do some nuclei from rat kidney, from normal and from tumor bearing liver, from the small intestine and from the pancreas (Thompson, 1953). Nuclei from rat liver (Frazer & Davidson, 1953; Fukuda & Sibatani, 1953a) and from normal and regenerating rat liver (Thompson & Frazer, 1953) show some tetraploid values. It may be seen from the histograms (Figs. 18, 20, 22, 24, 26, 28, 30, 32, 34, 35, 37) of individual DNA values that guinea-pig adrenal nuclei are almost entirely diploid, a finding similar to that of Frazer & Davidson (1953) and of myself (Figs. 17 to 37) for kidney nuclei.

If the results are a reflection of the conditions present in adrenals of other laboratory animals, it is then permissible as Fiala, Sproul & Fiala (1956) have done, to estimate the number of cells present in the adrenal after ACTH administration, by measuring the total DNA content of the adrenal and then dividing the total by the mean DNA value per cell of the normal gland. This method of estimating the total number of nuclei may be more accurate and less

tedious than the method which uses tissue sections of "known" thickness (Brues, Drury & Brues, 1936).

It is also much less time-consuming than the technically difficult method devised by Carnes, Weissman & Goldberg (1952), which involves estimation of the relative volumes of nuclei and of cytoplasm in the total volume of an organ and of the mean absolute nuclear volume. The method used by Fiala, Sproul & Fiala (1956), if applied to guinea-pig adrenal, has the drawback that it may lead to some error due to destruction of nuclei during homogenization.

The present results also suggest that it may be justifiable, after ACTH administration, to express results of estimations of RNA, of lipid P etc. per cell or per unit DNA content of guinea-pig adrenal tissue as has been done by Symington & Davidson (1956) and by Fiala, Sproul & Fiala (1956).

The conclusion come to from statistical analyses of variance (Tables, 4, 5) that specimens on the ~~same~~ slide only are comparable is in agreement with the finding of Swift

(1950) that comparatively large differences sometimes occurred between specimens on different slides even though these are fixed and stained together. This variability is in contrast to the findings of Moses (1950) and of Leuchtenberger (1950) who claim that Feulgen values for nuclear DNA are reproducible from one experiment to the next. The number of nuclei which should be examined was stated by Swift (1950a) to be approximately fifty. I found that this number was adequate, and approximately forty to fifty analyses gave results to which the application of a simple t test, as indicated by an analysis of variance, was adequate for statistical treatment. The variation in mean DNA values from slide to slide is also evident from Figs. 17 to 37 and Tables 1 and 2, and it is evident that a greater variation occurred when nuclei were isolated by the citric acid method (Table 1 and Figs. 17 to 26) than when isolated in a sucrose-calcium chloride medium (Table 2, Figs. 27 to 37). Nuclei isolated in sucrose-calcium chloride were more homogeneous.

The scatter found in nuclear DNA values for each specimen from an arbitrary value x to $2x$ is consistent with the synthesis of new DNA prior to mitosis as suggested by the work of Alfert (1950), Price & Laird (1950), Peterman & Schneider

(1951) and Walker & Yates (1952). It seems from the work of these authors that formation of DNA for the new sets of chromosomes occurs in interphase, in nuclei which show no morphological changes known to be associated with mitosis. This finding was confirmed by Howard & Pelc (1951) using autoradiography.

The inverse relationship between mean nuclear extinction value and mean projected area in groups of adrenal nuclei which were found to contain different concentrations of DNA shown in Table 6 may be considered to indicate that our cytophotometric technique reveals variation in concentration of nuclear DNA in Feulgen-stained nuclear smears. The ratio $\frac{\text{extinction}}{\text{area}}$ for adrenal nuclei isolated by the citric acid method shows an almost linear relationship (Fig. 8); with decrease in mean nuclear concentration of DNA there is an increase in nuclear size.

Mean extinction values are plotted against mean projected areas in Fig.7. Although these specimens isolated by the sucrose-calcium chloride method are strictly not comparable

as pairs of specimens were mounted on different slides, the reciprocal relationship between extinction and area is evident.

The increase in pooled adrenal weights which occurred after ACTH administration indicates an initial hypertrophy of the adrenal cells. Hyperplasia was occurring at 5 and 7 days, as evidenced by a marked increase in mitosis. These findings are similar to those of Selye & Stone (1950), Symington & Davidson (1956) and Fiala, Sproul & Fiala (1956). The size of the adrenal nuclei had not returned to normal after prolonged (21 days) ACTH administration (Table 6). This is similar to the persistent hypertrophy observed by Selye & Stone (1950) in rat adrenal cells after prolonged exposure to stress.

An investigation of the increase in nuclear volumes in different zones of guinea-pig adrenal after administration of ACTH for 3, 7, 14 and 21 days by Jenkinson (1957) in this department has shown that the increase occurs in the zona fasciculata and zona reticularis. The maximum increase was found in the outer zona fasciculata in the adrenals at 7

days. Jenkinson reports a peak in mitotic figures at 7 days, also maximal in the outer zona fasciculata. He found no changes in nuclear volume or in mitotic counts in the zona glomerulosa. These results indicate that the effect of ACTH on guinea-pig adrenal DNA content and concentration are confined to the zona fasciculata and zona reticularis, and are maximal in the outer zona fasciculata after administration of ACTH for 7 days.

No evidence for or against a role for DNA in secretory processes in adrenocortical cells accrues directly from these experiments. It is possible that the increase in nuclear size and the diminished concentration of DNA observed may be concerned directly or indirectly in the formation of adrenocortical hormones.

SUMMARY AND CONCLUSIONS

1. The effect of Adrenocorticotropin (Armour ACTHar gel) on the mean amount of deoxyribonucleic acid (DNA) per nucleus, and the scatter about this mean, in adrenal nuclei from mature female guinea-pigs was studied using cytophotometry of Feulgen-stained nuclear smears. Results are compared with those from

chemical analysis of the same material.

2. The mean adrenal nuclear DNA content was similar to that in a control specimen mounted on the same slide in each instance, except after administration of ACTH for five and seven days. Cytophotometric analyses showed a highly statistically significant increase ($P < 0.01$) in mean adrenal nuclear DNA content after administration for five and seven days. Chemical analyses gave values for adrenal nuclear DNA which were slightly higher than those for pooled control kidney nuclei. The significance of these findings is discussed.

3. Administration of ACTH was found to cause an increased projected area of the adrenal nuclei and a diminution in the mean nuclear extinction values. The mean nuclear DNA content, however, remained approximately constant in almost all cases.

4. Kidney nuclei from two groups of test animals were found by cytophotometric analyses to have a slightly higher mean nuclear DNA content than the kidney nuclei of controls. This difference was statistically significant ($0.01 < P < 0.05$).

This finding is discussed.

5. A general scatter of diploid nuclear DNA values from an arbitrary amount x to $2x$ was found for all specimens. Few diploid nuclear DNA values were found outside this range.

6. Cytophotometric results indicate that adrenal and kidney nuclei from normal and ACTH treated guinea-pigs are almost entirely diploid.

7. Statistical analysis of the results indicates that specimens on the same slide only are to be compared when using cytophotometry of the Feulgen reaction. If specimens on different slides are used for comparison, a large error is introduced.

8. The results suggest that it is justifiable to estimate the number of nuclei per adrenal gland after ACTH administration by dividing the total amount of DNA in the gland by the mean DNA value per resting adrenal nucleus. However, this procedure may lead to some error if active proliferation of adrenal cells is taking place.

9. Absolute proof of an increase in mean adrenal nuclear DNA content during hyperplasia will be difficult to establish until a cytochemical reaction for DNA rests on a surer footing. Improved techniques too, for isolation of fragile nuclei are greatly to be desired.

PART II

THE EFFECT OF ADRENOCORTICOTROPIN ON RIBONUCLEIC
ACID AND OTHER HISTOCHEMICAL SUBSTANCES IN THE
GUINEA-PIG ADRENAL CORTEX .

RIBONUCLEIC ACID: HISTOCHEMISTRY AND OCCURRENCE IN ADRENAL.

Cytoplasmic basophilia is now accepted to be generally due to the presence of ribonucleic acid. This property of cytoplasm was described as early as 1899 by Matthews who studied the morphological changes which occurred in pancreatic cells undergoing secretory activity. He described nebenkerns or mitosomes in the cytoplasm of cells from the pancreas of the frog, fowl and the cat. These were said to occur as a cytoplasmic reticulum of threads in a variety of forms. They had been described in other tissues by previous workers. Matthews (1899) concluded from their staining with acid fuchsin-methyl green that they were formed of 'nucleo-albumin'.

Observations similar to those of Matthews were made by Garnier (1900) in the salivary and lachrymal glands and pancreas of various laboratory animals and man. They were called 'accessory nuclei' by this author who observed that they frequently occurred as nucleus-like spherical bodies beneath the true nuclear membrane. Guieysse (1901) described similar structures in the adrenal cortex of the guinea-pig, and called them 'corps siderophiles' or siderophilic bodies as they stained with iron haemalum. These tissue components were described later by Kolmer (1912, 1918) in the guinea-pig suprarenal.

This author found that they were more numerous in the male than in the female guinea-pig, and suggested that they were possibly a secondary sexual character of the male.

Fibrillar structures similar to the nebenkern of previous authors were described by Saguchi (1925) in the cytoplasm of cells from frog pancreas. This author observed the similarity of their staining reactions to those of nuclear chromatin, but stated that when stained they could be decolorized more easily. He considered they were formed by fragmentation of degenerated cells. They were particularly numerous in the zona reticularis of the normal guinea-pig, but not prominent in the normal rat adrenal cortex. Where their occurrence was marked he frequently found groups of agglutinated erythrocytes in the sinusoids of the inner zona reticularis and medulla. Siderophilic bodies were also described by Takechi (1925) in guinea-pig tissues. They were more marked in male than in female or castrate male guinea-pigs. This author demonstrated them by staining with molybdenum-haematoxylin. Basophilic granules were described by Santee (1936) in adrenal and liver cell cytoplasm of patients who died with severe infection. Their chemical nature was stated to be obscure.

Cytoplasmic 'chromidia' formed of fibres were stated by

by Monne (1946) to possess all the characters of "biogenmolecules" i.e. they were centres of growth and metabolism of the cytoplasm.

The basophilia of the reticulum of reticulocytes was found by Dustin (1947) to disappear after these were exposed to ribonuclease. This author concluded that the prime histological characteristics causing staining of this reticulum and of the cytoplasm surrounding vacuoles in various cells was the presence of ribonucleic acid.

The work of Dustin seems to offer the first acceptable evidence that these basophilic cytoplasmic granules were formed mainly, if not entirely, of ribonucleic acid.

Ribonucleic acid was found to form the main component of the granules described by Santee (1936) by Rich & Berthrong (1949) in the same laboratory. These authors succeeded in removing, by means of ribonuclease, the basophilic material observed in adrenal and liver cell cytoplasm. Treatment with lanthanum chloride, which renders RNA insoluble, prevented removal of the basophilic granules with the enzyme.

The cytoplasm of lipid-rich adrenal cortical cells was stated to be strongly basophilic by Dempsey (1948). The cytoplasm in these cells was compressed into thin strands by

the lipid. The cytoplasmic basophilia was more marked at the peripheral zones of the adrenal than in the zona reticularis where the cytoplasmic strands were found to be markedly eosinophilic. The basophilia was considered to suggest the presence of nucleoprotein and rapid protein synthesis.

Chromidia similar to the ~~nebe~~ kern and 'corps siderophiles' of the literature were observed by Selye & Stone (1950) in the rat and guinea-pig adrenal cortex. These authors stated that chromidiosis was not marked in the rat adrenocortical cells. They were called 'chromidia' since they resembled nuclear chromatin. They sometimes stained like the nucleolus, and were particularly abundant after stress (during the stage of resistance) or after prolonged administration of pure adrenocorticotropin. These structures were stated to be formed as a chromatin cushion on the nuclear side of the nuclear membrane, and to be extruded into the cytoplasm. They seemed to have an affinity for cell membranes.

A method of purification of ribonuclease from "pancreatin" was described by Dubos & Thompson (1938). The optimum temperature for the action of the purified "enzyme" was 70 °C, and it did not lose its activity when subjected to boiling over a wide range of pH. The protein was inactivated by

small amounts of purified pepsin. The products of degradation of ribonucleic acid (RNA) after exposure to ribonuclease were soluble in mineral acids. Ribonuclease did not have any demonstrable effect on other substrates tested. It changed pneumococci from Gram-positive to Gram-negative by removal of the basophilic ribonucleic acid. A crystalline enzyme capable of digesting ribonucleic acid was also isolated by Kunitz (1940) from beef pancreas. It was a soluble protein of albumin type. It was stable after boiling over a wide range of pH. Some loss of activity, however, did occur after heating, exposure to alkali or digestion with pepsin. It broke up RNA into small fragments which diffused readily through a collodion membrane.

It was concluded by McDonald (1948a) that ribonuclease, as prepared by previous authors, was capable of hydrolysing proteins, and that this ability was due to impurities in the preparations rather than to an intrinsic property of the enzyme itself. This author emphasized the dangers of relying on the specificity of ribonuclease, and evolved a method (McDonald 1948b) for the preparation of crystalline ribonuclease which was free from measurable proteolytic activity.

The possibility of using the Unna-Pappenheim methyl

green-pyronin mixture in conjunction with ribonuclease was suggested by Brachet (1940).

A method evolved by Brachet (1940) is described in detail in a later publication (Brachet, 1953) and critically evaluated. Serra's fluid is recommended for fixation, though it is advised that the fixative for a given tissue should be chosen by preliminary investigation. It is concluded that there is a general parallelism between depth of staining with pyronin or toluidine blue and RNA content, but that insufficient evidence was available for the confident cytophotometric determination of the quantities of this compound present in tissue sections. It is affirmed by Brachet that it is the specificity of ribonuclease which determines the specificity of the staining method. The methyl green-pyronin method for RNA was reinvestigated by Taft (1951). Various tissues of the mouse were examined, control sections being pretreated with ribonuclease or hot trichloroacetic acid for extraction of nucleic acids. Staining of various normal and pathological cellular constituents was also studied. Though complete proof could not be achieved, the evidence was highly suggestive that pyronin was specific for RNA providing ribonuclease was used for control specimens, and that methyl green stained DNA independently of the degree

of its depolymerization.

In the opinion of Kurnick (1950a) the selective staining of the nucleic acids by methyl green-pyronin is an indication of the degree of depolymerization of these compounds.

One of the earlier studies of cellular RNA using the methyl green-pyronin mixture in conjunction with ribonuclease was that of Biesele (1944). This author studied cytoplasmic RNA in methylcholanthrene-induced skin carcinoma in mice. RNA was increased in 12hr following the initial application of this compound, was maximal from the third to the tenth day, following repeated application, and declined thereafter to an intermediate value by two months.

The distribution of RNA in the human anterior pituitary gland and placenta was examined by Dempsey & Wislocki (1945). Sections from these organs were stained with eosin-methylene blue, control sections being pretreated with ribonuclease prepared by the method of Kunitz (1940). RNA was stated to be abundant in the cytoplasm of the basophils of the anterior pituitary and in the cytotrophoblast of the human placenta. Alkaline phosphatase was also demonstrated in high concentration in these sites ; and the results were said to indicate that

a functional relationship probably existed between these two compounds.

Cytophotometric analysis of RNA in various human tumours stained by methyl green-pyronin was undertaken by Stowell (1946). An increase in RNA , per unit volume and per cell, was found in the majority of these, which were compared with adjacent normal tissues. In half of the tumours the increase was more than 50%. Tumour cells had a smaller mean size, and most had a lesser cytoplasmic - nuclear ratio. It was concluded that a disturbance in nucleoprotein metabolism existed.

The functional state of rat thyroid, regulated by exposure of the experimental animals to different environmental temperatures, was correlated with the RNA content of thyroid epithelium and colloid by Dempsey & Singer (1946). These authors used methylene blue staining in conjunction with ribonuclease extraction. A variation occurred with functional activity, diminished concentration of RNA accompanying increased thyroid activity.

Bone marrow cells were examined for their RNA content by White (1947) using staining with methyl green-pyronin. RNA was found to be abundant in young marrow cells, and to be responsible for their cytoplasmic basophilia. A rapid dimin-

ution in amount was stated to occur with maturation of the erythropoietic series, and in the granulocytic series with the appearance of granules.

Cytochemical findings were stated by Caspersson (1950) to indicate that RNA takes part in the synthesis of protein. RNA was said to be always more abundant where protein synthesis is intense and where cell multiplication is taking place. Cytoplasmic RNA was implied to stimulate the synthesis of cytoplasmic protein.

Electron microscopy enabled Palade & Porter (1954) to demonstrate an endoplasmic reticulum in avian cells cultured in vitro, and in various rat and rabbit tissues. According to Porter (1954) this is a universal component of cytoplasm. It is composed of a complex system of strands and vesicles, and is stated to form the basophilic component of cytoplasm. Its structure varies in different cell types, and in different physiological conditions. Studies on different cell fractions showed that this cytoplasmic component is extremely rich in RNA.

Ribonucleic acid was studied in mouse liver and pancreas under normal and pathological conditions by Fiala, Sproul, Blutinger & Fiala (1955). Chromidia composed of a heterogeneous population of granules were separated by differential centrifugation. These granules corresponded to chromidia observed in tissue sections. They stained with pyronin Y

and this stainability was reversed by treatment with ribonuclease. These structures were rich in ribonucleic acid. They were also associated with considerable succinoxidase activity.

An examination of homogenized rat adrenal by Fiala, Sproul & Fiala (1956) showed that in this animal also basophilic sedimentable chromidia were rich in RNA content as shown by chemical analysis.

Ribonucleic acid content and distribution in human adrenals in normal patients and others who died after a variety of pathological conditions were studied by Symington, Currie, Curran & Davidson (1955), Symington & Davidson (1956) and Symington, Duguid & Davidson (1956). In the normal gland RNA was present in the zona glomerulosa and zona reticularis. Fine granules stained with pyronin were evident in the zona reticularis. The clear cells of the zona fasciculata did not contain stainable RNA. After the stress due to acute infections or burns, 'compact' cells, normally evident in the zona reticularis, extended from the inner to the outer border of the cortex, or a narrow rim of 'clear' lipid-laden cells, devoid of RNA, remained in the outer zona fasciculata. Where degenerative changes were found, no stainable RNA was evident.

Biochemical estimations confirmed an increase in the RNA/DNA ratio. Simultaneous production of corticosteroids was shown to occur. Symington & Davidson (1956) suggested the possibility that RNA may play a biological role in the formation of corticosteroids, directly, or indirectly by elaboration of enzymes which were also shown to be increased following stress.

LIPID : HISTOCHEMICAL TECHNIQUES AND OCCURRENCE IN THE ADRENAL

Ciaccio (1910) reviewed the work in the 19th and early 20th centuries on steroid producing organs. The earliest study on the lipid droplets of the adrenal cortex seems to have been conducted by Ecker (1846). Many observations on the staining properties of adrenal lipids were made around 1900, and these showed some differences between the staining reactions of adrenal lipids and those of fat in storage depots. Thus, adrenal lipid showed only secondary staining (after immersion in alcohol) with osmium tetroxide ; adrenal lipid was insoluble in alkalies, and staining with Weigert's haematoxylin occurred after mordanting in potassium dichromate (Plecnik 1902).

Various compounds, including triglycerides, fatty acids, phrenosin, phosphatides and cholesterol esters have been isolated from adrenals of many species of animals. The differences in staining properties of adrenal lipids and neutral fat were shown to be due to the presence of cholesterol esters in adrenal lipids (Hays & Steelman, 1955). Since then many studies have shown that lipid extracts of the adrenal exert the physiological effects of that organ, and a number of methods for the bioassay of the active adrenal have been developed. Many biologically active corti-

costeroids have been isolated and the literature on the adrenal cortex is vast indeed.

Various histochemical methods for lipids have been investigated by different authors (Pearse, 1953) and most of these have been applied to the study of the cytology of the adrenal cortex. It is concluded by Dempsey (1948) that Sudanophilia does not serve to differentiate between the various types of lipid in tissue sections, and that, for want of any better designation, material stained with Sudan is regarded as neutral fat. It was shown by Kaufmann & Lehmann (1926) that the Sudan stains demonstrate triglycerides of fatty acids, free fatty acids, cholesterol esters and free and combined lecithin. According to Gatenby & Beams (1950) Sudan and Scharlach R stain fatty acid esters and cholesterol esters, and Mallory (1938) is of the opinion that Sudan stains almost all fat in the body. It is concluded by Sayers (1950) that those stains do not selectively combine with any class of lipids.

Black (1938) studied the effects of fixation of various tissues with formalin, mercuric chloride and Muller's fluid on staining for fat with Sudan III, Scharlach R, Nile blue sulphate, indophenol and osmic acid. He concluded that

formalin fixation gave the best routine results and resulted in deeper staining. Fresh frozen unfixed tissues, however, were highly satisfactory. Vegetable fats were found to stain more deeply than animal fats, and unsaturated neutral fats stained more deeply than those which were comparatively saturated. The results indicated that a partial hydrolysis of neutral fat occurred during fixation.

The possibility of distinguishing between 'neutral lipids' (esters and hydrocarbons) and 'acidic lipids' (phospholipids and fatty acids) was investigated by Cain (1947a) using Nile blue sulphate. He concluded that the distinction was feasible with this dye. His view ran counter to that of Lison (1936) who considered Nile blue sulphate had no histochemical value apart from the fact that the red coloration was specific for lipids in general. On re-investigation of this problem Cain (1948) decided that this dye could not be used to distinguish between various members of the lipids.

Baker (1946) conducted an investigation into the staining of lipid with acid haematein. This dye was found to stain some proteins also, but Baker claimed that pyridine extraction of control tissue sections made possible specific histochemical recognition of lipid, including lecithin. Further investigation

of this staining technique by the same author (Baker 1947) gave results which showed that the stain is a specific histochemical test for phospholipid. The specificity of the test was investigated by Cain (1947b) who concluded that specificity depends on the relatively greater affinity of phospholipid among lipids, for the mordant used (potassium dichromate-calcium chloride solution). He thought that a dark blue coloration following staining was an indication of the presence of phospholipids.

Reduction methods in which a silver salt or osmic acid is reduced to a black precipitate are stated by Dempsey & Wislocki (1946) to be capricious and non-specific. Sudan stains, however, gave less precise localization of lipid, but were not soluble in phospholipids and cholesterol. Sudan black is believed by Dempsey & Wislocki to overcome some of the limitations of Sudan III and Sudan IV, and to give more precise localization of lipid, and to show small lipoidal inclusions. The Smith Dietrich test for lipids (Dietrich 1910) was found by these authors to stain lecithin, cephalin and sphingomyelin also. In their opinion the use of methods involving saponification has not received the attention which they deserve.

Many studies on adrenocortical lipid have been conducted

under normal, experimental and pathological conditions.

Alexander Watson (1923), when he was a lecturer in Histology in this Institute (Institute of Physiology, Glasgow University), studied adrenocortical lipid distribution in the male mole. He correlated adrenal lipid content, using Sudan III staining, with different phases of the oestrous cycle. An increase in spermatogenetic activity in March was accompanied by a marked diminution in adrenal lipid content. No comparable changes were observed by this author (Alexander Watson, 1927) in the rat adrenal during different phases of spermatogenesis.

A high concentration of lipid in cells of the elasmobranch interrenal was demonstrated by Fraser (1929) using osmic acid staining. Lipid was frequently found in the capillaries of the interrenals and he suggests that this lipid represents a secretory product of the interrenal cells.

The first systematic study of the variations in adrenal cortical lipid in the guinea-pig with sex and age was conducted by Whitehead (1934) using Sudan III. In most adrenals minute droplets of fat were found in the 'subcapsular rim' of cells. A lipid-laden area was evident immediately adjacent to this zone, marked by a sharp outer border. A progressive slight

decrease in cellular lipid was found towards the inner zones. It is apparent from the photographs that little lipid was found in the zona reticularis. Some scattered groups of cortical cells were found in the medulla and almost all of these contained a variable amount of lipid. The proportion of cortex occupied by lipid decreased progressively from birth to 168 days. In animals older than two weeks the proportion of cortex occupied by lipid was slightly greater in females than in males. All sudanophilic substances present were found to give the Schultz reaction for cholesterol.

Age and sex variations in adrenocortical lipid of the albino rat were studied by Tobin & Whitehead (1942). Lipid was found to be abundant in the outer half of the adrenal cortex, and the range of its distribution was similar for both sexes. No evidence was found for an alteration of its distribution during the oestrous cycle. At birth the zona reticularis was found to contain abundant lipid, but at two and four weeks of age this zone was fat free. Later, however, the zona reticularis always contained a variable amount of sudanophilic material. A marked similarity in lipid distribution in the left and right adrenal from the same animal was invariably observed. Tobin & Whitehead suggested that the

best method to study the experimental distribution of adrenocortical lipid was unilateral adrenalectomy followed by experiments on the remaining adrenal which could be compared with the gland previously removed. Without such control it was necessary to work with quite large groups of animals to ensure the existence of normal adrenals for control purposes.

The distribution of lipid in the adrenal cortex of the rat under various experimental conditions was studied by Sarason (1943). Depletion of adrenocortical lipid was observed following administration of deoxycorticosterone acetate (DOCA), after hypophysectomy along with DOCA administration, after castration with DOCA administration, after starvation, after feeding on a protein-rich diet and after injection of stilboestrol. Sarason suggested that adrenal cortical hypertrophy and lipid depletion indicated increased protein catabolism in acute inanition.

As everyone knows the "General Adaptation Syndrome", which occurs after a large variety of "alarming" stimuli, was studied by Selye (1946, 1950). Resulting enlargement of the adrenal cortex, due to hypertrophy of the individual cells, and to a lesser extent to hyperplasia, was accompanied by discharge of cytoplasmic lipid granules. These changes

took several hours to develop, and the 'alarm-reaction' phase lasted for a few minutes to 24 hours. The lipid changes subsided gradually during the following phase of resistance, but reappeared, generally in a few weeks, during the phase of exhaustion. Fatty metaplasia occurred in rats treated with lyophilized anterior pituitary and 'testoid' hormones. Changes similar to those occurring during the "General Adaptation Syndrome" followed prolonged administration of ACTH. Similar findings are reported by Selye & Stone (1950) for the rat adrenal, and changes similar to those reported by these authors are described by Zamcheck (1947) in the adrenal cortex of patients dying of acute disease.

Acute stress or administration of 'folliculoid' hormones caused discharge of adrenocortical lipid granules in the rat. Purified ACTH, however, or crude anterior pituitary extracts, when administered, did not cause lipid depletion even in acute experiments.

Administration of pure (Armour) ACTH did, in fact, inhibit the loss of lipid which normally occurred during the 'alarm-reaction'. It was concluded that factors other than the production of endogeneous ACTH were involved in the causation of the adrenocortical lipid depletion which followed acute stress. Stress did not cause depletion of adrenal lipid in hypophysectomized animals (Selye 1946). The

possibility was put forward that other anterior pituitary hormones may have been involved (Selye & Stone 1950).

Considerable variation in the quantity and distribution of lipid in the adrenal cortex of the albino rat was observed by Harrison & Cain (1947). Three main regions were described by these authors. An outer zone composed of the zona glomerulosa and some of the zona fasciculata contained a variable amount of sudanophilic material. The outer zona fasciculata contained a large amount of lipid, whereas the inner zona fasciculata and the zona reticularis were usually poor in lipid. Intra-litter variations in lipid content and distribution were less marked than inter-litter variations, a finding similar to that of Tobin & Whitehead (1942).

Biochemical estimates of adrenal cholesterol and the amount of sudanophilic material in human adrenal cortices were found to give parallel results by Rogers & Williams (1947). This finding differs from that of Harrison & Cain (1947), who did not find variations of cholesterol in spite of variations in sudanophilic material.

The reaction of the rat adrenal cortex to adrenaline stress was studied by Symington (1951) using histological and biochemical methods. Repeated injections of adrenaline in various doses for different periods of time were followed by varying degrees of adrenocortical lipid depletion. The

changes were correlated with secretion of glucocorticoids assayed by analyses of liver glycogen content and blood sugar levels.

Lipid depletion in the adrenals of children who died from systemic disease were described by Stoner, Whitely & Emery (1953). The adrenals were compared with those from normal children who died suddenly without previous illness. The loss of adrenal lipid which occurred during disease was less marked in children under two weeks old than in older children, and was not found in children less than four days old. Much less sudanophilic material was present in the adrenals of normal children than in normal adults.

The pattern of lipid distribution and content in normal and in stressed patients is described by Symington & Davidson (1956) and by Symington, Duguid & Davidson (1956). The stress was a systemic disease e.g. coronary thrombosis, acute infection, prostatic or mammary carcinoma, or extensive burns. Normal adrenals were procured at postmortem from patients who died suddenly without disease, or in the first stage of a bilateral adrenalectomy for malignant disease. Histological results were compared with chemical findings. In the normal gland the zona glomerulosa consisted of small cells with an ill-defined border, and fine lipid droplets.

The cytoplasm of cells in the zona fasciculata was clear and contained abundant lipid. The zona reticularis was composed of "compact" cells, small, with fine droplets of lipid. The adrenals of the 'stressed' patients showed lipid depletion which was focal, or extreme and diffuse. Good agreement was found between chemical analyses and stained lipid in tissue sections. The histological and chemical findings were similar to those found after administration of human adrenocorticotropin.

PLASMALOGENS AND THE ADRENAL

The first authors to notice that some cytoplasmic substances stained with Schiff's (1866) leucofuchsin solution without initial exposure to the hydrolysing effects of hydrochloric acid were Feulgen & Rossenbeck (1924). In the same year Feulgen & Voit (1924) demonstrated the blocking effect of pretreatment of sections with phenylhydrazine or sodium bisulphite. These authors therefore concluded that aldehydes were responsible for giving the reaction. They named the reacting substances 'plasmals'. Most of these were found to be removable by lipid solvents. Mild hydrolysis with mercuric chloride intensified the staining reaction, or the acidity of Schiff's (1866) reagent resulted in a similar though less marked intensification. The adrenal cortex and corpus luteum were found by these authors to be rich in plasmals. Feulgen & Bersin (1939) studied the chemistry of the plasmal reaction, and demonstrated that the fatty aldehydes of plasmalogen were joined to glycerol by an acetal linkage. They claimed to show that the substance responsible for the plasmal reaction was acetal phosphatide. In their opinion mercuric chloride solution split acetal phosphatides into higher aldehydes and other substances, and these higher aldehydes were responsible for restoring colour to Schiff's (1866) reagent. The main compound involved was stated to be a cyclic acetal of an aldehyde, R-CHO, with glycerol-phosphoryl-choine.

In an investigation concerning the histochemical localization of amine oxidase, Oster & Schlossman (1942) studied initially the localization of plasmal in fresh frozen sections of fixed tissues from various organs and species. Scission of plasmalogen was found to take place with gold chloride as well as with mercuric chloride or with Schiff's solution as used in Feulgen staining. No staining of plasmalogens occurred if sections were treated initially with phenylhydrazine or sodium bisulphite, confirming this finding of Feulgen & Voit (1924); also condensation products of aldehydes did not react with Schiff's reagent.

A battery of reactions were employed by Dempsey & Wislocki (1944) to demonstrate ketosteroids in the human placenta. These included the plasmal reaction, birefringence, fluorescence, the phenylhydrazine reaction and the sulphuric acid reaction. It was stated by these authors that it was apparent from their results that the plasmal reaction showed certain fat soluble substances in organs which play an active part in synthesizing biological steroids. Control sections were not exposed to hydrolysis with mercuric chloride, or were treated with lipid solvents before staining. Where a positive reaction occurred without prior hydrolysis, the substances which caused recoloration of leucofuchsin were considered to include steroids. A positive plasmal reaction was thought

to be due to unsaturated ketosteroids. Application of this staining reaction to the human placenta resulted in localization of the purple colour to the syncitium which, therefore, was considered to be the site of production of placental oestrogen and progesterone. A later investigation led Dempsey (1946) to voice the opinion that reactions including the plasmal reaction, which had been used to demonstrate ketosteroids, were not capable of differentiating between tissue aldehydes and ketones.

The distribution of substances showing the plasmal reaction was compared with that of substances reacting with dinitrophenylhydrazine. This work was carried out by Albert & Leblond (1946) in normal, adrenalectomized and castrated rats. Some of the experimental animals were deprived of two important sources of ketosteroids i.e. adrenals and testes. Adrenalectomy did not modify the plasmal or the dinitrophenylhydrazine reactions in the corpus luteum, mammary gland, myelin, fat cells, thyroid, muscle or liver. Only in the case of the prostate and seminal vesicles did castration cause a diminution in depth of staining by these methods. A strict parallelism was found between these two staining reactions, and it was concluded that plasmalogens were more likely to be responsible for a positive plasmal reaction than

were ketosteroids.

Wislocki & Wimsatt (1947) studied the placentae of two North American shrews for birefringent fat droplets which exhibited green fluorescence and stained with the plasmal reaction. The chorionic epithelium was rich in such droplets and it was inferred that it was a site of active ketosteroid formation.

Variations in the method of application of the plasmal reaction are criticized by Cain (1949a). This author stated that the only factors common to all of them were the use of leucofuchsin solution and a positive reaction with some cytoplasmic components. These variations were stated to affect the results, and this author was of the opinion that the plasmal reaction, as usually performed, does not demonstrate aldehydes specifically. Oxides and peroxides, the autoxidation products of unsaturated lipids, were said to be shown by use of the plasmal reaction as usually practised. An improved technique for the demonstration of acetal linkages was suggested and described. He advised comparison, in all cases, of material which is plasmal-positive with fresh unfixed tissue stained by Schiff's reagent. He concluded that there was no completely satisfactory method for the demonstration of 'plasmal'. Unsaturated lipid would, in his

opinion, give rise to substances which react positively with Schiff's reagent if exposed to atmospheric oxygen, acid media, or prolonged fixation (Cain 1949b).

The possibility of distinguishing between free aldehydes, acetals and aldehydes liberated by oxidation was investigated by Danielli (1949). The use of supplementary tests for aldehydes is advised. There ought to be confirmation of the reacting group by pretreatment with hydroxylamine, and demonstration of solubility in lipid solvents of fatty aldehydes, in order that reactions with other chemical groups may not lead to misinterpretation of positive results.

In the opinion of Hayes (1949) the duration of tissue fixation in 10% neutral formalin should be limited to 1 - 6 hr and the time of exposure of tissue sections to mercuric chloride solution should not exceed 2 - 10 min. Under these conditions acetal lipids alone are said to show a positive plasmal reaction. Fixation for the short time prescribed is said to cause only minor destruction of some acetal lipids. Fixation for a longer period of time is stated to result in progressive destruction of these compounds and to unmask aldehyde groups from other cytoplasmic components, which then react. This author also advises that control sections be always used and treated

in all steps similarly to the sections used for demonstration of acetal lipids, but the control sections should not be exposed to the oxidizing action of HgCl_2 . The importance of this step is further emphasized by the findings of Leblond (1949) and Knouff (1949) who found that adrenocortical lipids from mice and hamsters react directly with Schiff's reagent. It is inferred by Hayes (1949) that such lipids cannot be shown, using the plasmal reaction, to be acetal lipids.

Elimination of tissue aldehydes by preliminary treatment of tissue sections with sulphanilic acid or sulphonamide by Boscott & Mandl (1949) was followed by a negative plasmal reaction. These authors also treated deoxycorticosterone and deoxycorticosterone acetate with mercuric chloride and failed to hydrolyze either of these compounds to an aldehyde in vitro. They conclude that the plasmal reaction demonstrates aldehydes in human and rat adrenal.

A marked difference was sometimes encountered in the distribution of 'plasmalogens' and of substances stained with phenylhydrazine by Yoffey & Baxter (1949). This variation was associated with different functional states of the gland, and was inferred to show that these two reactions were demonstrating different substances. These results run counter to those of Gomori (1942).

Gomori (1950) states that in his experience floating frozen sections unprotected from air for a few days became increasingly plasmal positive. Waelsh (1950) contends that the age of the preparation influences the depth of staining with this reaction, and that this may be related to oxidation of unsaturated fatty acids. He advises, therefore, that results be accepted with caution. Bennett (1950) stated that the plasmal reaction could be produced by aliphatic ketones as well as by aldehydes, and it may be inferred that in his opinion the plasmal reaction may not be used to distinguish between ketonic corticosteroids and plasmalogens.

In a critical discussion of previous work on the plasmal reaction Pearse (1953) states that it may be used to show the presence of ester phosphatides providing neutral fats are shown to be absent. The present interest in this reaction centres, in his opinion, around the problem concerning tissue ketosteroids giving a positive reaction.

Chemical investigation of higher fatty aldehydes freed by the action of mercuric chloride from the lipids of beef muscle and brain, and rat muscle and brain by Anchel & Waelsh (1942) showed that the 'plasmals' consisted mainly of stearaldehyde and palmitaldehyde. These were isolated as hydra-

zones and methoximes. A variety of other aldehydes were considered to be probably present, and in the opinion of these authors these may have included derivatives of ketosteroids.

TECHNIQUES FOR THE HISTOCHEMICAL DEMONSTRATION OF
KETOSTEROIDS IN THE ADRENAL CORTEX.

The first attempt to demonstrate ketosteroids by a histochemical method was undertaken by Bennett (1939). This author used the formation of hydrazines with phenylhydrazine hydrochloride and was of the opinion that the yellow band in the outer zona fasciculata of fixed sections of cat adrenal was due to the presence of steroids. Substances in this region were stated to reduce ammoniacal silver nitrate solution and to be soluble in acetone. They also showed birefringence. The outer zona fasciculata was regarded, therefore, by this author as the secretory zone. Further evidence of the ketonic nature of the substance purported to be shown was said to accrue from the negative reaction following treatment of sections with semicarbazide solution which blocks ketone groups. More detailed study of similar material by this author (Bennett 1940) led to similar conclusions. Since ketones were, presumably, present in small amounts, thick sections (80 - 120 microns) were found necessary. Ascorbic acid was removed initially by oxidation in an alkaline buffer solution, with indophenol or iodine. The phenylhydrazine hydrochloride solution was buffered at pH 6 - 6.5. As well as the above methods reaction with digitonin to form

birefringent crystals and Sudan staining were also used. These methods were utilized to postulate four zones in the cat adrenal. With reference to ketosteroid content there seem to be pre-secretory, secretory, postsecretory and senescent zones if one accepts the theory of migration of cells inward from the capsule.

Gomori (1942) investigated the phenylhydrazine reaction of Bennett (1939, 1940) and compared the distribution of positively staining substances with that of plasmalogens in normal and pathological tissues. The phenylhydrazine and plasmal reactions were stated to give results which were identical. Fresh tissue, or tissue fixed in formalin under anaerobic conditions, did not react, and oxidation or hydrolysis was considered a necessary step before a positive reaction could be obtained with the hydrazine or plasmal techniques. A progressive increase in staining intensity with both techniques was evident as fixation was prolonged from 6 hours to 5 - 6 days. Tubercles, infarcts and tumors were found to give positive reactions with both techniques, and it was concluded that Bennett's (1939, 1940) reaction was not specific for ketosteroids.

A battery of reactions were employed by Dempsey & Wislocki (1944) to demonstrate ketosteroids in the human placenta.

The use of the plasmal reaction for the histochemical recognition of ketosteroids did not receive support from the work of Boscott, Mandl, Danielli & Shopper (1948). These authors studied the plasmal reaction with three samples of deoxycorticosterone in vitro. No evidence was found for the oxidation of deoxycorticosterone to an aldehyde which could react with Schiff's reagent. Alpha hydroxyketones were therefore considered to give a negative plasmal reaction.

Boscott & Mandl (1949) criticized the deductions of Dempsey & Wislocki (1944). In the opinion of these authors the various criteria used by Dempsey & Wislocki (1944) for identification of ketosteroids could be given by a mixture of neutral fats, cholesterol, carotenoids, aldehydes and phospholipids without ketosteroids being necessarily present. After elimination of aldehydes by condensation with valeric aldehyde or isobutyric aldehyde, these authors found a negative diphenylhydrazine reaction. Failure, therefore, was reported in the demonstration of ketosteroids, using this technique.

A notable success was considered to have been attained in the histochemical recognition of ketosteroids by Dempsey (1946). The methods of Bennett (1939, 1940) were considered by Dempsey to demonstrate ketosteroids. Reactive substances

were said to occur in sites where steroids are formed and were therefore considered to indicate steroid hormones. The study of the distribution of steroid hormones by these techniques in different physiological states, however, was stated to be fragmentary. Dempsey found an increase in staining of the adrenal cortex with phenylhydrazine in moderate 'cold' stress, but a depletion of reactive substances if the stress were severe. An improvement in Bennett's (1940) technique was suggested, i.e. the use of 2,4 - dinitrophenylhydrazine sulphate in a phosphate buffer at pH 6 - 6.8. A deeper coloration was said to be thus obtained.

The histochemical technique of Bennett (1939, 1940) was investigated by Albert & Leblond (1946). The reaction was found to be given by tissues in the body which had a widespread distribution, and in almost all organs the depth of staining was not appreciably affected by the removal of two important sources of ketosteroids i.e. adrenal and testes. The persistence of the phenylhydrazine reaction after removal of these organs was said to run counter to the view that ketosteroids were being demonstrated by this technique. In the opinion of Albert & Leblond plasmalogens were more likely responsible for a positive result.

Camber (1949) advised coupling of hydrazones formed with 2-hydroxy-3-naphthoic acid hydrazide with a diazonium salt.

A greater depth of staining was said by this author to result, and to enable recognition of smaller amounts of stained material. The best hydrazide among those tested by Ashbel & Seligman (1949) using the hydrazine reaction for ketosteroids, was also found to be 2-hydroxy-3-naphthoic acid hydrazide.

The resulting compound was coupled with tetrazotized diorthoanisidine to give an azo dye. The blue colour of this substance developed rapidly and was intense. The hydrazide used reacted in vitro with 3, 17 and 20 ketosteroids, but not with 11 - ketosteroids. Satisfactory results were obtained with adrenals, ovaries, placenta and testes, whereas other tissues gave a negative result. Schiff's reagent did not react in vitro with the monoketosteroids tested.

In the opinion of Sayers (1950) the battery of specialized reactions applied to ketosteroids had added little more to our knowledge of the content and localization of these hormones in the adrenal cortex or in other steroid producing organs. He considered the Ashbel-Seligman (1949) reaction little better than the hydrazine reaction of Bennett (1940). He stated that it gives a blue colour with non-lipid material, and advises caution in drawing conclusions from results accruing from its use.

Gomori (1950) reaffirmed his opinion, previously voiced

in 1942, in view of the work of Feulgen and his school, and of the serial publications of Verne. Bennett, in the discussion following Gomori's (1950) paper, states that he did not claim in 1940 that the phenylhydrazine reaction was specific for ketosteroids, and said that all the reactions used by him then were characteristic of aldehydes and ketones in general. Since, however, initial treatment of sections with indophenol did result in a positive reaction in the adrenal cortex, and there was no evidence for oxidation of lipid compounds to plasmalogens in the course of this treatment, he did consider it likely that the hydrazine method did enable localization of ketosteroids in some specified cases.

Formalin was said by Wolman & Greco (1952) to combine during fixation with unsaturated lipids, and the resulting compound was stated to contain free carbonyl groups. Staining of the resulting compounds could be obtained using Schiff's reagent or the Ashbel-Seligman (1949) technique. The latter technique was therefore considered to depend, at least in part, on reaction with nonsaturated ketosteroids, presuming these compounds were being demonstrated.

A view similar to that of Sayers (1950) was expressed by Yoffey (1953). This author could not accept the specificity of Bennett's (1939, 1940) phenylhydrazine reaction for ketosteroids, or the specificity of the technique later

proposed by Ashbel & Seligman (1949). These reactions were found by Yoffey (1953) to behave similarly to the Sudan stains, to the plasmal reaction, and to the Schultz reaction.

Yoffey (1953) concentrated on the Schultz reaction for cholesterol, as this compound is generally thought to be a precursor of steroid hormones.

Deane & Seligman (1953) state that the hydrazide reaction of Bennett (1939,1940) and the technique proposed by Ashbel & Seligman (1949) are not specific for ketosteroids, or even for ketones. Aldehydes are said to have more reactive carbonyl groups, and to react more easily with these reagents than do ketones. In their opinion, if it is shown that the material being examined is lipid, and if chemical methods have demonstrated the presence of ketosteroids, it may be presumed that ketosteroids or their ketonic precursors are reacting. In their experience the hydrazide reactions could be more sensibly correlated with different physiological and pathological states than the Sudan stains for lipid. They contended that the hydrazide reactions are worth studying on an empirical basis.

A similar opinion was put forward by Pearse (1953), insofar as investigation of the hydrazide reactions in different physiological conditions was thought to be worth pursuing.

This author got results from use of the hydrazine reactions which differed from those from use of the plasmal reaction. He considered use of the coupling azo dye techniques for alkaline phosphatase more profitable in assaying the functional state of the adrenal cortex.

A number of phenolic hydrazides were tested by Camber (1954) for their ability to distinguish between aldehydes and ketones, since use of 2-hydroxy-3-naphthoic acid hydrazide (Camber 1949) did not enable this distinction. Preliminary results with salicylohydrazide were said to have been promising. Aldehyde hydrazones were said to fluoresce brightly in different colours in ultra-violet light, whereas ketonic hydrazones emitted a uniform dull blue fluorescence. Only the blue fluorescence of ketones was intensified by treatment with zinc acetate. The detailed use of these compounds for cytological recognition of ketosteroids is being studied by Camber.

Bourne (1955) considered the hydrazide technique of Ashbel & Seligman (1949) to be the most useful yet devised. Bourne states that final assessment of its value awaits further investigations. The discovery of aldosterone, which contains ketone and aldehyde groups, further complicates this controversial problem.

ASCORBIC ACID : HISTOCHEMICAL METHODS AND
DISTRIBUTION IN THE ADRENAL.

A factor, hexuronic acid, characterized by its reducing power was isolated by Szent-Gyorgi (1928) from greens and vegetables. The adrenal cortex was found to contain a high concentration of a similar reducing substance. To hexuronic acid was ascribed the function of a catalytic carrier of hydrogen between peroxidase and other oxidizing or reducing systems. It seems that this author was the first to isolate ascorbic acid from greens and from the adrenal cortex, and to obtain evidence for its biological role.

The high reducing power of ascorbic acid was used by Bourne (1933a) for its histochemical localization. Fixation with formalin vapour was followed by impregnation in silver nitrate solution. Aqueous formalin was considered unsuitable as ascorbic acid is water soluble. Adrenals of mice, cats and guinea-pigs were studied. Black granules of (reduced) metallic silver indicated ascorbic acid. Control slices of adrenal were placed in water and no staining was evident. Aggregation of silver granules tended to occur at membranes, and results were stated to be sometimes erratic. A positive staining reaction occurred in the cortex and medulla of mouse, cat and guinea-pig adrenals, most cortical granules occurring

in the zona glomerulosa and zone fasciculata. Ionization or ability to reduce surface tension were considered to cause the ascorbic acid present to run together into droplets which stained as granules with the silver nitrate solution (Bourne, 1933b). Adrenal slices were impregnated directly with alcoholic silver nitrate solution in this instance. In some cases a somewhat diffuse staining occurred even although macroscopic examination of stained adrenal slices showed a black coloration. Adrenal ascorbic acid content was said to vary with the physiological state of the organ.

The use of acid silver nitrate solution for the selective localization of ascorbic acid was proposed by Giroud & Leblond (1935). This technique was used for histochemical demonstration of ascorbic acid in chromophil cells of the anterior pituitary, in luteal cells from the ovary, in interstitial cells of testes and in cells from the zone fasciculata of the guinea-pig adrenal.

A microchemical technique involving removal of cylindrical plugs of tissue, and estimation of ascorbic acid by titration with 2,6 dichlorophenol indophenol was used by Glick & Biskind (1935) for the estimation of the amount of ascorbic acid per cell in the different zones of beef adrenal. The greatest mean amount per cell was found in the zona

fasciculata. An extension of this investigation was reported by these authors in the following year (Glick & Biskind 1936). Maximal mean cellular ascorbic acid content was confirmed in the zona fasciculata. Lesser quantities were found in the zona reticularis, and the zona glomerulosa and medulla were said to contain much smaller amounts. The concentration varied in different stages of development, and with age. In cattle ascorbic acid increases in the adrenal with age from the embryo to the calf and then decreases in the adult.

The acid silver nitrate technique was discussed by Barnett & Bourne (1941) and used for the study of ascorbic acid distribution in the chick embryo. The high reducing power of ascorbic acid was said to determine the specificity of the method. Other cellular substances were said not to reduce acidified silver nitrate in the dark after treatment for the short periods of time used to show ascorbic acid. A negative reaction obtained by a variety of authors for adrenals of scorbutic guinea-pigs was stated to be important confirmatory evidence of the specificity of the reaction. Melanin, however, was stated to be capable of giving a positive reaction. Dehydroascorbic acid did not induce a precipitate of metallic silver.

Artifacts were said to be produced in some tissues when using the acid silver nitrate technique (Barnett, Bourne & Fischer 1941). This was particularly so in the case of bone. This complication was claimed to be obviated by treatment of sections with 5% ammonium hydroxide after impregnation with the silver salt and the specificity of the technique for ascorbic acid was said to be thus increased.

Chemical determination of adrenal ascorbic acid under a variety of experimental conditions in rats was performed by Mulinos, Pomerantz & Lojkin (1942). Complete starvation, or administration of non-specific toxic substances (tissue emulsions), caused an increase in adrenal ascorbic acid and adrenal weight, whereas chronic underfeeding caused adrenal atrophy accompanied by a fall in ascorbic acid content and concentration. The results were considered to further indicate the similarity between chronic underfeeding and hypophysectomy.

The possibility of precise localization of cellular ascorbic acid using silver-reduction methods was investigated by Barnett & Fischer (1943). It was considered that silver precipitates might tend to accumulate near interfaces and thus lead to artifacts suggesting localization of ascorbic acid at e.g. the Golgi apparatus of cells. Mixtures of

ascorbic acid, gelatine and olive oil were emulsified and treated with acid silver nitrate solution. A tendency for silver granules to accumulate at interfaces was found, and it was concluded that cytological localization was not possible using the techniques of Bourne (1933a, 1933b) or of Giroud & Leblond (1935).

The effects of various stresses on the ascorbic acid and cholesterol levels in rat and guinea-pig adrenals were the object of an investigation by Long (1947). In the stressed rat a fall in adrenal ascorbic acid was followed by a return to normal amount in 6 hours. In the guinea-pig, however, a rapid and sustained fall in adrenal ascorbic acid content was found, which persisted as long as these animals were exposed to stress (24 hr).

The changes in ascorbic acid content and distribution in rat adrenals, ovaries and livers, during the phases of the oestrous cycle were followed by Hogh-Ligetti & Bourne (1948) using histochemical and chemical methods. Whereas cyclical changes occurred in ovarian and liver ascorbic acid, no significant changes were found in the adrenals. Chemical and histochemical results were similar and complementary in these instances.

Adrenal secretion of ketosteroids was studied in conjunction with ascorbic acid levels in the blood from the adrenal vein in the dog and cat by Vogt (1948). No ascorbic acid was found in cat adrenal veins, and in the dog the levels of adrenal arterial and venous blood ascorbic acid were similar. She found no correlation between adrenal secretion of ketosteroids and the level of ascorbic acid in adrenal venous blood.

Further study concerning the intracellular localization of adrenocortical ascorbic acid was pursued by Bourne (1950). He used a new technique for crushing small pieces of tissue between cover-slips and allowing acid silver nitrate solution to penetrate from the periphery. He considered that his experiments showed localization of this substance in the region of the Golgi apparatus and criticized the work of previous authors, who, he claimed had mistakenly identified fixation artifacts as Golgi material. His views run counter to those of Barnett & Fischer (1943), of Chayen (1953) and of Hagen (1954).

The extensive literature concerning adrenal ascorbic acid was reviewed by Sayers (1950). This author stated that the role of this substance in the adrenal is unknown, but a number of studies indicate that it plays a part in the function of the adrenal cortex. It seemed, however, that there was

little evidence to support a direct participation by this compound in the synthesis of adrenocortical steroids. Increased adrenal cortical activity was generally associated with diminution in adrenal ascorbic acid, and stress caused depletion in the rat and in the guinea-pig adrenal unless the hypophysis was first removed. A parallelism was generally found between adrenal ascorbic acid and cholesterol contents, and in Sayer's opinion adrenal ascorbic acid content was a reliable index of adrenocortical function in healthy rats.

Chayen (1953) was of the opinion that existing histochemical methods generally did not accurately localize ascorbic acid in tissue sections. His view was thus similar to that of Barnett & Fischer (1943), but opposed to that of Bourne (1950). He stated also that other cellular substances may cause reduction of acid silver nitrate. He proposed a modified technique, designed more especially for plant tissues.

The distribution of ascorbic acid in the particulate and non-particulate material of ox adrenal and liver was studied by Hagen (1954) using high-speed centrifugation of homogenized tissue, followed by indophenol titration. The ascorbic acid was found to be confined, almost entirely, to the non-particulate fraction, and the author concluded that localization of

reduced silver particles does not necessarily indicate the site of cellular ascorbic acid.

The acid silver nitrate technique was reinvestigated by Bourne (1955). Rat, ox and human adrenals were studied. Unspun homogenized tissue treated with silver nitrate solution did not show granules similar to those found in frozen sections of stained tissue. A very finely granular precipitate was, however, observed. Repeated centrifugation of homogenized adrenals gave similar results. Further experiments with 'squash preparations' (Bourne 1950) led the author to conclude that granules do, in fact, exist in vivo, and that ascorbic acid is associated with them but removed from them during centrifugation of homogenized adrenal.

Yoffey (1955) found that blood in the blood vessels of the adrenal frequently gave an intense reaction, following impregnation of adrenal slices with acid silver nitrate. This author thus doubted the specificity of the reaction, and abandoned it in favour of the study of alkaline phosphatase.

The function of ascorbic acid was discussed by Meiklejohn, Passmore & Stewart (1953). The evidence available indicated, in their opinion, that the function of ascorbic acid has some relationship to that of glutathione, and, possibly, therefore, to enzymes which contain a sulphhydryl radicle. The reversible

oxidation/reduction property of ascorbic acid was said to be probably a good indication of its function in biology.

Evidence from which it may be inferred that ascorbic acid is not required for the secretion of ketosteroids was put forward by Prunty, Clayton & McSwiney (1955). These authors found a marked increase in urinary ketosteroids in scorbutic guinea-pigs before death in spite of an extremely low adrenal ascorbic acid content. The question arises whether the sudden liberation of preformed stored adrenal ketosteroids was responsible for the finding of these authors.

ALKALINE AND ACID PHOSPHATASE; CYTOCHEMICAL
DEMONSTRATION; DISTRIBUTION IN ADRENAL.

Gomori (1939) must be credited with the first attempt at cytological localization of alkaline phosphatase. His technique was later criticized by a number of authors including himself (Gomori 1951), and has now been largely superceded by azo dye coupling methods. The principle of the original technique depended on the hydrolysis by the phosphatase of sodium glycerophosphate or other phosphate compound with the liberation of phosphate ions. An insoluble precipitate is formed by salt formation with a metal, whose phosphates are insoluble. In the original method the phosphate initially liberated reacted with calcium nitrate to give calcium phosphate. This compound was hardly visible so cobalt nitrate was used to form cobalt phosphate; a precipitate of black cobalt sulphide was finally produced by treatment of the sections with yellow ammonium sulphide solution. Alternatively, calcium phosphate in the sections was treated with silver nitrate solution in ultra-violet light or sunlight and a brown deposition of metallic silver resulted. A variety of normal and pathological tissues were studied, and staining of adrenal sections showed a positive reaction in the 'deeper parts of the cortex'.

Chemical estimation of phosphatase activity was said to show good agreement with the results from the histochemical method. A slight modification of this technique was described by Gomori (1946). Thin slices of tissue were fixed in cold acetone: he studied osteogenic sarcoma, Ewing's tumor and normal and pathological kidney tissues.

Gomori's (1939) method for alkaline phosphatase was applied to sections of rat liver by Palade (1951) and the results compared with those from chemical analysis of liver homogenates separated into nuclei and cytoplasm by differential centrifugation. Gomori's methods gave deepest staining in the nuclei, whereas chemical analysis showed that 95% of the alkaline phosphatase was present in the cytoplasm. Palade concluded that the histochemical technique demonstrated only differences in the affinity of various cellular components for lead phosphate.

The action of phosphatases was studied in vitro by Kroon, Neumann & Krayenhoff Sloot (1944). These phosphatases were classified on the basis of their activity at different pH levels, and divided into alkaline phosphatase which had an optimum hydrolytic activity at pH 9 and above, and acid phosphatase whose activity was maximal in the region of pH 5. No other peak of phosphatase activity was found.

Re-examination of the technique originally proposed, using a variety of substrates, was conducted by Gomori (1949). The distribution of alkaline phosphatase was found to be entirely independent of the substrate used. The rates of phosphate production and its different cellular components were said to affect results, and nonenzymatic impregnation of cells with the metal used was stated possibly to occur. Precaution was advised in the interpretation of results, and in the opinion of Gomori only marked differences in staining intensity should be interpreted as indicating a variation in enzyme content. Minor differences alluded to by a number of workers were probably unimportant.

Nuclear staining with the calcium-cobalt sulphide method was investigated by Gomori (1951) and the results compared with those from a modified Menten-Junge-Green (1944) azo dye coupling method. A conspicuous difference was found between the two methods; nuclear staining generally occurred with the calcium-cobalt sulphide technique, whereas it was not evident in sections stained by the azo dye method. Nuclear staining by the calcium-cobalt sulphide method was considered to be an artifact, due to secondary absorption of calcium phosphate, at least in part. A similar comparison (of the older and the azo dye coupling techniques) by Lorch (1947), however, led this author to conclude that the azo dye tech-

niques were inferior in that they failed to demonstrate the low concentration of alkaline phosphatase believed to be present in nuclei.

Danielli (1950) believed that artifacts are produced by the use of Gomori's (1939) technique. Danielli believes that most of the work with Gomori's technique is of questionable value: it suffers from a deceptive simplicity. Gomori's method should be used in conjunction with the azo dye staining reaction proposed by Danielli (1946). A critical evaluation of results would then be available.

Nuclear staining by Gomori's (1939) technique was further studied by Novikoff (1952). His experiments involved incubation of sections with a medium containing alkaline phosphatase. This treatment resulted in nuclear staining. Differential centrifugation of rat liver, however, enabled him to demonstrate a low concentration in rat liver nuclei. Novikoff's attitude agrees substantially with that of Danielli (1950) and that of Gomori (1951).

According to Pearse (1953) the considerable amount of work with the older techniques is largely of historical interest. This author is confident that the newer methods, which involve azo dye coupling, will replace all other methods.

Acid phosphatase demonstration in tissue sections was considered feasible by Gomori (1941) using a technique adapted

from that originally devised for histochemical demonstration of alkaline phosphatase (Gomori 1939). The phosphates of lead and of uranium were found to be insoluble at pH 5.0, and these were therefore used as substrates. Thin slices of tissue were incubated in a buffered solution (pH 5.0) of sodium glycerophosphate and lead nitrate. Treatment with yellow ammonium sulphide solution was stated to result in the deposition of a dark precipitate of lead sulphide at the sites of acid phosphatase activity. This enzyme was found to be present in high concentration in human prostate, spleen and various carcinomata. Further studies, using a slightly modified technique, were conducted by this author (Gomori 1946) on prostatic carcinoma and giant cell tumours in which this enzyme was found to be present in comparatively large amounts.

Inconsistency and non-reproducibility of results using techniques previously advocated (Gomori 1941, 1946) led Gomori (1950) to reinvestigate these methods. The ratio of buffer to substrate was stated to be an important factor, and to affect results materially. Various substrates were investigated, but no adequate reason was found for doing away with the older and cheaper sodium glycerophosphate.

A new approach to the problem of histochemical localization of alkaline phosphatase was made by Menten, Junge &

Green (1944). The organic (alcoholic) instead of the phosphate moiety of a phosphate ester can be used histochemically. When a monoaryl organic phosphate is hydrolysed by the enzyme, the aryl residue can be coupled directly to a diazotized amine to precipitate an insoluble dye at the locus of enzyme activity. Beta-naphthol, derived from the hydrolysis of calcium alpha-naphthol phosphate, can be coupled with diazotized alpha-naphthylamine. The results, for kidney convoluted tubules, agreed substantially with those of previous authors using the older techniques. Use of the azo dye-coupling method is, in the opinion of Pearse (1953) a stroke of genius, and considerable advancement may be expected in the science of enzyme histochemistry from its utilization.

A technique based on a principle similar to that devised by Menten, Junge & Green (1944) was proposed by Danielli (1946). Various phenol phosphates were used as substrates and simultaneous coupling was achieved with a diazotized aromatic amine. Results with older techniques were also considered to be satisfactory.

Difficulty in the preparation of diazotized naphthyl amines as used by Menten, Junge & Green (1944) were overcome by Manheimer & Seligman (1948), who prepared a stabilized alphanaphthyl diazonium salt which could be stored in the

cold for months. A variety of normal and neoplastic tissues were studied.

The sodium salt of p-nitro-benzene-4alpha-naphthol phosphate was synthesized by Loveless & Danielli (1949) and used as substrate for azo coupling. Localization of alkaline phosphatase was said not to be as precise as with use of beta-glycerophosphate as substrate.

Improvement in enzyme localization was claimed using cold formalin fixation and 5-chloro-o-toluidine as substrate by Grogg & Pearse (1952a). Enzyme diffusion was found to be negligible, and artifacts few. Nuclear staining was never observed in frozen sections. Simultaneous coupling-azo dye methods were considered to be the ones of choice for the histochemical demonstration of alkaline phosphatase.

Attempts to apply processes for showing alkaline phosphatase to the localization of acid phosphatase proved unsuccessful in the hands of Seligman, Nachlas, Manheimer & Friedman (1949). An azo dye technique was developed about the same time by Seligman & Manheimer (1949) to show the acid variety in sections. The substrate used was calcium alpha-naphthyl phosphate and simultaneous coupling was achieved by using anthraquinone-1-diazonium chloride. A method was also evolved for the demonstration of alkaline and acid phosphatase in the same section.

An azo dye technique for acid phosphatase demonstration was described by Grogg & Pearse (1952b). Sodium alpha-naphthyl phosphate was used as substrate, and diazotized o-dianisidine was utilized for simultaneous coupling. Cold formalin fixation of frozen sections gave better results than cold acetone fixation followed by paraffin embedding of tissues. Fine intracellular localization was achieved and results agreed very well with chemical estimations.

Alkaline phosphatase distribution in the rat adrenal was investigated by Bourne (1955) using a variety of substrates. A positive reaction was frequently found in nuclei and in sinusoids. The enzyme does not seem to have been localized in any particular zone or zones. The enzyme in the sinusoidal walls was considered possibly to provide energy for the transfer of material into and out of these vessels. Azo dye coupling techniques do not seem to have been used.

Distribution of alkaline phosphatase in the guinea-pig adrenal was studied by Yoffey (1955) using Gomori's (1939) method. Chemical methods of analysis were used for similar material.

The zona glomerulosa gave an intense positive reaction, the zona fasciculata a moderate reaction, and the zona reticularis showed no stainable enzyme. Chemical estimation of the enzyme showed wide variation in different animals.

Starvation or variations in dietary salt content did not cause an appreciable change. The human adrenal showed an enzyme distribution which was diametrically opposite to that found in the guinea-pig. The deepest staining occurred in the zona reticularis; less staining was evident in the zona fasciculata, and a negative reaction occurred in the zona glomerulosa.

Results similar to those of Yoffey (1955) were reported by Symington, Currie, Duguid & Davidson (1955) and by Symington, Duguid & Davidson (1956) for the normal human adrenal cortex. These authors, however, used the newer azo coupling techniques. Alkaline phosphatase was normally found in the zona reticularis. The enzyme was abundant also in the 'compact cells' in adrenals where lipid depletion due to the stress of acute disease was evident. These cells were believed to be actively producing steroids. In stress conditions the compact cells extended from the inner zona reticularis to the outer zona fasciculata. The basement membranes of blood vessels in all zones gave a positive reaction for alkaline phosphatase.

THE EFFECT OF ADRENOCORTICOTROPIN ON THE
ADRENAL CORTEX.

The first observation on the dependence of the adrenal cortex for its structural integrity on the anterior pituitary gland was made by Smith (1930). This author noted a decrease in adrenal weight following hypophysectomy in the rat, and this was accompanied by atrophic changes in the adrenal cortex. Replacement therapy with pituitary transplants prevents these changes. Since the pioneer observations of this author many workers have studied the pituitary-adrenal relationship using normal or hypophysectomized animals and administered crude pituitary preparations or pure adrenocorticotrophic hormone. Some results have indicated a variation in the dependence of the different adrenocortical zones on pituitary hormones for the maintenance of their normal structure and their secretory ability.

Hypophysectomized mice were studied by Chester Jones (1949), and changes resulting in the histochemistry of the adrenal cortex noted following this operation. He concluded that the zona glomerulosa in this animal could exist independently of an intact pituitary. A variety of techniques aimed at recognition of ketosteroids in sections of adrenal cortex were applied. These included Sudan staining, the reaction

with Schiff's reagent, the Schultz reaction, fluorescence and acetone extractibility. Steroids were considered to be present in normal amounts in the zona glomerulosa after hypophysectomy in this and in a later study (Chester Jones 1950). Similar conclusions for mouse adrenal were reached by Miller (1950) who studied cytoplasmic lipid and mitochondria following pituitary ablation. These substances remained normal in the zona glomerulosa following the operation.

Female guinea-pigs were hypophysectomized by Schweizer & Long (1950) and the adrenals studied histologically. Depletion of lipid was observed in all zones, the depletion being least marked in the outer zona fasciculata. Cholesterol, as shown by the Schultz reaction, had a distribution similar to sudanophilic material in normal and experimental animals. Intraocular implantation of anterior pituitary tissue resulted in maintenance of normal adrenal morphology.

Hypophysectomy in the dog was found to affect all zones of the adrenal cortex by Lane & de Bodo (1952). The zona reticularis and zona fasciculata did, however, show atrophy and loss of sudanophilic material before the zona glomerulosa. In the opinion of these authors all adrenocortical zones were dependent on the anterior pituitary in this species.

The importance of the zona reticularis as an actively

functioning zone was stressed by Yoffey (1953). In his experience this zone always showed the first signs of atrophy and loss of sudanophilic and Schultz-positive material following hypophysectomy in a number of species.

Hypophysectomized rats were used for histochemical study of the adrenal by Cater & Stack-Dunne (1953). A widening of the sudanophilic zone which exists between the zona glomerulosa and zona fasciculata was noted in the experimental animals. Abundant alkaline phosphatase was present in the zona reticularis which also showed abundant P.A.S. - positive granules. The granules were believed to be composed of pigment. The inner zona reticularis showed many granules in the sections stained for ascorbic acid. Granules in the capillary walls in the inner zona fasciculata and outer reticularis were present in normal but absent in hypophysectomized animals. RNA was found to a variable extent in a sudanophobic zone. The zona glomerulosa also contained cytoplasmic RNA, as found in control rats. The plasmal reaction did not give satisfactory results, and acid phosphatase did not show any appreciable variation in the experimental animals (using Gomori's 1941 technique).

Methods of isolation of adrenocorticotropin were evolved independently by Li, Evans & Simpson (1943) and Sayers, White

& Long (1943) using sheep and hog pituitary respectively. The isolated hormone had a molecular weight of approximately 20,000 in each instance, and criteria applied to investigate its purity indicated that it was a single substance. Its iso electric point was 4.7 and boiling of a solution of the isolated hormone did not alter its activity. Biological tests indicated that it was free from other pituitary hormones.

Methods of ACTH isolation, its physicochemical properties and methods for its biological assay have been reviewed by White (1946), Li & Evans (1948), Stack-Dunne & Young (1954) and Hays & Steelman (1955).

Using the isolation method described by Sayers, White & Long (1943), Stack-Dunne & Young (1951) isolated two factors from ACTH. One was almost devoid of, and one was rich in ascorbic acid depleting activity. It was considered that hydrolysis was unlikely to have occurred, and these authors believe that two pituitary adrenocorticotropic factors do, in fact, exist. Two corticotropins, designated A and B were described by Hays & Steelman (1955). If no hydrolysis occurred during the process of isolation corticotropin A was said to be found, whereas hydrolysis with pepsin resulted in isolation of corticotropin B. The terminal amino-acid sequence was, however, the same in both, and, if it was

destroyed, loss of physiological activity resulted. Activity was thus associated with a similar molecular pattern in these two preparations.

The effect of ACTH, isolated by the method of Sayers, White & Long (1943), on adrenal content of ascorbic acid and cholesterol in the guinea-pig and rat was investigated by Sayers, Sayers, Liang & Long (1946) using chemical methods. A fall in the adrenal content of both these compounds followed a single injection of ACTH. Cholesterol had returned to normal levels after 24 hr but ascorbic acid was still present in subnormal amount after this lapse of time. It was considered that both these compounds were concerned with release of ketosteroids, and that cholesterol was a precursor of these compounds.

The specific effect of ACTH on these adrenal compounds was further shown by Long (1947) in the rat and guinea-pig. A single injection of 4 mg ACTH per 100 g body weight resulted in a more rapid fall in ascorbic acid than in cholesterol in both animals, and ascorbic acid levels in the guinea-pig adrenal showed a slower return to normal than in the rat. This finding was consistent with the fact, generally known, that guinea-pig (like human) tissues are unable to synthesize ascorbic acid. Concentrations of the substances examined were found not to be affected by the hormone.

Sayers, Sayers & Woodbury (1948) devised a biological assay method for ACTH. This method depends on adrenal ascorbic acid depletion following injection of ACTH into hypophysectomized rats. The ascorbic acid concentration was determined in one adrenal removed at operation. The rat was later given an intravenous injection of ACTH. The difference in concentration of ascorbic acid was the basis for the assay technique. This method was claimed to be highly sensitive and specific. A drawback may have been the estimation of only one of the biological actions of ACTH.

The histochemistry of the rat adrenal after ACTH (Armour) administration for varying periods of time was studied by Bergner & Deane (1948). The hormone was administered in doses of 2.5, 5 and 10 mg six hourly up to 18 hr and the animals killed six hours after the final injection. Long term experiments were also conducted in which animals received injections for 4, 8 and 12 days. The adrenals were examined for sudanophilia, for staining with the plasmal reaction, for birefringence, and for fluorescence and acetone extractibility. These methods were all considered to demonstrate ketosteroids. In the short term experiments the zona fasciculata was found to have broadened but to show varying degrees of 'ketosteroid' depletion. The diminution in reactivity with the battery of reactions applied was maximal

at 18 hr, and at 24 hr 'ketosteroids' began to be replenished. No effect was observed on the zona glomerulosa. After 8 days ACTH administration the zona fasciculata had broadened and a maximal content of 'ketosteroids' was found (resistance phase). At 12 days, some depletion of ketosteroids in the inner zona fasciculata was evident, and hypertrophy of the zona reticularis took place.

Evidence supporting the concept that mineralocorticoids are secreted by zona glomerulosa in the rat was put forward by Nichols (1948). Feeding of animals with diets deficient in sodium or potassium led to changes in demonstrable lipid and cholesterol in this zone. A similar conclusion was reached by Bacchus (1950) for this animal. She used sodium and potassium 'loading' and observed histochemical changes in the zona glomerulosa of experimental animals. 'Ketosteroids' were demonstrated by the variety of reactions used by Bergner & Deane (1948) as well as by the hydrazine reaction (Bennett 1939, 1940). Atrophy of the zona glomerulosa, accompanied by depletion of 'ketosteroids', followed administration of excessive amounts of sodium chloride, whereas hypertrophy of this zone with accumulation of 'ketosteroids' resulted from excessive potassium chloride intake. Increased glucocorticoid formation in rats, made diabetic by alloxan administration, was found by Applegarth (1949) to be accompanied

by indications of increased 'ketosteroid' formation and storage by the zona fasciculata of the adrenal. He based his opinion on sudanophilia, on plasmal staining, on fluorescence, on birefringence and on acetone extractibility of material in this zone. Good correspondence was found between these various reactions.

A single injection of ACTH was given to groups of rats by Yoffey & Baxter (1949) and the adrenals studied using Sudan staining, the plasmal reaction and the phenylhydrazine reaction. The animals were killed at 1, 3, 6 and 12 hr and after 1, 2 and 3 days. At 1 hr depletion of material stained by these methods had occurred in the zona reticularis, and at 3 hr intensification of staining was evident in the outer zona fasciculata. At 2 days, staining with all reactions was intense and extended from the inner reticularis to the outer zona fasciculata. After other periods of ACTH administration a response, variable in different animals, was encountered. This could not be accounted for. It was also stated that the plasmal and phenylhydrazine reactions did not always coincide.

Histochemical examination of the mouse adrenal cortex after ACTH administration was pursued by Chester Jones (1949), using hypophysectomized animals. Methods used for the demonstration of 'ketosteroids' were similar to those employed

by Yoffey & Baxter (1949) with the addition of the Schultz reaction for cholesterol. Loss of 'ketosteroids' in all zones following hypophysectomy was prevented substantially by administration of ACTH, and it was concluded that the presence of lipid (and 'ketosteroids') in the zona glomerulosa as well in the other zones depended on adrenocorticotropin. The zona glomerulosa could, however, exist independently of anterior pituitary hormones.

Chromidia were said by Selye & Stone (1950) to be particularly numerous in the 'cortical' cells of the rat after prolonged administration of ACTH, whereas they were not prominent in the adrenals of control animals, and were invariably absent following hypophysectomy. It is now accepted that these structures are rich in RNA. ACTH administration in actual experiments did not result in depletion of sudanophilic material, even when a total of 48 mg was given in divided doses over a period of two days and adrenal hypertrophy was found to have resulted. Long continued administration of ACTH was followed by increased lipid deposition in the cortical cells. Increased secretion was presumably accompanied by a greater storage of ketosteroids. Staining with the plasmal technique gave results which were in general similar to those using Sudan staining, though differences were frequently found.

Miller (1950) found that an increase in adrenocortical secretory activity was induced by injections of formalin or of insulin as well as by unilateral adrenalectomy. Associated cytological findings were an increase in mitochondria and a discharge of sudanophilic granules.

The cytology of the human adrenal was studied by O'Donnell, Fajans & Weinbaum (1951) after administration of ACTH in various amounts for 1 to 23 days. A varying degree of lipid depletion was found in all cortical zones, and it was generally more marked after prolonged treatment of patients with larger doses. Changes in the zona glomerulosa were least evident. Hypertrophy, and sometimes hyperplasia, of the zona fasciculata and zona reticularis were observed.

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Armour ACTH labelled with I was administered to adult rats by Sosenberg, Keston & Money (1951), and localized by autoradiography in the 'inner zones' of the adrenal cortex only. Its rapid entrance into, and disappearance from the adrenal cortical cells was noted. Tepperman (1950) and Nichols & Little (1951) investigated the oxygen consumption by the dog adrenal. After addition of ACTH to the incubation medium the metabolism of all zones was stimulated.

An extensive histochemical investigation of adrenals from hypophysectomized rats after administration of a single injection of 0.5 mg of ACTH was conducted by Cater & Stack-

Lunne (1953). Sections were examined for RNA, lipid, plasmalogens, ketosteroids (using the phenylhydrazine reaction), cholesterol, polysaccharides, ascorbic acid, alkaline and acid phosphatase and esterase. The phenylhydrazine and plasmal reactions were found unsatisfactory in their laboratory. ACTH caused reappearance of sudanophilic material in the sudanophilic zone which resulted from pituitary removal. An increase in RNA was noted in the zona glomerulosa, and in alkaline phosphatase in the zona reticularis. Depletion of ascorbic acid seems to have occurred in all zones except in the inner reticularis, where an increase in stained material was evident following injection of the hormone. Increased staining with the periodic acid-Schiff technique occurred in the inner zona reticularis. No notable changes were observed in acid phosphatase or esterase content or in their distribution.

Deane & Seligman (1953) discussed the secretion of glucocorticoids by the zona glomerulosa and of mineralocorticoids by the zona fasciculata and reticularis, and concluded that the evidence from hypophysectomy and from ACTH administration indicated differential secretion of hormones by these zones.

ACTH was stated to have a primary effect on the zona reticularis by Yoffey (1953) who noted that depletion of material stained by the Schultz and phenylhydrazine reactions

occurred earliest in this zone following injection of the hormone in the rat. This zone was therefore considered to be active, and not a zone of degeneration as proposed by Bennett (1940). In the opinion of Bishop (1954) it is impossible to demonstrate ketosteroid hormones in situ, since secretion of these at a normal rate, unaccompanied by their formation, would lead to their depletion in adrenocortical tissues in 6 to 12 sec. This author, however, investigated sudanophilic material in the rat after ACTH administration, and found depletion in all zones. He further stated that cholesterol and 'Schiff positive' material had a distribution similar to that of lipid. Perfusion adrenal studies were stated to support the belief that cholesterol is a precursor of adrenal ketosteroids.

The effect of ACTH on the plasma and urine levels of ascorbic acid in human beings and in guinea pigs was studied by McSwiney, Clayton & Prunty (1954). An increase in plasma and urine levels of this vitamin resulted, and it was concluded that a shift of this compound occurred from cells to extracellular fluid. In guinea pigs, urinary levels of ascorbic acid remained elevated as long as ACTH administration was continued.

The dosage level of ACTH administered to hypophysectomised rats affected sudanophilia differentially in different

adrenal zones in experiments performed by Wexler & Rinfret (1955). Administration of less than one unit of Wilson or Armour ACTH per 100 g body weight per day resulted in depletion of sudanophilic material in the zona glomerulosa, whereas doses above one unit caused lipid depletion in the zona fasciculata and zona reticularis also. The results were said to support the concept that the zona glomerulosa was under pituitary control in this animal.

Alkaline phosphatase in the human adrenal and in guinea pigs after ACTH administration was studied by Yoffe, (1955) using histochemical and chemical methods. The enzyme in human adrenals was distributed in the zona reticularis and to a lesser extent in the zona fasciculata. Distribution of the enzyme was diametrically opposite to that found in the human, being concentrated in the zona glomerulosa and the outer fasciculata. Intraperitoneal injection of 5 mg ACTH was found to have no effect on adrenal alkaline phosphatase content of animals killed 1/2 to 24 hr after the injection. Studies on ascorbic acid distribution in the rat adrenal were said to indicate a zonal distribution of this compound. The highest concentration was found in the zona fasciculata; the zona reticularis and medulla contained moderate amounts, and the zona glomerulose least.

The effects of (Armour) ACTH on the human adrenal were

studied by Symington & Davidson (1956), using histochemical and chemical methods. Unilateral adrenalectomy allowed examination of a control adrenal, and removal of the second adrenal was preceded by daily injections of ACTH_{ar} gel (100 mg) for four days. Sections were examined for lipids, alkaline and acid phosphatase and RNA. Extreme diffuse lipid depletion followed ACTH administration, and compact cells rich in RNA and alkaline phosphatase extended throughout the width of the cortex, or more frequently a layer of 'clear cells' laden with lipid remained in the outer zona fasciculata. Similar results were reported by Symington, Duguid & Davidson (1956), who also stated that the 'compact cells' were rich in dehydrogenases. No variation in non-specific esterase was noted following injections of ACTH. Chemical analyses confirmed an increase in the RNA/DNA ratio, and a loss of lipid phosphorus.

An increase in mean adrenal cellular RNA following ACTH administration in the rat was described by Fiala, Sproul & Fiala (1956). These authors analysed homogenized tissue subjected to differential centrifugation for chemical analysis of this compound. They assumed, however, in common with Symington & Davidson (1956) that mean adrenal cellular DNA content is unaltered by ACTH administration. In the opinion of Fiala, Sproul & Fiala (1956) the primary effect of ACTH on the rat adrenal was on basophilic cytoplasmic granules rich in RNA.

MATERIALS AND METHODS USED IN PART II OF THE
PRESENT WORK

Animals were similar to those used in Part I of this investigation. Mature female guinea-pigs weighing 350-500g were divided into a control group of 6 animals and 12 groups of 3 animals each. The mean weight of animals from each group was similar. The guinea-pigs were fed a regular commercial diet supplemented by adequate greens. Groups of experimental animals were treated with ACTH for 3, 6, 12, and 18 hr and 1, 3, 5, 7, 10, 14, 21 and 28 days as described in the table on page 220. The first two groups (3hr and 6hr) received a single injection of 5 mg soluble ACTH per 500 g body weight and were killed after 3 hr and 6 hr respectively. The third and fourth groups (12 hr and 18 hr) received similar injections at 0 and 6 hr and 0, 6 and 18 hr respectively. The remaining groups received an injection of 5 mg ACTH ar gel (Armour) per 500 g body weight morning and evening for periods varying from 1 to 28 days, and were killed on the morning following the last injection. Animals were killed by stunning and immediate bleeding from the neck. The adrenals were removed immediately, less than four minutes having elapsed before the completion of this procedure.

The right adrenal was cut across its transverse axis

Administration of ACTH to Groups of Guinea-pigs used for Experiments described in Part II of the present work.

| Group No. | Duration of ACTH Treatment. | Form of ACTH injected. | Total No. of injections. |
|-----------|-----------------------------|------------------------|--------------------------|
| 3 hr | 3 hr | Armour Soluble | 1 |
| 6 hr | 6 hr | " " | 1 |
| 12 hr | 12 hr | " " | 2 |
| 18 hr | 18 hr | " " | 3 |
| 1 | 1 day | Armour ACTHar gel | 2 |
| 3 | 3 days | " " | 6 |
| 5 | 5 " | " " | 10 |
| 7 | 7 " | " " | 14 |
| 10 | 10 " | " " | 20 |
| 14 | 14 " | " " | 28 |
| 21 | 21 " | " " | 42 |
| 28 | 28 " | " " | 56 |

and approximately one fourth of it removed. The remainder was fixed in 12% neutralized formalin, dehydrated, and embedded in paraffin wax. Removal of one quarter of the adrenal ensured that all zones of the cortex were present in sections. Sections, 6 microns in thickness, were cut and stained in one of the following ways :- with methyl green-pyronin (m.g.p.), with m.g.p. after treatment of sections with ribonuclease, with m.g.p. after exposure of the sections to distilled water without the enzyme, with periodic acid-Schiff (P.A.S.), with P.A.S. after diastase, with P.A.S. after ribonuclease, with P.A.S. after lipid extraction and with haemalum and eosin.

The left adrenal was cut across its transverse axis and a terminal one fourth removed. A thin slice was immediately impregnated with acid silver nitrate solution for demonstration of ascorbic acid, the remainder being fixed in cold 12% neutral formalin. Frozen sections, approximately 15 microns in thickness, were cut and stained using Sudan III, the plasmal reaction, and the Ashbel-Seligman reaction (1949) for ketosteroids, control sections being treated with acetone for 30 min at room temperature before staining with these methods. Frozen sections were also used for demonstration of alkaline and acid phosphatase.

The methyl green-pyronin method used was that described by Brachet (1953). Ribonuclease was prepared by the method of Mac Donnell (1948a). Periodic acid-Schiff staining was conducted by the method of Hotchkiss (1948). The leucofuchsin solution was prepared by the Coleman (1938) modification of the de Tomasi (1936) technique. Control sections were treated with ribonuclease, with acetone or with diastase prior to celloidin coating and staining in order to minimize possible staining of RNA or acetal phosphatides. The plasmal reaction employed was that advocated by Hayes (1949). For Sudan staining the routine described by Mallory (1938) was followed.

Alkaline phosphatase was demonstrated by a simultaneous azo coupling method (Pearse 1953) using the stabilized diazonium salt of o-dianisidine ("Fast Blue B salt", I.C.I.) or of 5 chloro-o-toluidine ("Fast Red TR salt", I.C.I.). Acid phosphatase was shown by the method of Grogg & Pearse (1952b). "Fast Red RC salt" (I.C.I.) was substituted for the stabilized diazonium salt of o-dianisidine as suggested by Pearse (1953). Ascorbic acid staining was after Barnett & Bourne (1941).

Details of the techniques used are given in Appendix 1.

R E S U L T S.

Normal Guinea-pig Adrenal.

Ribonucleic Acid. RNA is present in all zones of the guinea-pig adrenal cortex. It is present in greatest concentration in the zona glomerulosa and zona reticularis. (Fig.38). The zona fasciculata contains very much less. It is present in fine granular form in the zona glomerulosa and zona reticularis but is present as a fine reticulum surrounding lipid droplets in the zona fasciculata. In the zona fasciculata fairly deep staining is found in cell membranes. 'Compact cells' rich in RNA are distributed in groups in the zona reticularis. The term 'clear cells' would not appropriately describe those in the zona fasciculata.

Lipid. Sudanophilic material is present in all zones of the normal guinea-pig adrenal (Fig. 45). In the zona glomerulosa it is present as fine droplets, evenly distributed. No sudanophobic zone is evident. The zona fasciculata contains abundant lipid droplets which vary considerably in size, and are large and medium sized. The border between the zona fasciculata and the zona reticularis is not sharp, and this area (zona intermedia) shows irregular extensions of cortical cells containing abundant lipid into the outer zona reticularis. The zona reticularis cells show fine lipid droplets and a

diffuse light pink staining with Sudan III. A few 'islands' of cortical cells are found in the medulla and these stain similarly to zona reticularis cells. No sudanophilic material is evident in capillaries or in sinusoids. Lipid droplets tend to be of approximately one size in the inner zona fasciculata, but lack of uniformity in their size is evident in the outer zona fasciculata.

Plasmalogens The distribution of these substances is very similar to that of lipid (Fig. 49). They are present in high concentration in the zona fasciculata and in much smaller amounts in the zona glomerulosa and zona reticularis. After lipid extraction almost all positively staining material is removed, but a slight diffuse staining of all cortical zones remains.

'Ketosteroids Use of the Ashbel-Seligman (1949) reaction gave unsatisfactory results in my hands. In normal cavies slight blue coloration of stained frozen sections was distributed similarly to the distribution of sudanophilic material. Staining of adrenals from the experimental animals also gave results which were unsatisfactory, and staining was hardly evident.

Phosphatases Use of diazotized o-dianisidine ("Brentamine Fast Blue B salt", I.C.I.) gave a localization of alkaline

phosphatase (Fig. 58) which differed somewhat from that resulting from use of "Brentamine Fast Red TR" (I.C.I.) (Fig. 53). Use of the former dye (abbreviation FBB) resulted in a positive reaction in all zones of the cortex. The zona glomerulosa was coloured a dense black ; cells in the outer zona fasciculata brown, and cytoplasm in the zona reticularis a light diffuse brown (Fig. 58). Nuclear staining was not evident, and an intense staining of cell membranes resulted in the outer zona fasciculata and zona glomerulosa. Blood vessel walls did not give a positive reaction. Azo coupling with "Brentamine Fast Red TR salt" (abbreviation FRTR) resulted in deepest staining in the zona glomerulosa, moderate staining in the outer zona fasciculata and slight staining in the zona reticularis (Fig. 52). Medullary cells and blood vessel walls gave a negative reaction. Nuclear staining, or staining of cell membranes, did not occur. Acid phosphatase was found in very small concentrations in sections from normal adrenals, using "Fast Red RC salt" for azo coupling.

Ascorbic Acid A positive staining was observed in all cortical zones. Silver granules were present in moderate amounts in the zona glomerulosa (Fig. 63) ; they were somewhat

more numerous in the outer zona fasciculata and generally increased in concentration as cells were located further away from the capsule. They were most numerous in the inner zona reticularis (Fig. 64). Their concentration in the medulla was similar to that found in the outer zona fasciculata. A single row of granules frequently surrounded red blood cells in medullary blood vessels, but in general no aggregations of granules occurred at nuclear or cell membranes, and these were approximately uniformly distributed throughout the cell cytoplasm.

Glycogen. Very fine granules were scanty in the zona glomerulosa and zona fasciculata. They were more numerous in the zona reticularis, especially the inner part of that zone, although still present only in small amount. No confusion was evident due to P.A.S. staining of pigments in this region.

Adrenals from Experimental Animals.

Ribonucleic Acid. At 3 hr (Fig. 39) and at 6 hr the cytoplasm of small groups of cells in the zona reticularis and inner zona fasciculata stained more deeply than in control animals.

At 12 hr and 18 hr this feature was evident generally in almost all cells in the zona reticularis.

At 1 day nearly all cells in the zona reticularis contained abundant RNA, and 'compact cells' occurred singly or in small groups in the zona fasciculata.

At 3 days all zones of the cortex except the outer zona fasciculata were rich in cytoplasmic RNA. No alteration in nuclear RNA content was evident.

At 5 days (Fig. 40) and 7 days (Fig. 41) a similar and slightly more marked appearance was found. Maximal staining of compact cells occurred in the zona reticularis especially in the inner parts of this zone (Fig. 42). Distribution of compact cells elsewhere in the cortex was focal, but almost all cells contained abundant RNA.

At 10 and 14 days the depth of staining of cytoplasm in the zona fasciculata and zone reticularis was somewhat lighter than at 5 and 7 days, and a uniform rather than a focal distribution of RNA was found.

At 21 days (Fig.43) and 28 days (Fig.44) cytoplasmic RNA was distributed approximately uniformly in the zona fasciculata and zona reticularis and was present in amounts greater than in normal adrenals. At 28 days (Fig.44) the deepest staining was found in the zona glomerulosa.

Lipid. Marked lipid depletion had occurred after 3 hr. This was most obvious in the inner zona fasciculata, but was present in all parts of this zone. Slight depletion of lipid had occurred in the zona reticularis but no obvious change had occurred in the zona glomerulosa. Lipid distribution was similar at 6 hr. At 12 hr the zona fasciculata showed moderate lipid depletion and an increase in lipid distribution in fine droplets was evident in the zona reticularis. At 18 hr medium sized lipid droplets were evident in small groups of cells in the zona reticularis (Fig.46).

The width of the cortex was obviously greater than normal, this being due mainly to an increase in width of the zona reticularis which showed little lipid depletion; what depletion there was appeared chiefly in its inner parts.

At 1 day the zona fasciculata was slightly wider than in control animals, and contained a normal amount of lipid. The zona reticularis showed an amount of lipid greater than normal, distributed mainly as fine droplets (Fig.47).

No change was evident in the zona glomerulosa.

At 3 days a marked increase in sudanophilic material had occurred in the zona reticularis. This was mainly found as diffusely distributed medium-sized droplets, although large globules were found in some cells, and a light background of diffuse cytoplasmic staining was generally evident. The zona fasciculata showed a moderate increase in lipid content.

At five and at seven days lipid distribution was essentially similar.

At 14 days a further intensification of staining was evident in the zona fasciculata.

At 21 days maximal staining was evident in the zona fasciculata and in the zona reticularis.

At 28 days obvious depletion of medium and large lipid droplets had occurred in the zona reticularis and inner zona fasciculata (Fig.48) although staining of the rest of the zona fasciculata was of approximately normal intensity.

Plasmalogens. At 3 hr the distribution of these substances (presumably acetal phosphatides) was similar to that in control animals.

At 6 and 12 hr a marked depletion was evident in the zona fasciculata especially in its outer portion. In some

small groups of cells in the zona reticularis an increase in staining intensity was evident. A variation occurred in different areas and in some sections the middle zona fasciculata showed much less positively staining material than the outer or inner zona fasciculata. Lipids reacted positively, as shown by acetone extraction, but there was a slight diffuse cytoplasmic staining in all zones which remained after this treatment.

At 18 hr (Fig.50) the distribution of lipids was similar to that found at 6 and 12 hr, but an increase in diffuse cytoplasmic staining was evident in the zona reticularis. Small groups of cells in the zona reticularis showing deep staining were found more frequently. The zona glomerulosa showed no change.

At 1 and 3 days the distribution was similar to normal, but increase in plasmalogen content of the zona reticularis was somewhat more marked.

At 5, 7, 10 and 14 days no further alteration in distribution of material was evident, but a marked variation frequently occurred in the same slide, variable widths of the zona fasciculata showing some loss of staining intensity. Plasmalogens in the zona reticularis were present in concentration greater than at 1 or 3 days.

At 21 days (Fig.51) the zona reticularis stained a fairly deep red colouration, and staining was generally uniform in this zone.

At 28 days a patchy depletion of plasmalogens was evident in the zona reticularis (Fig.52).

Phosphatases. At 3 hr a definite increase in alkaline phosphatase had resulted in the zona glomerulosa and outer zona fasciculata. This increase was more evident with F.R.T.R. salt.

At 6 hr (Fig.54) the increase was slightly more marked and a wider region of the outer zona fasciculata gave a positive staining reaction. Due to the greater intensity of staining of control sections with F.B.B. salt an increase in stained material was less obvious (Fig.59).

At 12 and 18 hr a further increase in staining was evident in the zona glomerulosa and outer zona fasciculata. Cells occurring singly and in small groups in the zona reticularis gave a positive reaction. This was found in slides stained by each of the two methods employed. Sections stained for acid phosphatase showed a very light brown diffuse staining reaction. These and the control specimens stained for acid phosphatase were not suitable for photomicrography.

At 24 hr a definite increase in staining intensity

occurred in the zona fasciculata.

At 3 days a further increase in depth of staining occurred in the zone of highest enzyme content (zona glomerulosa and outer zona fasciculata).

At 5 days (Fig.55) a further increase in depth of staining occurred in the zona fasciculata. Staining of the zona reticularis was slight.

At 7 days (Fig.56) a further increase was evident in staining of the zona reticularis.

At 10 and 14 days a marked diminution in enzyme content occurred, especially in the zona glomerulosa and the zona fasciculata. At 28 days the concentration was almost normal (Fig.57).

Acid phosphatase was found in increased concentration in all adrenocortical zones of the 3 day test animals (Fig.60). The enzyme was present in greatest amount in the zona glomerulosa, and in small amounts in the other zones.

At 7 days acid phosphatase was present in high concentration in the zona glomerulosa (Fig.61). Depth of staining in the other zones was similar to that found at 3 days.

At 14 days acid phosphatase distribution and concentration (Fig.62) were similar to that found in the 3 day test animals.

At 21 and 28 days concentration had diminished and was similar to normal. Staining intensity was very light, and sections were not suitable for photomicrography.

'Ketosteroids'. Extremely light staining of sections resulted from use of the Ashbel-Seligman (1949) technique. Results were disappointing and stained sections were unsuitable for photomicrography.

Ascorbic Acid. At 3 hr (Figs. 65, 66) a marked uniform depletion of ascorbic acid had occurred in all zones of the cortex. Staining in the medulla was similar to that found in normal adrenals. Very few scattered granules remained, however, in the cytoplasm of cells in the zona reticularis.

At 6, 12 and 18 hr almost uniform depletion of ascorbic acid granules was still evident. Few granules were evident in medullary cells. A slight increase in ascorbic acid content was evident at 18 hr. A granular precipitate was evident in the medullary vein in some sections.

At 1 day similar observations were made. The medullary cells showed a greater than normal content of silver granules.

At 3 days (Figs. 67, 68) ascorbic acid distribution and content was slightly subnormal in the zona glomerulosa and zona fasciculata. The zona reticularis contained a normal content of reduced silver granules. Medullary content of stained material was similar to that found in control animals.

At 5, 7, 10, 14, 21 and 28 days adrenal ascorbic acid content and distribution were similar to that found in control guinea-pigs.

Glycogen. At 3, 6 and 12 hr the distribution of this polysaccharide was similar to that observed in normal guinea-pigs. At 18 and 24 hr moderate depletion of granules had occurred in all zones. This depletion was focal and most evident in the zona reticularis. At 3, 5, 7, 10, 14, 21, and 28 days glycogen content and distribution was similar to normal.

DISCUSSION.

The distribution of RNA in the adrenal cortex of normal guinea-pigs is similar to that of the 'Corps Siderophiles' observed by Guieysse (1901), Kolmer (1912, 1918) and Takechi (1925), and the 'Chromidia' described by Selye & Stone (1950) in this organ. It is also similar to the distribution of RNA described in the human adrenal by Symington & Davidson (1956), Symington, Duguid & Davidson (1956) and Symington, Currie, Curran & Davidson (1955). Some stainable RNA was, however, always found in cells rich in lipid, and it is considered that the term 'clear cells' employed by these authors to describe these cells is not entirely suitable for lipid-laden cells in the guinea-pig adrenal cortex.

No direct evidence is available from my work that the 'Corps Siderophiles' or the 'Chromidia' of the guinea-pig adrenal contain a high concentration of RNA. It is probable, however, that these cellular constituents are similar to those described in the human adrenal by Santee (1936), and it has been shown by Rich & Berthrong (1949) that the cytoplasmic basophilia in this organ is due to RNA. Cytoplasmic basophilia in various types of cells has been shown to be due mainly to RNA by a number of authors (Dustin, 1947; White 1947;

Taft, 1951; Brachet, 1953; Palade & Porter, 1954; Porter, 1954; Fiala, Sproul & Fiala, 1956). The most conclusive evidence seems to have been put forward by Fiala, Sproul, Blutinger & Fiala (1955) who demonstrated a reversible stainability of basophilic chromidia (separated by differential centrifugation of homogenized tissue) with Pyronin Y by pre-treatment with ribonuclease. Purification of ribonuclease by Dubos & Thompson (1938), Kunitz (1940), and more satisfactorily by McDonald (1948a) facilitated the demonstration of a high concentration of RNA in the 'basophilic component' of cytoplasm. A slight residual staining of cytoplasm with Pyronin Y, after enzymatic removal of RNA, was observed by me and this finding is in agreement with the observation of Brachet (1953) that the specificity of the Methyl-Green Pyronin method for RNA depends on the purity of the enzyme preparation.

My finding that there is an increase in RNA in the adrenal cortex of guinea-pigs, treated with ACTH, is in keeping with a similar finding for the adrenals of patients who died following the stress of disease (Rich & Berthrong, 1949; Symington & Davidson, 1956; Symington, Duguid & Davidson, 1956). It is also in keeping with an increased RNA content of the rat adrenal following ACTH administration found by Fiala, Sproul

Miala (1956), using chemical methods. An increase in adrenal RNA in the zona glomerulosa of ACTH treated-hypophysectomized rats was observed by Cater & Stack-Dunne (1953). Their results and my own suggest that adrenal RNA plays a direct or an indirect role in the biosynthesis of steroids.

The distribution of lipid in normal guinea-pig adrenals, as shown by staining with Sudan III, is similar to that described by Hoerr (1937) using Sudan black. I feel that Sudan III was more suitable for the experiments I undertook since marked variations in lipid concentration after ACTH administration could be observed more easily using Sudan III. Lipid distribution in the different zones of the guinea-pig adrenal is also similar to that reported by Whitehead (1934) using Sudan III staining. It is in general similar to that described for the rat adrenal by Tobin & Whitehead (1942), by Sarason (1943), by Harrison & Cain (1947), by Selye & Stone (1950) and by Symington (1951). A similar distribution has been described for the human adrenal by Zamcheck (1951), by Stoner, Whitely & Emery (1953), and by Symington & Davidson (1956).

In view of the findings of Kaufman & Lehman (1926), Dempsey (1948), Sayers (1950) and Pearse (1953), the various types of lipid were probably not distinguished by staining

with Sudan III. Since, however, sudanophilia gives an indication of the functional state of the adrenal cortex (Sarason, 1943; Selye, 1946, 1950; Dempsey, 1948; Selye & Stone, 1950; Sayers, 1950; Symington & Davidson, 1956), staining for lipid in general was considered to be of primary importance.

The finding that most substances which gave a positive plasmal reaction were removable by a lipid solvent is in agreement with a similar finding of Feulgen & Voit (1924). It is evident from the results of Feulgen & Bersin (1939) that acetal phosphatides are responsible for a positive staining reaction. The view of Cain (1949a) that substances other than acetal phosphatides react with Schiff's reagent in the course of staining with this technique is counter to that of Hayes (1949). Hayes considers that the plasmal reaction, as described by him, demonstrates the acetal phosphatides only. Chemical data may be expected to give the most reliable results concerning this problem, and results from the chemical analyses of Anchel & Waelsh (1942) indicate that plasmals from some beef and rat tissues consist mainly of stearaldehyde and palmitaldehyde.

Use of the plasmal reaction, in conjunction with other cytological techniques, for the cytochemical demonstration

of ketosteroids by Dempsey & Wislocki (1944), Wislocki & Wimsatt (1947) and Bergner & Deane (1948) was justifiably criticized by Boscott & Mandl (1949). Use of the hydrazide reaction of Bennett (1939,1940) to show ketosteroids did not receive support from the work of Gomori (1942), of Albert & Leblond (1946), of Gomori (1950) and of Wolman & Greco (1952). The use of this technique, or of that later developed by Ashbel & Seligman (1949), met with criticism from Deane & Seligman (1953) and Yoffey (1953). If the view of Bishop (1954) is correct, i.e. that depletion of adrenocortical ketosteroids can occur in 6 to 12 sec then there is little prospect of a reliable histochemical method for the demonstration of these compounds in the adrenal. The lack of success in the Glasgow laboratory with the Ashbel-Seligman (1949) reaction may conceivably have been due to a normal rapid secretion of these substances.

The adrenal lipid depletion observed in acute experiments (3, 6, 12 and 18 hr) is similar to that found by Selye (1946) to accompany the general adaptation syndrome caused by stresses of various kinds. It is also comparable to a similar finding of Sarason (1948) for the rat adrenal in a variety of experimental conditions, of Symington (1951) in adrenaline stress in the rat, of Stoner, Whitely & Emery (1953) in children dying after

acute disease, and of Symington & Davidson (1956) and Symington, Duguid & Davidson (1956) for adrenals of adult patients dying after various pathological stresses. The lipid and plasmalogen depletion observed is similar to that found by Bergner & Deane (1948) for the rat adrenal after ACTH administration. In their experiments depletion was maximal at 18 hr whereas in my series a maximal loss of staining material was evident at 3 hr. A maximal depletion of lipids and plasmalogens in the rat adrenal after ACTH administration was observed at 1 hr by Yoffey & Baxter (1953).

Sudanophilic material and plasmalogens were unaffected in the zona glomerulosa of the rat adrenal after ACTH administration in the experiments described by Bergner & Deane (1948). My findings in the guinea-pig adrenal were similar. All zones of the human adrenals studied by O'Donnell, Fajans & Weinbaum (1951) showed depletion of lipid after ACTH treatment. The zona glomerulosa, however, was least affected. In the experience of these authors, ACTH-induced lipid depletion was more marked following prolonged administration of ACTH. This finding is in marked contrast to those of Bergner & Deane (1948) and of Yoffey & Baxter (1953) for the rat, and to my own findings in the guinea-pig. These findings for intact animals cannot be compared directly with those of Cater & Stack-Dunne (1953) who

studied the effect of a single injection of ACTH on adrenals of hypophysectomized rats, though the reappearance of lipid in the sudanophobe zone of these hypophysectomized rats indicates a pituitary-adrenal relationship affecting the outer zona fasciculata, a finding similar to my own for the guinea-pig.

The occurrence of lipid depletion in all adrenocortical zones following ACTH administration was noted by O'Donnell, Fajans & Weinbaum (1951), by Symington & Davidson (1956) and by Symington, Duguid & Davidson (1956) in the human adrenal, and by Bishop (1954) and Wexler & Rinfret (1955) in the rat adrenal. Wexler & Rinfret (1955), however, noted lipid depletion in the zona glomerulosa only of adrenals from animals which received less than one unit of ACTH per day. Selye & Stone (1950) did not find an appreciable lipid depletion in the adrenal of the rat after administration of ACTH, a finding which may possibly be attributable to the duration of the experiments (2 days).

The increase in guinea-pig adrenal lipid and plasmalogen content after prolonged ACTH administration resembles the increase found during the phase of resistance of the General Adaptation Syndrome (Selye, 1946; Selye & Stone, 1950). This increase is also seen following intraocular implantation of anterior pituitary tissue in hypophysectomized female

guinea-pigs (Schweizer & Long, 1950), after administration of ACTH to hypophysectomized rats (Cater & Stack-Dunne, 1953), following administration of ACTH for 8 days to rats (Bergner & Deane, 1948), during treatment of rats with ACTH (Yoffey & Baxter, 1949), and accompanying ACTH administration to hypophysectomized mice (Chester Jones, 1949). This increased lipid deposition is in marked contrast to the continued lipid depletion observed in human adrenals by O'Donnell, Fajans & Weinbaum (1951) and by Symington, Duguid & Davidson (1956). The lipid depletion observed by Symington, Duguid & Davidson (1956) was accompanied by an increase in the level of blood corticoids, and was presumably associated with an increase in tissue utilization of steroids.

The similarity in the distribution of sudanophilic material and plasmalogens which I observed is comparable to the findings of Bergner & Deane (1948), of Yoffey (1949) and of Chester Jones (1949, 1950). In the experience of Selye & Stone (1950), however, the distribution of these materials does not always coincide.

Azo coupling methods were used in preference to the older techniques (Gomori 1939, 1941, 1946) for the demonstration of alkaline and acid phosphatases in view of the findings of Gomori (1949, 1950, 1951), Danielli (1950), Palade (1951) and Novikoff (1952), which indicate that accurate localization of these enzymes could not be achieved by means of the older methods. The absence of nuclear staining, which I found

when using azo coupling methods, is in keeping with the findings of Gomori (1951). Let us hope it indicates that fewer artifacts occur with the newer techniques.

Alkaline phosphatase distribution in the normal guinea-pig adrenal is in general similar to that described by Yoffey (1955) using the original technique proposed by Gomori (1939). This distribution is diametrically opposite to the distribution found in the normal human adrenal by Yoffey (1955) using the older method, and by Symington & Davidson (1956), and Symington, Currie, Duguid & Davidson (1955) who used an azo coupling method. Acid phosphatase was found to have a similar distribution to alkaline phosphatase. Staining intensity of the acid enzyme was, however, very light in the adrenals of the control animals.

The increase in alkaline and acid phosphatase activity observed by me is comparable to the increase found in the human adrenal by Symington, Duguid & Davidson (1956). It is in contrast to the findings of Yoffey (1955) for the guinea-pig. The early increase in enzyme content of the zona reticularis after injection of the hormone is a finding similar to that reported by Cater & Stack-Dunne (1953) for the ACTH treated hypophysectomized rat. The maximum increase in alkaline and acid phosphatase in my series (after 5 and 7 days ACTH administration) occurred when hyperplasia was most marked. This finding is consistent with the widely held view that phosphatase is concerned in protein synthesis. Alkaline phosphatase was,

however, present in increased amount in the adrenals of the 3 hr test animals when hyperplasia was not evident: it is possible that alkaline phosphatase plays a part in the synthesis or secretion of biologically active steroids. The increase in adrenal alkaline phosphatase was accompanied by an increase in RNA in the zona glomerulosa and zona fasciculata. In the first four groups, where the animals were given ACTH for 3, 6, 12 and 18 hr, an increase in RNA in the zona reticularis was evident but the histological methods did not show an increase in phosphatase content.

Although the specificity of the acid silver nitrate technique for ascorbic acid (Bourne, 1935; Barnett & Bourne, 1941) is "generally accepted" (Bourne 1955), agreement concerning the precise localization of ascorbic acid by means of this technique has not been reached. According to Barnett & Bourne (1941), moreover, melanin will reduce acid silver nitrate. The tendency of silver granules to aggregate at interfaces (Barnett & Fischer, 1943), and the finding of Hagen (1954) that ascorbic acid was confined mainly to the non-particulate fraction of homogenized ox adrenal are the main criticisms of the technique. The experiments of Barnett & Fischer (1943) were conducted under conditions which are quite different from those under which the technique is normally practised; the findings of Hagen (1954) may have been due to removal,

by homogenization and centrifugation, of ascorbic acid from granules which exist in vivo (Bourne, 1955). The occurrence of reduced silver granules in adrenal blood vessels, noted by Yoffey (1955) and of aggregations of similar granules around erythrocytes in my own preparations, suggests that the present technique is inadequate insofar as localization is concerned. Studies aimed at the estimation of acid silver nitrate diffusion rates into adrenal slices and perfusion impregnation of whole adrenals should help to decide if ascorbic acid has diffused from its normal site prior to silver impregnation. Melanin will induce precipitation of metallic silver when this technique is used, and may lead to some confusion (Barnett & Bourne, 1943).

The distribution of ascorbic acid in my preparations of the guinea-pig adrenal is diametrically opposite to that found in beef adrenal by Glick & Biskind (1935, 1936) who used a microchemical technique. The results of these authors and my own results are, however, not strictly comparable. The variation observed by Bourne (1955) in ascorbic acid content in neighbouring guinea-pig adrenal cells is in contrast to the uniformity I found in ascorbic acid distribution in adjacent cells. The results of Yoffey (1955) for the rat adrenal differ from mine in the guinea-pig. Yoffey (1955) found the highest concentration of ascorbic acid in the zona fasciculata.

The initial depletion of ascorbic acid which I observed

in acute experiments agrees with the findings of Sayers, Sayers, Liang & Long (1946). They worked with guinea-pigs and used chemical methods. Depletion of ascorbic acid also occurred in ACTH-treated hypophysectomized rats (Cater & Stack-Dunne, 1953). My findings are consistent with the elevated urinary ascorbic acid levels observed by McSwiney, Clayton & Prunty (1954) during ACTH administration to guinea-pigs.

Distribution of polysaccharides in normal guinea-pig adrenals is similar to that described in the rat adrenal by Cater & Stack-Dunne (1953). The depletion observed in my short-term experiments is consistent with the probable role of these substances in the synthesis of steroids in the adrenal (Brownie & Grant 1954).

SUMMARY AND CONCLUSIONS.

1. Mature female guinea-pigs weighing 350-500 g were treated with ACTH (Armour) for 3, 6, 12 and 18 hr, and 1, 3, 5, 7, 10, 14, 21 and 28 days, and the effects on the histochemistry of the adrenal were noted. A group of 6 animals acted as controls.
2. Sections from the adrenals of these animals were stained for ribonucleic acid, lipid, plasmalogens, ketosteroids, alkaline and acid phosphatases, ascorbic acid and glycogen.
3. Depletion of lipid and of plasmalogens occurred in the zona fasciculata and zona reticularis at 3, 6, 12 and 18 hr. In the other experimental groups of animals an increase in lipid and plasmalogens occurred with continued ACTH administration. At 28 days, however, some depletion of these substances was evident in the zona reticularis.
4. A gradual increase in adrenocortical RNA occurred with ACTH administration. This increase was found initially (3 hr) in the zona reticularis, later in the zona fasciculata and zona glomerulosa also.
5. The Ashbel-Seligman (1949) technique for ketosteroids gave unsatisfactory results in this laboratory.

6. Alkaline phosphatase was found to be increased in the zona glomerulosa and zona fasciculata, and later in the zona reticularis in the adrenals of ACTH treated cavies. The enzyme was present in greatest amount in the 5 and 7 day test animals, when hyperplasia was at a maximum. Acid phosphatase was also present in greatest amount in the adrenals of the animals which received injections of ACTH for 5 and for 7 days.

7. Ascorbic acid depletion was observed after ACTH administration for 3, 6, 12 and 18 hr and 1 day. The adrenal content and distribution of ascorbic acid was apparently normal in the guinea-pigs receiving ACTH for longer periods.

8. Glycogen depletion occurred at 12 and 18 hr only. Its distribution in all other animals was essentially the same as normal, maximal concentration being found in the zona reticularis.

9. The findings are discussed and compared with results of other authors who investigated the pituitary-adrenal relationship.

10. It is evident that RNA, alkaline and acid phosphatase and ascorbic acid have important roles in adrenocortical physiology. The results suggest that RNA and alkaline and

acid phosphatases are probably concerned with adrenocortical hyperplasia. It seems more likely, however, that ascorbic acid and glycogen are concerned with secretion of adrenal hormones, as depletion of these substances occurred when secretion of ketosteroids was probably at a maximum.

APPENDIX I STAINING METHODS.

The Feulgen Reaction (Feulgen & Rossenbeck, 1924, Modified)

Schiff's Reagent (de Tomasi, 1936)

Dissolve 1 g of basic fuchsin in 200 ml of boiling dist. water. Shake for 5 min and cool to exactly 50°. Filter and add to the filtrate 20 ml of N HCL. Cool to 25°, and add 1 g of potassium metabisulphite. Stand this soln. in the dark for 14 to 24 hr. Add 2 g of activated charcoal and shake for 1 min. Filter. Keep the filtrate in the dark at 0-4°. Allow to reach room temperature before use.

Method

Bring sections to water and remove mercury if necessary. Rinse briefly in cold N HCL. Place in N HCL at 60° for the optimum time of hydrolysis. Rinse briefly in cold N HCL and then in dist. water. Transfer to Schiff's soln. for the optimum time (1/2 to 1 hr). Drain and rinse in three changes of freshly prepared bisulphite soln. (5 ml 10% $K_2S_2O_5$, 5 ml N HCL, water to 100 ml). Rinse in water. Dehydrate in alcohols. Clear in Xylene and Mount in DePeX.

Results :- DNA appears in shades of reddish purple.

Methyl Green-Pyronin Method (Erachet, 1953, Modified)

Reagents

Solution A

| | |
|-------------------------|---------|
| 5% aqueous pyronin | 17.5 ml |
| 2% aqueous methyl green | 10 ml |
| Dist. water | 250 ml |

Solution B

| | |
|--------------------------|--------|
| M/5 acetate buffer pH4.8 | 200 ml |
| Dist. water | 30 ml |

For use :- Equal parts A and B. This will keep about 2 weeks.

Methyl Green-Pyronin Method Ctd.

Method

Bring sections to water.

Stain in working soln. 10 min to 24 hr (usually 30 min).

Rinse in dist. water 7 sec.

Blot dry.

Dehydrate rapidly with acetone, acetone and xylol, and xylol.

Mount in DePeX.

N.B. Methyl green soln. is repeatedly treated with chloroform to remove methyl violet (2-4 hr).

Pyronin solution (0.3%) is made up in acetate buffers pH 3.6, 4.8 and 5.2. Equal volumes of 0.1% orange G are added to each tube. There should be no ppt. at pH 4.8 or 5.2, and only a slight ppt. at pH 3.6. This indicates that a satisfactory batch of pyronin is being used.

Acetate buffer : A pH 4.8 buffer may be prepared from 2 parts M/5 acetic acid and 2 parts M/5 sodium acetate. This is then diluted with an equal volume of dist. water.

Sudan III

Fix in 12% neutral formalin. Cut frozen sections 15 μ thick.

Dip for an instant in 70% alcohol.

Stain in Sudan soln. (sat. soln. in 70% alc.) 30 min.

Wash in water.

Mount in glycerin.

Result :- Fat orange to red.

Plasmal Reaction (Hayes, 1949)

Fix in 12% neutral formalin. Cut frozen sections 15 μ .

Wash in several changes of dist. water.

Place one section in 1% HgCl₂ for 2-10 min.

Place this section and an untreated control section in Schiff's reagent for 5-15 min.

Wash in three changes of bisulphite water (5 ml 10% K₂S₂O₅, 5 ml N HCL, 100 ml dist. water), 2 min in each change.

Wash in water. Mount in glycerin.

Result :- Acetal lipids, reddish purple.

Method for Ketonic Steroids (Ashbel & Seligman, 1949)

Reagents :-

2-Hydroxy-3-naphthoic acid hydrazide (0.1%).

The hydrazide (1 g) is dissolved in 50 ml hot glacial acetic acid in a volumetric flask. To this is added 950 ml of 50% alcohol, prepared just before use from absolute ethyl alcohol and dist. water. In order to prevent formation of aldehydes, sealed bottles of absolute alcohol are stored in the refrigerator.

The reagent may be stored at room temperature for 2 weeks.

Alcohol buffer solution.

Phosphate buffer (pH 7.2 -7.5, 1/15 M) is mixed with an equal volume of absolute ethyl alcohol just before use.

Tetrazotized diorthoanisidine.

The powder (50 mg) is stirred into the alcohol buffer at the time of coupling, without prior solution in water, because the diazonium compound decomposes rapidly in pure aqueous soln, (and darkens).

Method :-

Wash formalin from frozen sections in several changes of cool water for several hr. Sections may be first mounted on slides and allowed to dry for 10 min.

Incubate sections in hydrazide soln. at room temp. for 2 hr.

50 ml of reagent may be used for 10 to 20 sections only.

Wash in several changes of 50% alcohol for 2 hr.

Incubate in 0.5 N HCL at room temp. for 1/2 hr.

Wash in dist. water.

Place in alcohol buffer soln.. Add tetrazotised diorthoanisidine powder (50 mg) and stir. Stain for 2 min.

Wash in several changes of dist. water.

Rinse in 50% alcohol, followed by dist. water, in order to dislodge gas bubbles.

Mount in glycerin.

Result :- Ketonic steroids, bluish purple.

Silver Method for Ascorbic Acid (Bourne, 1933, Barnett & Bourne 1941).

Treat thin slices of tissue with acid silver soln. (5 ml glacial acetic acid to 100 ml 5% aqueous silver nitrate) 5-10 min. Treat in the dark for 10 to 15 min with 5% ammonia soln. Wash in dist. water. Mount in glycerin.

Result :- Black silver granules indicate the presence of reduced ascorbic acid.

A Modified Coupling Azo Dye Method for Alkaline Phosphatase (Pearse, 1953)

Fix thin slices of tissue in 10% neutral formalin at 4° for 10 to 16 hr. Cut frozen sections 15 μ thick, and mount on clean slides without adhesive. Allow to dry in air for 1-3 hr to ensure adherence. Dissolve 10-20 mg sodium α -naphthyl phosphate in 20 ml of 0.1 M veronal acetate buffer (pH 9.2). Add 20 mg of the stable diazotate of o-dianisidine (Fast Blue B salt, I.C.I., Ltd.) or of 5-chloro-c-toluidine (Fast Red TR salt, I.C.I., Ltd.), and stir well. Filter on to the slides sufficient to cover each section adequately and incubate at room temp. (17-22°) for 15-60 min. Wash in running water for 1-3 min. Mount in glycerin Jelly.

Results :- The sites of alkaline phosphatase activity are coloured black with Fast Blue B salt, or brick red with Fast Red TR salt.

A Modified Coupling Azo Dye Technique for Acid Phosphatase (Grogg & Pearse, 1952)

Fix thin (2-4 mm) slices of tissue in 10% neutral formalin, at 4° for 10-16 hr. Cut frozen sections 15 μ thick and mount them on slides, without adhesive, drying for 2-3 hr at room temp. to ensure adherence. Immerse the sections at room temp. in the following mixture :- Dissolve 10-20 mg of sodium α -naphthyl phosphate in 20 ml of 0.1 M veronal acetate buffer at pH 5.0. Add approximately 20 mg of Fast Red RC salt (I.C.I., Ltd.). Shake well and filter the mixture on to the dry sections. Incubate for 30 min at room temp. Mount in glycerine jelly.

The Periodic Acid-Schiff Technique (Hotchkiss, 1948)

Reagents :- Schiff's reagent as for the Feulgen Reaction.

Periodic Acid. Dissolve 0.4 g of periodic acid ($\text{HIO}_4 \cdot 2\text{H}_2\text{O}$) in 35 ml of reagent ethyl alcohol and add 5 ml of M/5 sodium acetate (27.2 g of the hydrated salt in 1,000 ml dist. water). This soln. should be kept in the dark at 17-22° and used at this temp. It should be discarded if a brown colour appears.

Reducing Bath. Dissolve 1 g potassium iodide and 1 g sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) in 30 ml of reagent ethyl alcohol and 20 ml of dist. water. Add 0.5 ml of 2 N HCL (20% conc. HCL). A deposit of sulphur forms which can be ignored. Keep between 17 and 22°. The soln. lasts for about 14 days, not longer.

Method :- Celloidin coated sections are employed. When control sections are treated with diastase or other substance, the celloidin is applied after this treatment.

Bring sections to water and remove mercury if necessary.
Rinse in 70% alcohol.

Immerse in periodic acid soln. for 5 min.

Rinse in 70% alcohol.

Immerse in the reducing bath for 1 min.

Rinse in 70% alcohol

Immerse in Schiff's soln. for 20 min.

Wash in running water for 30 min.

Dehydrate in alcohols, clear in xylene, mount in DePeX.

Result :- Glycogen stains a particularly vivid red colour, distinct from the colour given by granules of other polysaccharide containing substances.

Haemalum and Eosin

Bring sections to water.

Stain in Haemalum soln. 3-4 min.

Wash in running water 5 min.

Stain in (1% alcoholic) Eosin soln. 1 min.

Dehydrate in alcohols, clear in xylene, mount in DePeX.

T A B L E 1

| TISSUE | DAYS ACTH | No. of NUCLEI | AVERAGE EXTINCTION | AVERAGE AREA | ARBITRARY UNITS DNA |
|-----------------------------------|--------------|------------------|-----------------------|-----------------|------------------------|
| Control Kidney Control Adrenal | 0 0 | 46 40 | 1.71 1.75 | 8.81 10.0 | 15.5 14.6 |
| Control Kidney Test Kidney | 0 1 | 44 43 | 1.72 1.64 | 8.72 9.77 | 14.5 15.4 |
| Control Kidney Test Adrenal | 0 1 | 46 44 | 2.17 1.95 | 8.9 10.68 | 18.4 20.3 |
| Control Kidney Control Kidney | 0 0 | 52 52 | 1.63 1.79 | 9.19 8.75 | 14.9 15.4 |
| Control Kidney Test Kidney | 0 3 | 48 54 | 1.79 1.95 | 9.20 10.3 | 18.4 20.3 |
| Control Kidney Test Adrenal | 0 3 | 50 50 | 1.61 1.48 | 9.49 10.90 | 15.2 15.6 |

T A B L E 1 (continued)

| TISSUE | DAYS ACTH | No. of NUCLEI | AVERAGE EXTINCTION | AVERAGE AREA | ARBITRARY UNITS DNA |
|----------------|--------------|------------------|-----------------------|-----------------|------------------------|
| Control Kidney | 0 | 49 | 1.67 | 8.10 | 13.3 |
| Test Kidney | 5 | 49 | 1.34 | 8.50 | 11.8 |
| Control Kidney | 0 | 48 | 1.62 | 9.31 | 14.6 |
| Test Adrenal | 5 | 50 | 1.62 | 11.52 | 17.9 |
| Control Kidney | 0 | 47 | 1.47 | 9.71 | 14.1 |
| Test Kidney | 7 | 49 | 1.50 | 8.80 | 13.0 |
| Control Kidney | 0 | 50 | 1.92 | 9.04 | 16.8 |
| Test Adrenal | 7 | 33 | 1.65 | 13.6 | 21.4 |

Table 1. Mean values obtained by cytophotometry for the relative DNA content in arbitrary units of kidney and adrenal nuclei from guinea-pigs treated with ACTH for varying periods of time. The difference between values for 3 day test kidney and corresponding control kidney is statistically significant at the 5% level ($0.05 > P > 0.01$). The differences between the values for 5 and 7 day test adrenals and those for the corresponding control kidneys are highly statistically significant ($P < 0.01$). No other difference between a principal and its corresponding control is statistically significant, using a simple t test. Nuclei were isolated in a citric acid medium.

Table 2. Mean values obtained by cytophotometry for the relative DNA content in arbitrary units per nucleus of the kidneys and adrenals from guinea-pigs treated with ACTH for varying periods of time. The lowest two values shown for test kidney and test adrenal (*) are for test tissues from animals which received a high dosage of ACTH (50 mg per 500 g body weight per day).

The differences between the 10 day test kidney value and that for the corresponding control kidneys is statistically significant at the 5% level (0.05 P 0.01). No other difference between a principal and corresponding control is statistically significant using a simple t test, as indicated by an analysis of variance. Nuclei were isolated in a sucrose-calcium chloride medium.

| TISSUE | DAYS ACTH | No. of NUCLEI | AVERAGE EXTINCTION | AVERAGE AREA | ARBITRARY UNITS DNA |
|-----------------|--------------|------------------|-----------------------|-----------------|------------------------|
| Control Kidney | 0 | 47 | 1.67 | 5.45 | 7.4 |
| Test Kidney | 3 | 51 | 1.24 | 5.90 | 7.2 |
| Control Kidney | 0 | 42 | 1.27 | 7.18 | 7.9 |
| Test Adrenal | 3 | 51 | 1.09 | 6.98 | 7.5 |
| Control Kidney | 0 | 45 | 1.37 | 5.46 | 7.2 |
| Test Kidney | 10 | 53 | 1.43 | 5.76 | 8.0 |
| Control Kidney | 0 | 52 | 1.77 | 5.17 | 7.7 |
| Test Adrenal | 10 | 55 | 0.87 | 9.22 | 7.7 |
| Control Kidney | 0 | 42 | 1.65 | 6.08 | 7.8 |
| Test Kidney | 14 | 35 | 1.01 | 7.87 | 7.6 |
| Control Kidney | 0 | 47 | 2.11 | 4.22 | 7.0 |
| Test Adrenal | 14 | 47 | 1.11 | 7.09 | 7.8 |
| Control Kidney | 0 | 38 | 1.21 | 7.78 | 7.7 |
| Test Kidney | 21 | 46 | 0.99 | 8.17 | 7.9 |
| Control Kidney | 0 | 34 | 1.33 | 6.90 | 8.9 |
| Test Adrenal | 21 | 45 | 0.81 | 10.86 | 8.3 |
| Control Kidney | 0 | 51 | 3.44 | 2.37 | 7.0 |
| Control Adrenal | 0 | 48 | 3.00 | 2.58 | 7.3 |
| Control Kidney | 0 | 49 | 3.55 | 2.25 | 6.1 |
| Test Kidney* | 3 | 52 | 3.77 | 2.21 | 5.9 |
| Control Kidney | 0 | 50 | 4.01 | 2.31 | 8.4 |
| Test Adrenal* | 3 | 46 | 2.18 | 3.29 | 7.8 |

TABLE 3

Mean Values obtained by chemical analysis for the absolute DNA content per nucleus of the Kidney and Adrenal Gland of guinea-pigs after treatment with ACTH for varying periods.

| DAYS OF ACTH ADMINISTRATION | DNA per NUCLEUS (pg.) | | | |
|--------------------------------|--------------------------|---|--------------------------|---|
| | KIDNEY | | ADRENAL | |
| | CITRIC ACID ISOLATION | SUCROSE CaCl ₂ ISOLATION | CITRIC ACID ISOLATION | SUCROSE CaCl ₂ ISOLATION |
| .0 | 6.5 | 5.9 | 7.2 | 7.7 |
| 1 | 6.7 | | 7.6 | |
| 3 | | 6.4 | | 7.6 |
| 5 | 6.4 | | 7.2 | |
| 7 | 7.0 | | 7.6 | |
| 10 | | 6.2 | | 7.4 |
| 14 | | 6.5 | | 7.4 |
| 21 | | 6.1 | | 7.7 |

T A B L E 4.

An Analysis of Variance of results obtained by cytophotometry of Feulgen-stained nuclei isolated by the Citric Acid method.

| SOURCE OF VARIANCE | Degrees of Freedom | Sum of Squares | Mean Square | F (Variation Ratio) |
|---------------------------------|--------------------|----------------|-------------|---------------------|
| Between Slides | 9 | 2,792.42 | 310.27 | 3.422 |
| Between Principals and Controls | 1 | 293.58 | 293.58 | 3.238 |
| Interaction | 9 | 815.97 | 90.663 | 8.054 |
| Error | 580 | 6,528.64 | 11.256 | 0.6125 |
| TOTAL | 599 | 10,430.61 | | |

T A B L E 5.

An Analysis of Variance of results obtained by cytophotometry of Feulgen-stained nuclei isolated by sucrose-calcium chloride Method.

| SOURCE OF VARIANCE | Degrees of Freedom | Sum of Squares | Mean Square | F (Variation Ratio) |
|---------------------------------|--------------------|----------------|-------------|---------------------|
| Between Slides | 7 | 59.94 | 7.85 | 1.402 |
| Between Principals and Controls | 1 | 1.02 | 1.02 | |
| Interaction | 7 | 39.20 | 5.60 | 3.279 |
| Error | 464 | 792.35 | 1.7076 | 0.2376 |
| TOTAL | 479 | 897.51 | | |

T A B L E 6.

Ratios between the average adrenal nuclear Extinction values in the kidneys of the experimental cavies and the average Extinction values for the corresponding control kidneys ($\frac{E.Adr.}{E.Kid.}$).

The duration of administration of ACTH is indicated.

Similar ratios for the mean projected areas ($\frac{A.Adr.}{A.Kid.}$).

| DAYS OF ACTH ADMINISTRATION | $\frac{E.Adr.}{E.Kid.}$ | $\frac{A.Adr.}{A.Kid.}$ |
|-----------------------------|-------------------------|-------------------------|
| 0 | 1.02 | 1.14 |
| 1 | 0.90 | 1.20 |
| 3 | 0.92 | 1.14 |
| 5 | 1.00 | 1.24 |
| 7 | 0.86 | 1.50 |
| 10 | 0.49 | 1.78 |
| 14 | 0.53 | 1.68 |
| 21 | 0.61 | 1.57 |

Fig. 1. Photograph of high speed recording microdensitometer used in the experiments described in Part I of this thesis. It was designed by P.M.B. Walker, Medical Research Council Biophysics Research Unit, King's College, London. The instrument was the first commercially made by Messrs. Joyce, Loebel & Co. Ltd., Newcastle-upon-Tyne, England. The front of the instrument is to the left in the photograph. The two upper controls on the front of the instrument are centring adjustments for the condenser, and above these is the condenser focussing adjustment. The switches below these controls are (from left to right) for mains supply, servomotor reverse and light supply. The lower controls on the front of the instrument are coarse and fine sensitivity adjustments. The specimen table is to the left and paper table, which carries the graph paper, to the right of the centre of the photograph. Control knobs on the right of the instrument allow control of direction and speed of movement of the paper table.

Fig. 1.

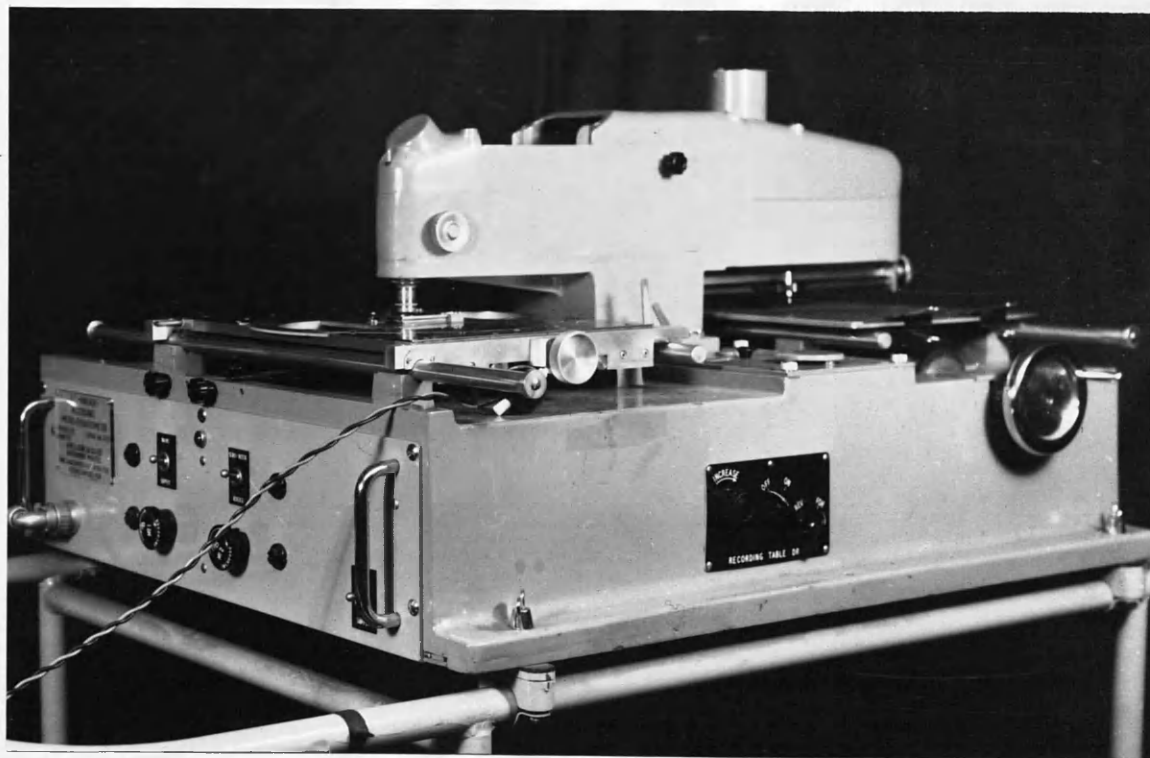


Fig. 2. Photomicrograph of adrenal nuclei isolated in sucrose-calcium chloride medium, fixed in acetic alcohol, and stained by the Feulgen method for DNA (X 1,000).

Fig. 3. Photograph of rotating step-wedge sector. Exposure times, from below upwards were 1/64, 1/32, 1/16, 1/8, 1/4, 1/2 and 1 second.

Fig. 2.

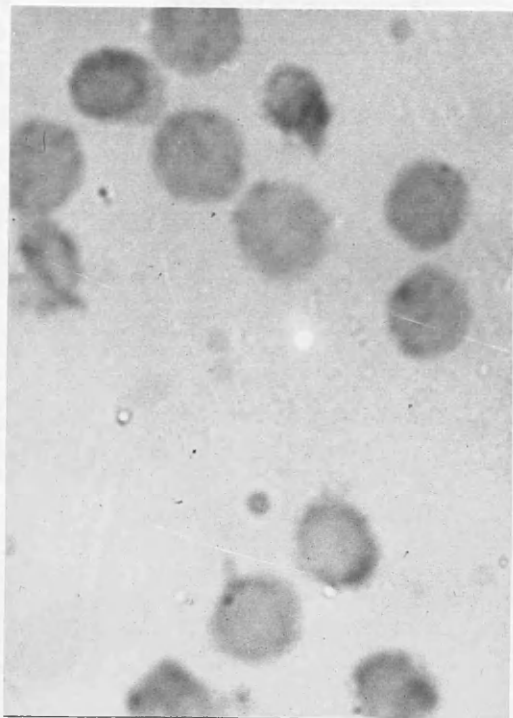


Fig. 3.

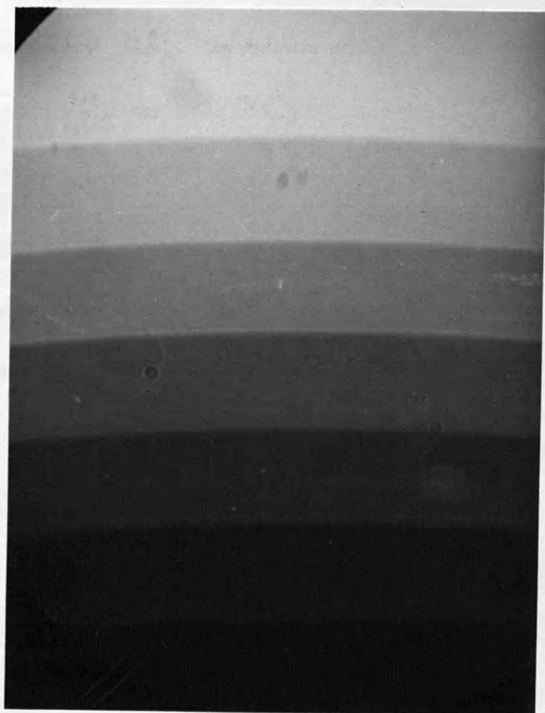


Fig. 4. Time-density (Gamma) curve of (Kodak) microfilm used. The exposure time of photomicrographs was arranged so that nuclear image extinctions were maximal within the linear part of the curve. Thus there is a direct relationship between the density of the object image on the film and the density of the object.

Fig. 4.

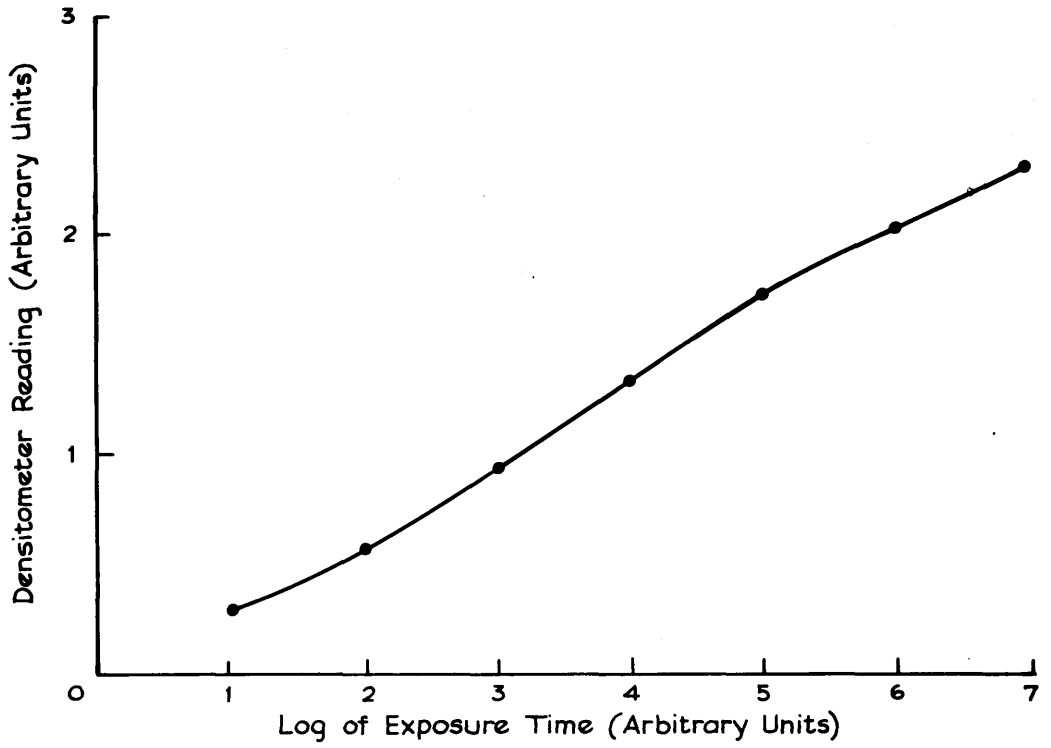


Fig. 5. The ratios between the mean adrenal nuclear extinction values after varying periods of ACTH administration and those found for corresponding groups of control kidney nuclei. An initial slight fall in this ratio is followed by a marked fall after the hormone was given for 7 and 10 days. A tendency for this ratio to return to normal is evident at 14 and 21 days.

Fig. 5.

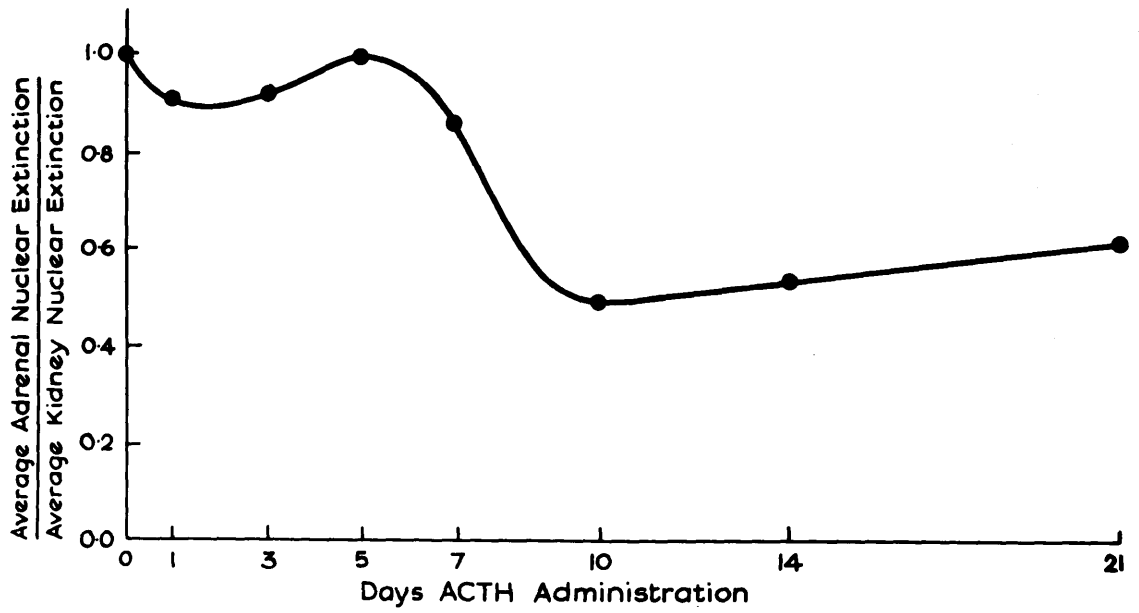


Fig. 6. The ratios between values for mean adrenal nuclear projected areas from animals receiving ACTH and those for groups of corresponding control kidney nuclei. A marked rise in this ratio is evident at 7 and 10 days, and later there is a tendency for this ratio to return to normal. There is a reciprocal relationship between values shown in this figure and those shown in Fig. 5, except at 5 and 7 days when a highly statistically significant difference was found between mean kidney nuclear DNA values and those for adrenal nuclei from ACTH treated guinea-pigs.

Fig. 6.

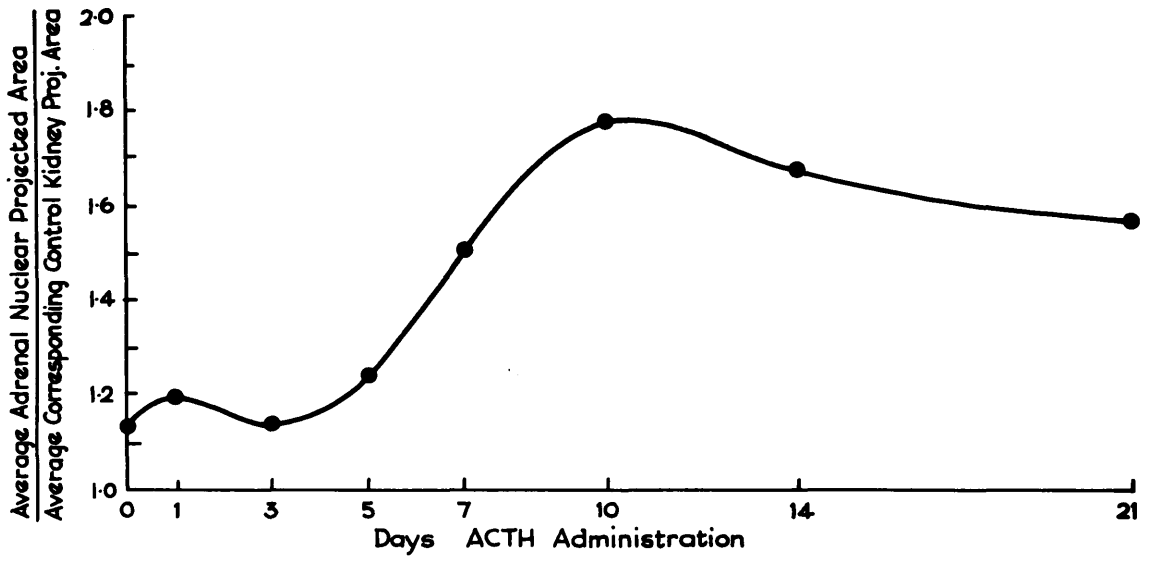


Fig. 7. Mean extinction values of groups of kidney and adrenal nuclei plotted against mean values for the projected areas of these. The deviation from a strict linear relationship is probably due to the specimens being mounted on different slides, and the various specimens are therefore not directly comparable.

Fig. 7.

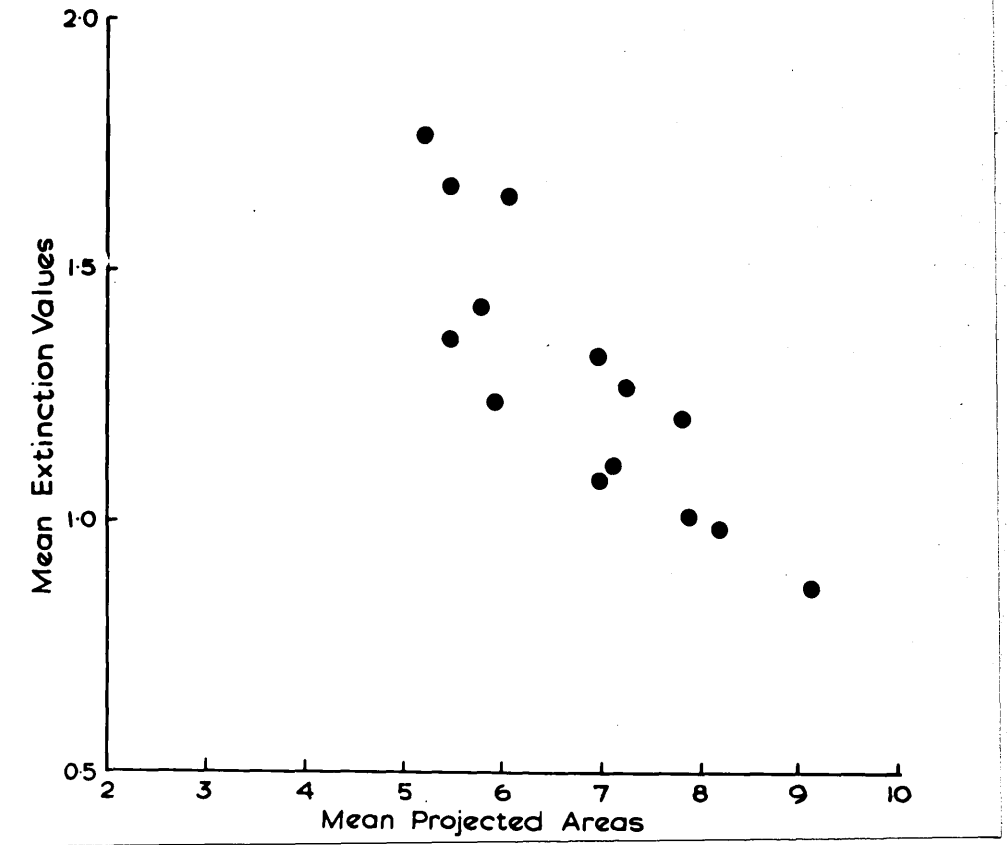


Fig. 8. Pooled adrenal weights of glands from groups of six animals, and nuclear concentrations of DNA ($\frac{\text{average extinction}}{\text{average area}}$) in arbitrary units after 1,3,5 and 7 days ACTH administration. There is a gradual increase for pooled adrenal weights and a fall in nuclear DNA concentration values as the administration of ACTH continues. Values for adrenal nuclei isolated in a citric acid medium are plotted, as the durations of ACTH administration to the groups of animals from which they were isolated varied much less than the durations of hormone treatment for groups of animals from which nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 8

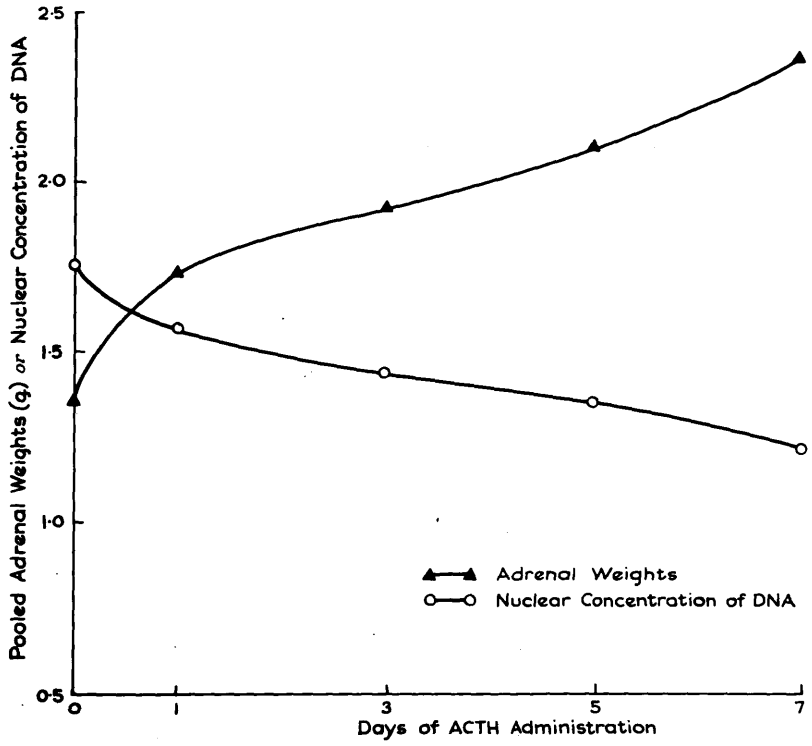


Fig. 9. Mean nuclear DNA content in arbitrary units of kidneys from animals which received ACTH for 0,1,3,5 and 7 days (open circles) and of corresponding control kidneys (filled circles) \pm standard error (S.E.). The difference between these DNA values for the 3 day test animals was statistically significant on the 5% level, using a simple t test. Nuclei were isolated in a citric acid medium.

Fig. 9.

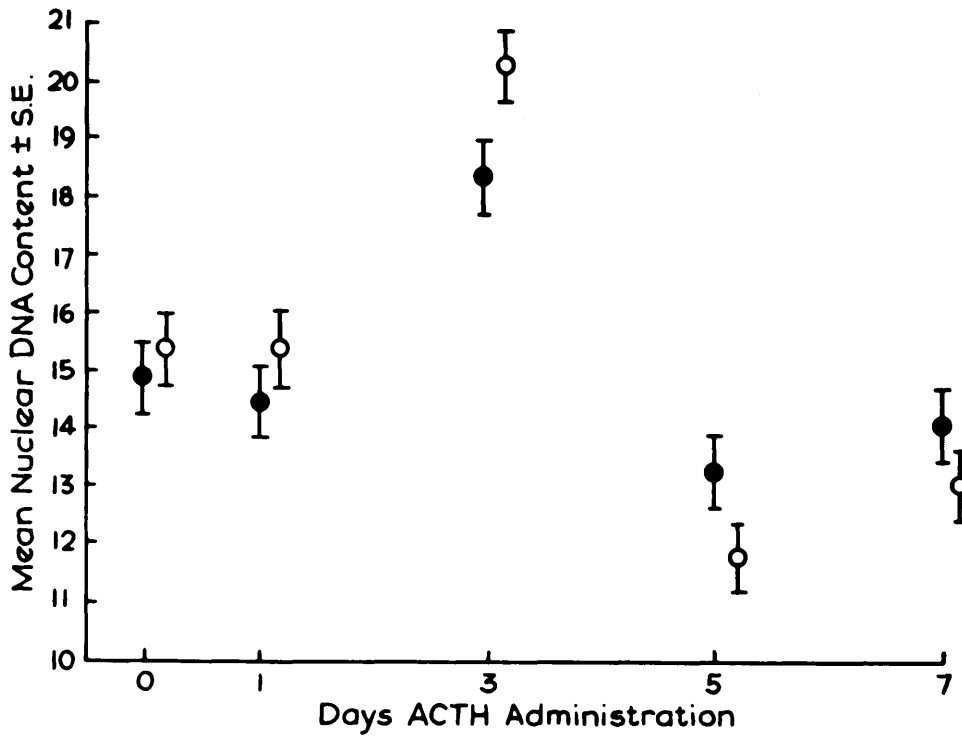


Fig. 10. Mean nuclear DNA content in arbitrary units of kidneys from animals which received ACTH for 3, 10, 14, and 21 days (open circles) and of corresponding control kidneys (filled circles) \pm standard error (S.R.). The difference between these DNA values for the 3 day test kidneys and control kidneys was statistically significant at the 5% level, using a simple t test. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 10.

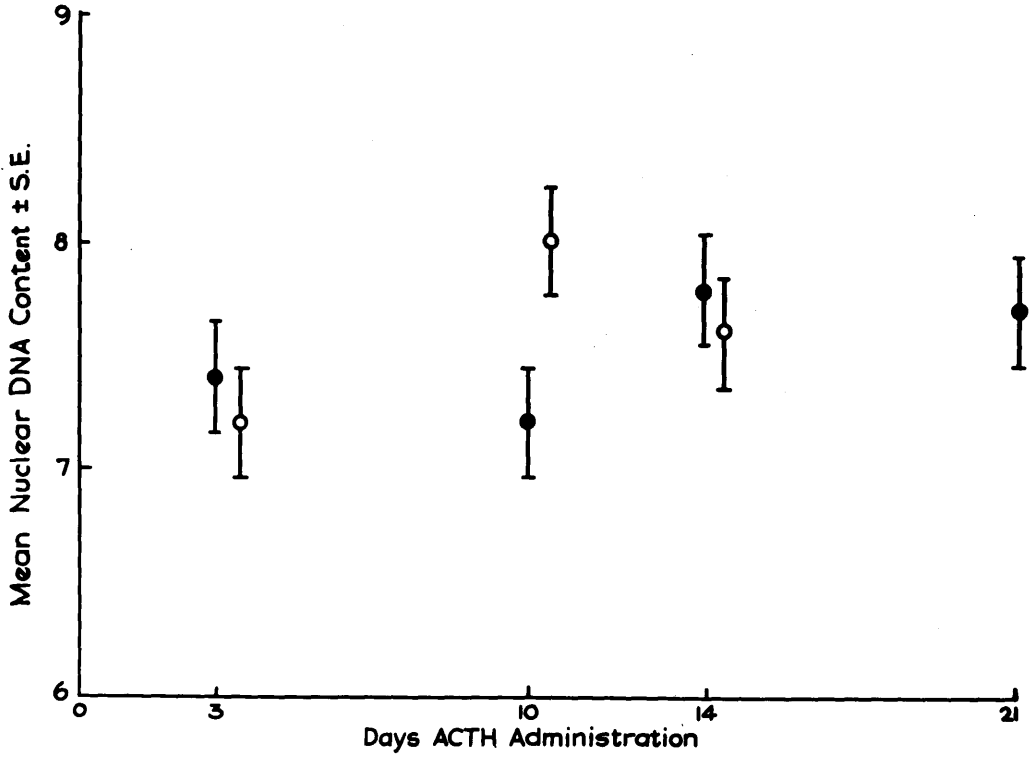


Fig. 11. Mean nuclear DNA content in arbitrary units of adrenals from animals given ACTH for 0, 1, 3, 5, and 7 days (open circles) and of corresponding control kidneys (filled circles) \pm standard error (S.E.). The difference between these DNA values for the 5 and 7 day test adrenals and control kidneys is highly statistically significant ($P < 0.01$). Nuclei were isolated in a citric acid medium.

Fig. 11.

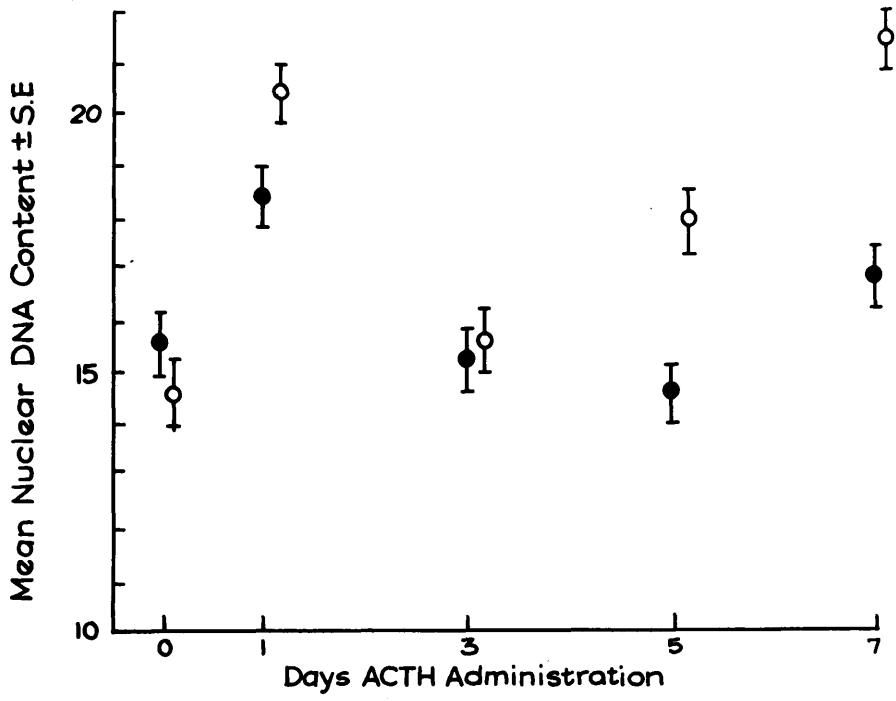


Fig. 12. Mean nuclear DNA content in arbitrary units of adrenals from animals given ACTH for 0, 3, 14 and 21 days (open circles) and of corresponding control kidneys (filled circles) \pm standard error (S.E.). None of the differences between these was statistically significant, using a simple t test. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 12.

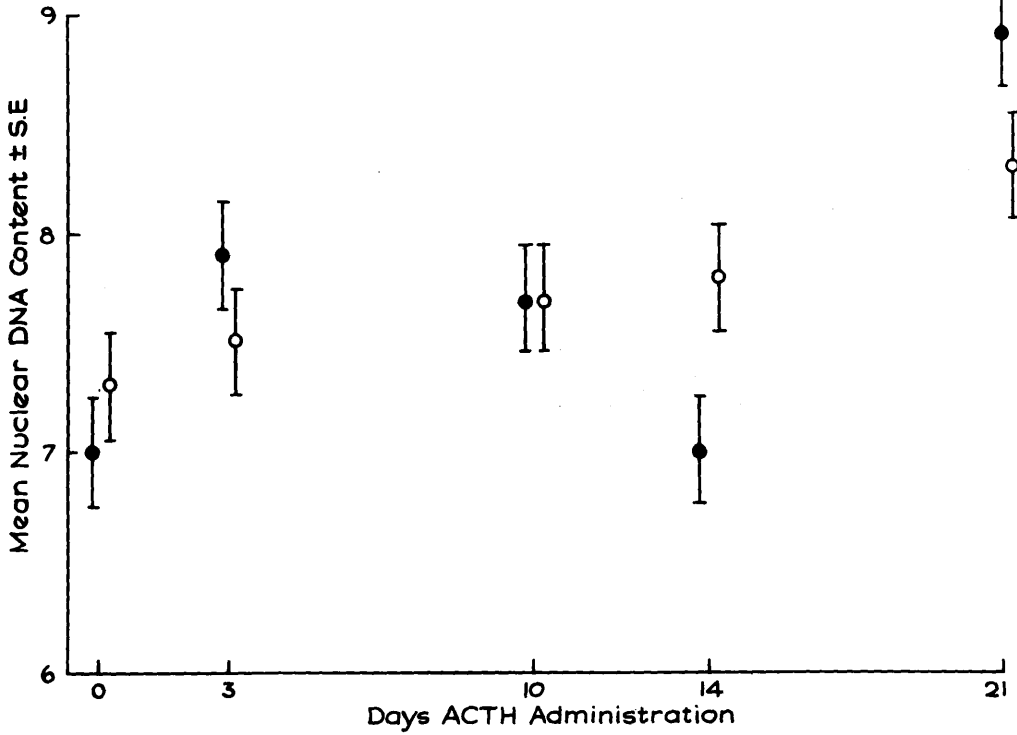


Fig. 13. Differences between mean nuclear DNA content of kidneys from animals which received ACTH for 0, 1, 3, 5 and 7 days, and mean nuclear DNA content of corresponding control kidneys + standard error (S.E.). The differences for 3 day test animals was statistically significant at the 5% level, using a simple t test. Nuclei were isolated in a citric acid medium.

Fig. 13.

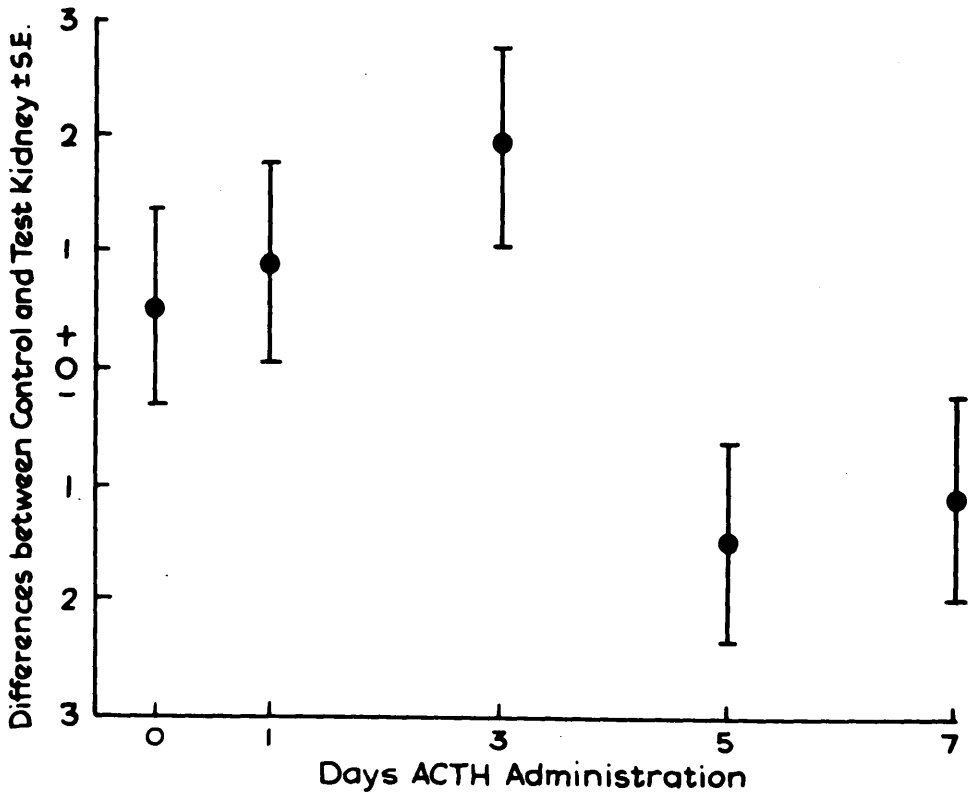


Fig. 14. Differences between mean nuclear DNA content of kidneys from animals which received ACTH for 3, 10, 14 and 21 days and mean nuclear DNA content of corresponding control kidneys ⁺ - standard error (S.E.). This difference for 10 day test animals was statistically significant at the 5% level using a simple t test. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 14.

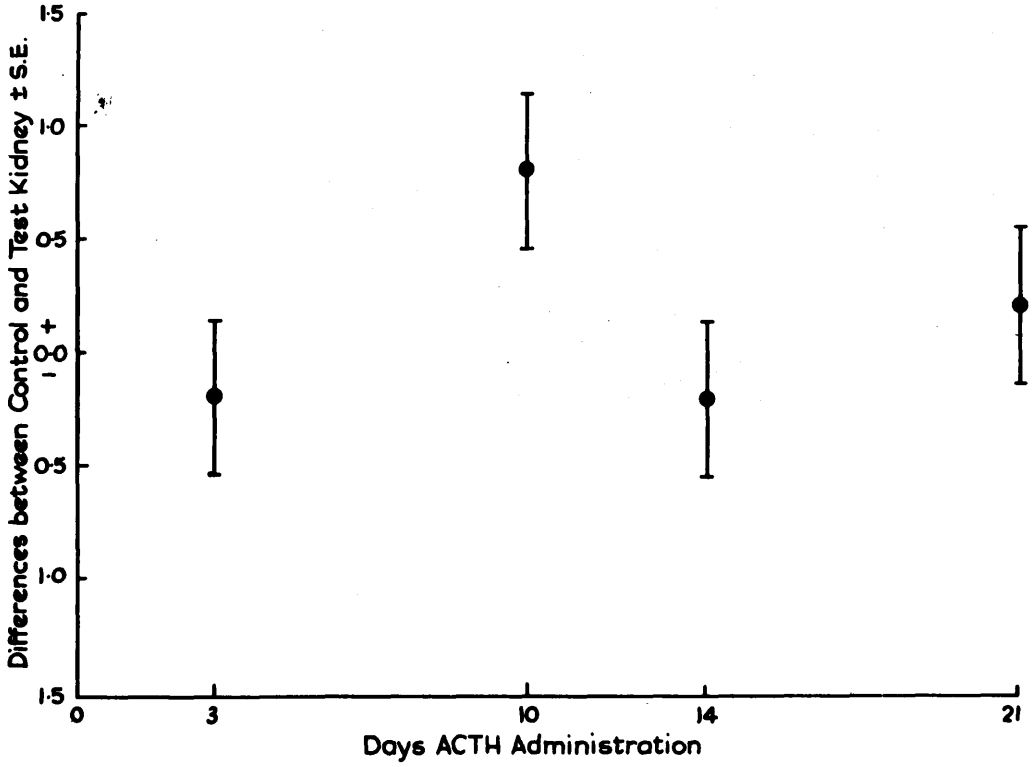


Fig. 15. Differences between mean nuclear DNA content of adrenals from animals which were treated with ACTH for 0, 1, 3, 5 and 7 days and mean nuclear DNA content of corresponding control kidneys - standard error (S.E.).⁺ The differences for the 5 and 7 day test animals were highly statistically significant using a simple t test ($P < 0.01$). Nuclei were isolated in a citric acid medium.

Fig. 15.

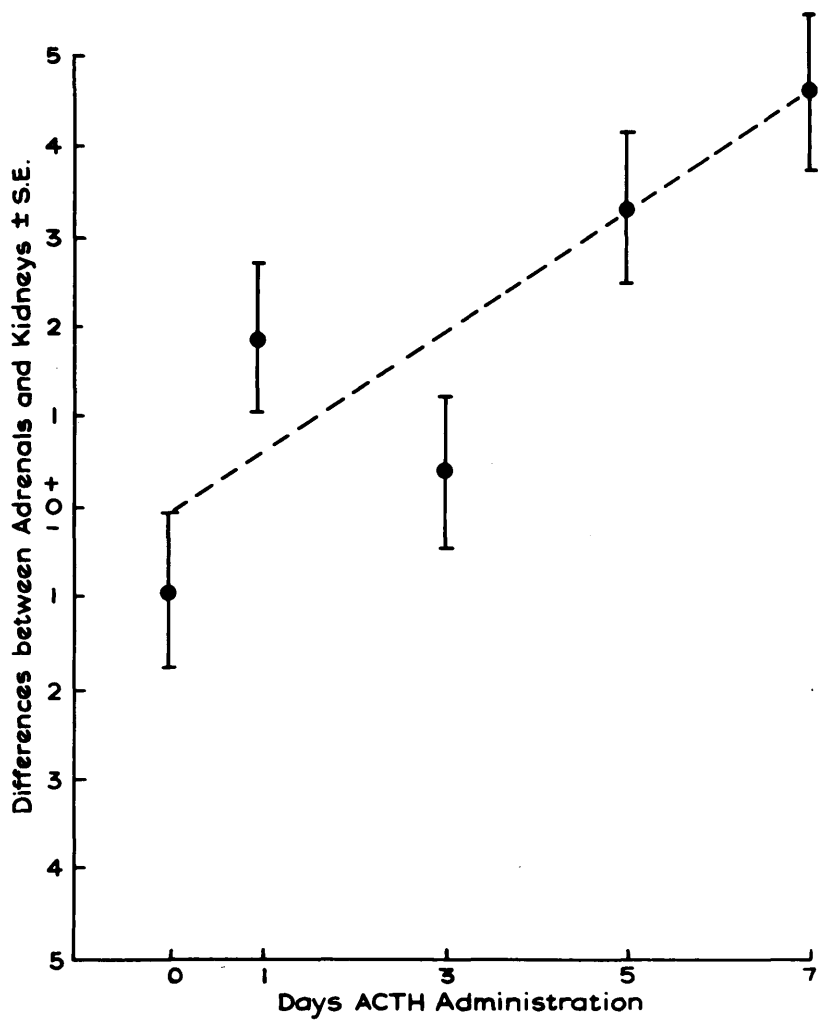


Fig. 16. Differences between mean nuclear DNA content of adrenals from animals which received ACTH for 0, 3, 10, 14 and 21 days, and mean nuclear DNA contents of corresponding control kidneys ⁺ - standard error (S.E.). None of these differences was statistically significant using a simple t test. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 16.

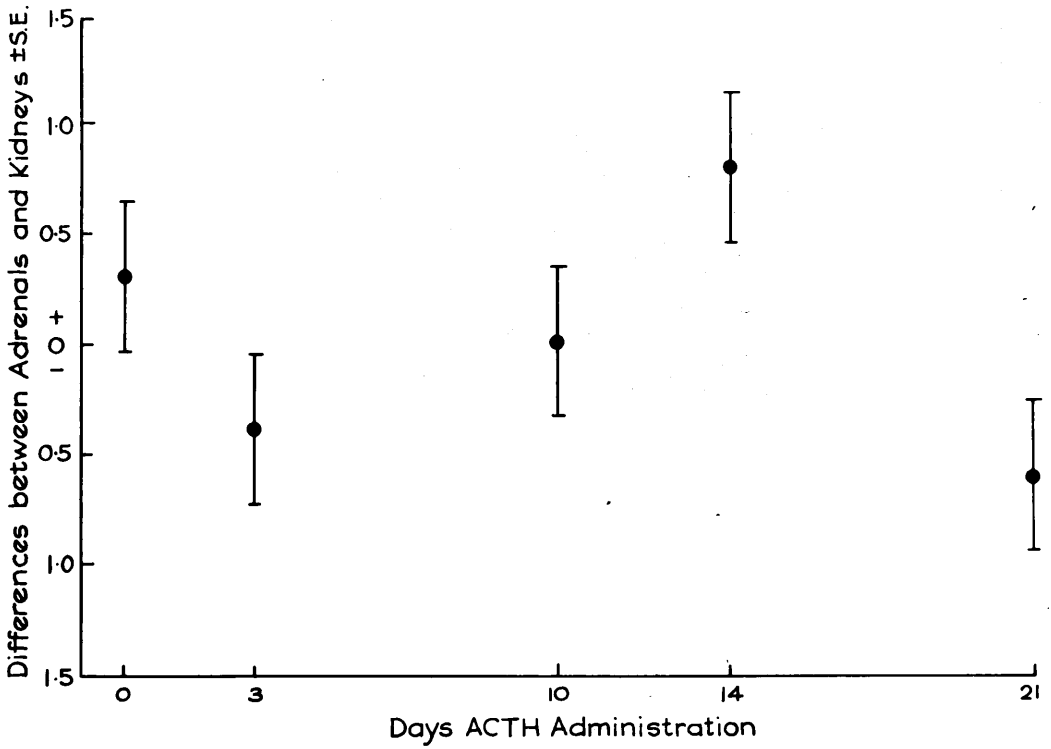


Fig. 17. Histograms showing the patterns of distribution of DNA content of nuclei isolated from kidneys of two groups (C 1 & C 2) of six guinea-pigs. All diploid values, except two, range from 10 to 20 arbitrary units DNA. One tetraploid DNA value was found for a kidney nucleus from group C 1. Nuclei were isolated in a citric acid medium.

Fig. 17.

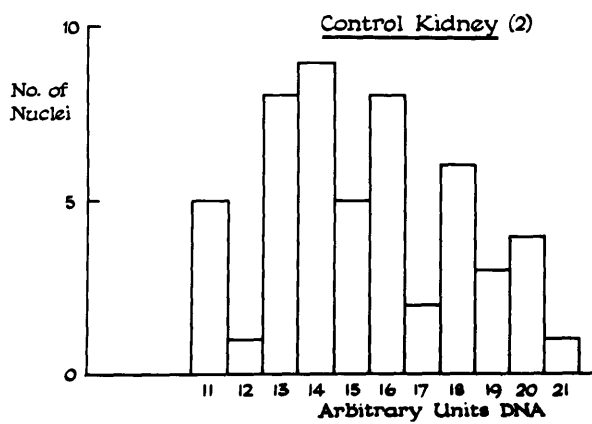
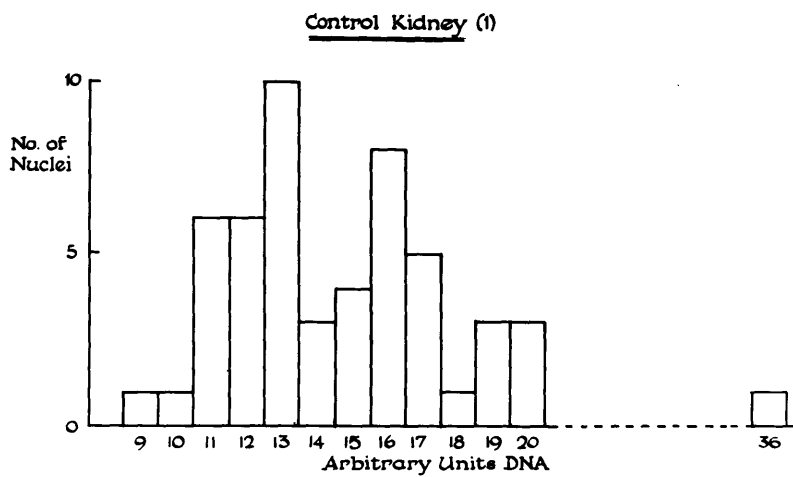


Fig. 18. Histograms showing the patterns of distribution of DNA content of nuclei isolated in a citric acid medium from kidneys and adrenals of a group of six guinea-pigs. All diploid values for control kidney nuclei range from 10 to 20 arbitrary units DNA, and all values except one (11 units) for control adrenal nuclei range from 13 to 25 arbitrary units. One kidney nucleus was found to have an approximately tetraploid value (25 A.U.).

Fig. 18.

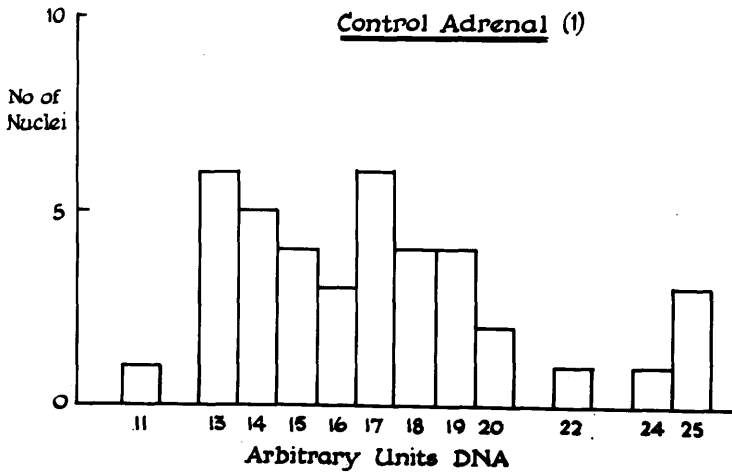
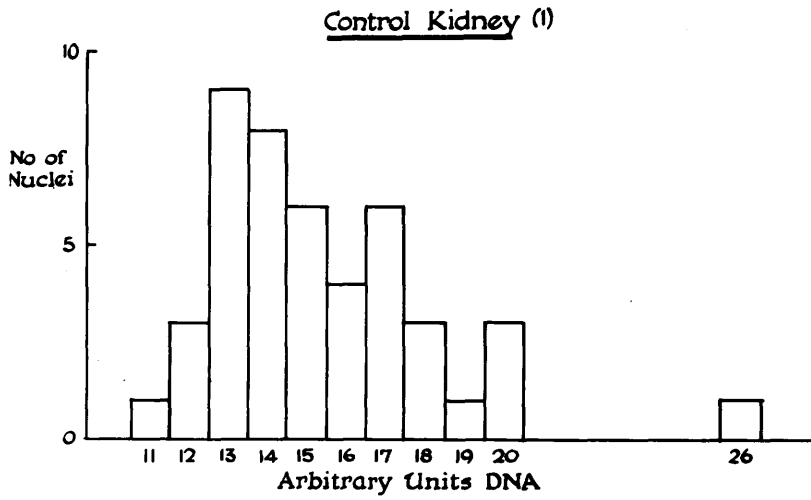


Fig. 19. Histograms showing the patterns of distribution of nuclear DNA values for one day test kidneys and control kidneys. All control kidney nuclear DNA values, except one, range from 10 to 20 units, and test kidney DNA values, with one exception, range from 12 to 24 arbitrary units. No tetraploid value was found. Nuclei were isolated in a citric acid medium.

Fig. 19.

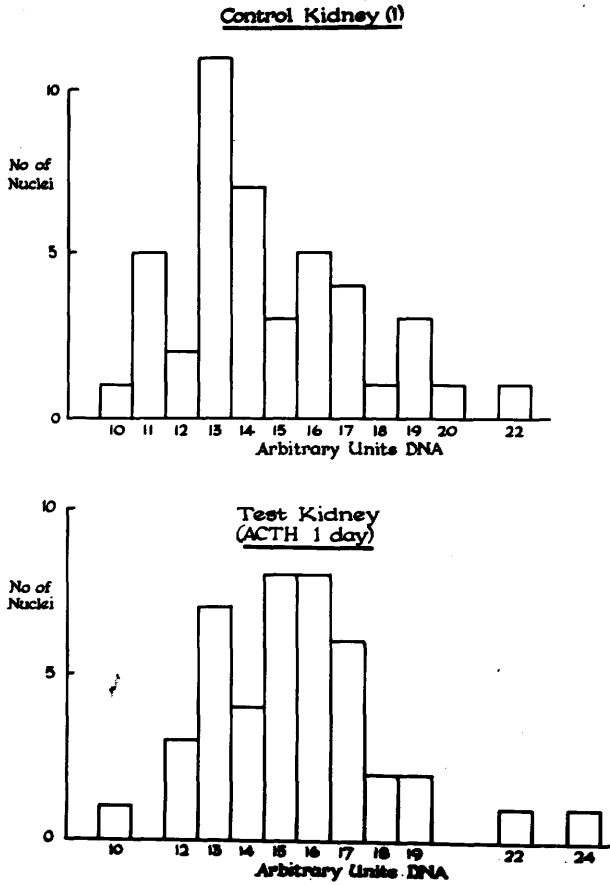


Fig. 20. Histograms showing the patterns of distribution of DNA content of control kidney nuclei and 1 day test adrenal nuclei. All except two diploid values range from 13 to 25 arbitrary units. One control kidney and three test adrenal nuclei were found to have tetraploid amounts of DNA. Nuclei were isolated in a citric acid medium.

Fig. 20.

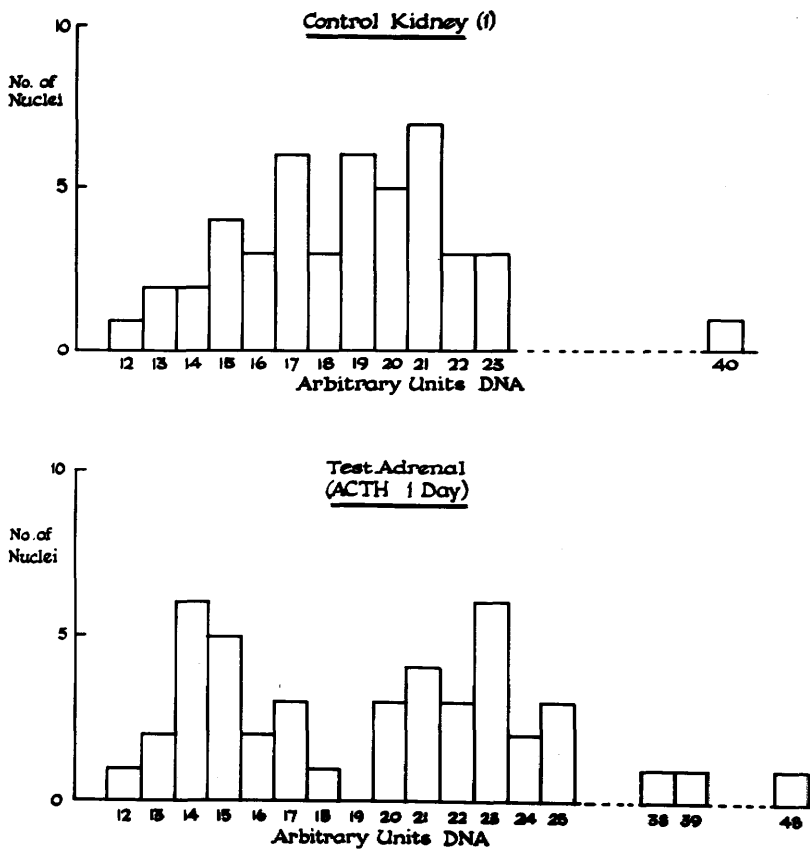


Fig. 21. Histograms showing patterns of distribution of nuclear DNA values for three day test kidney and corresponding control kidney nuclei. Diploid values for the former show a scatter from 13 to 26 units, and those for the latter a scatter from 12 to 24 arbitrary units DNA. The difference between the mean values of nuclear DNA for these control and test specimens is statistically significant at the 5% level. Nuclei were isolated in a citric acid medium.

Fig. 21.

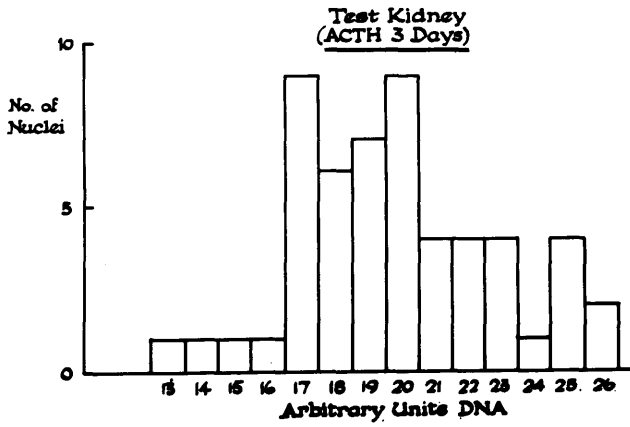
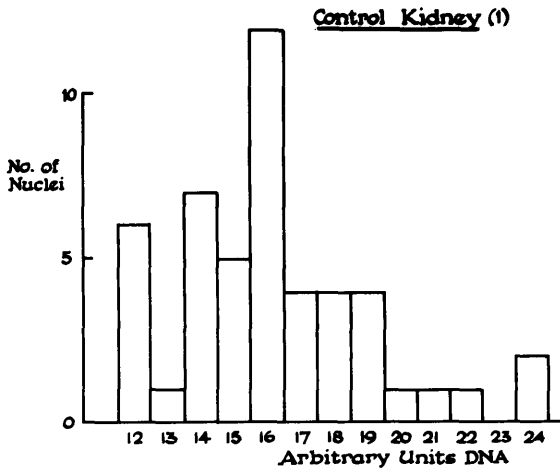


Fig. 22. Histograms showing patterns of distribution of nuclear DNA values for three day test adrenal and control kidney samples. All diploid values range from 11 to 22 arbitrary units DNA. Two tetraploid values were found for three day test adrenal nuclei. Nuclei were isolated in a citric acid medium.

Fig. 22.

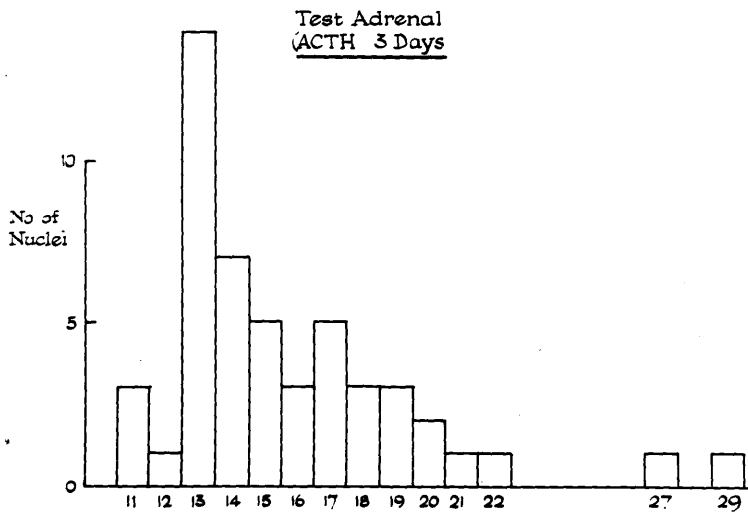
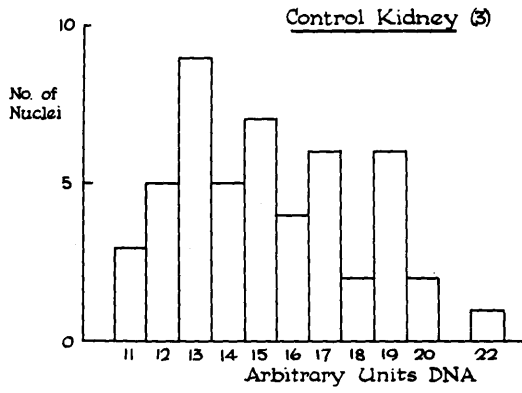


Fig. 23. Histograms showing patterns of distribution of nuclear DNA values for 5 day test kidneys and corresponding control kidneys. Almost all values range from an arbitrary value X to 2X. One tetraploid value was found for a test kidney nucleus. Nuclei were isolated in a citric acid medium.

Fig. 23.

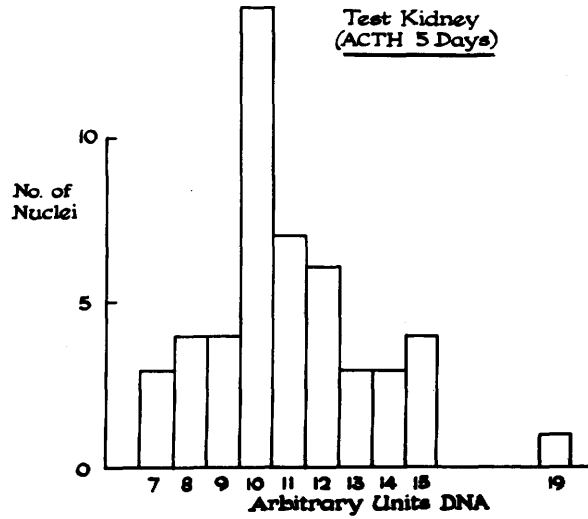
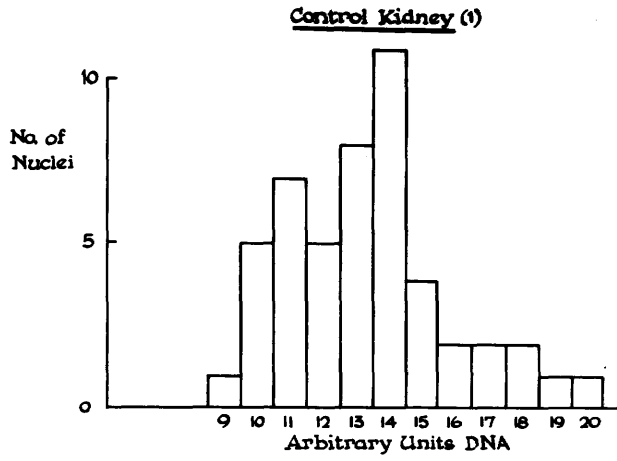
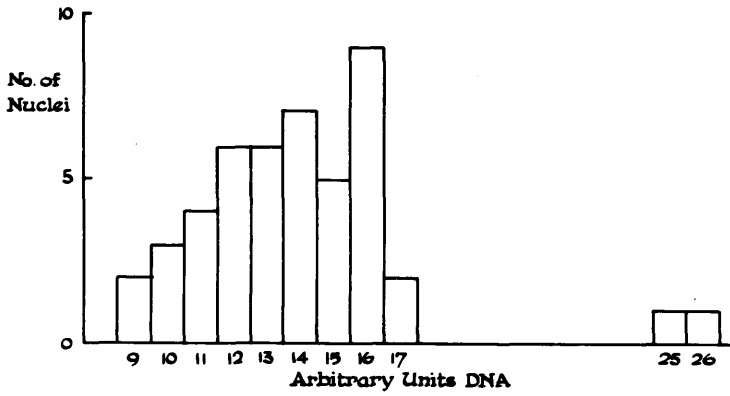


Fig. 24. Histograms showing patterns of distribution of nuclear DNA values for 5 day test adrenal and corresponding control kidney samples. Almost all values for the kidney nuclei range from 9, to 17 units, and those for the adrenal nuclei from 13 to 23 units. The difference between mean adrenal and kidney nuclear DNA values is highly statistically significant ($P < 0.01$). Two kidney nuclei and one adrenal nucleus were found to have tetraploid values of DNA. Nuclei were isolated in a citric acid medium.

Fig. 24.

Control Kidney (i)



Test Adrenal (ACTH 5 Days)

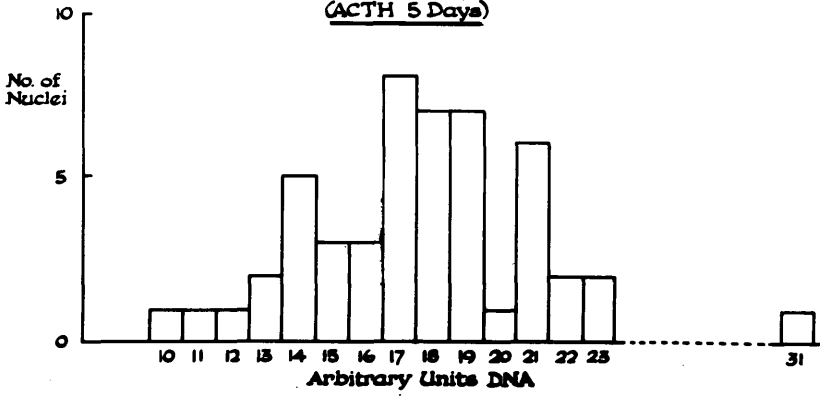


Fig. 25. Histograms showing patterns of distribution of DNA values in arbitrary units for 7 day test kidney and corresponding control kidney nuclei. All diploid values range from 9 to 19 arbitrary units. Two tetraploid values of DNA content were found for control kidney nuclei. Nuclei were isolated in a citric acid medium.

Fig. 25.

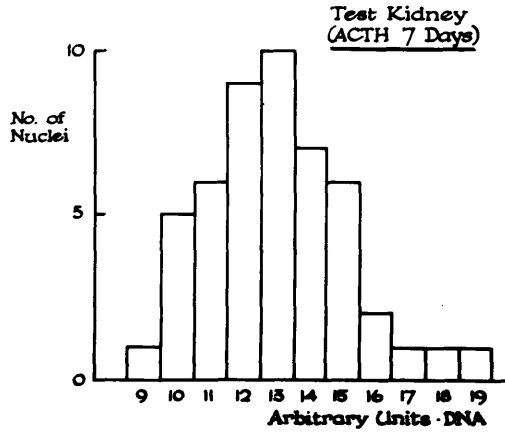
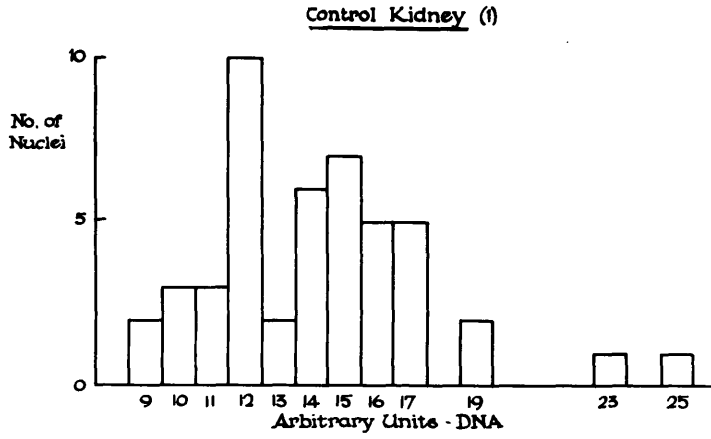


Fig. 26. Histograms showing patterns of distribution of nuclear DNA values for 7 day test adrenal and corresponding control kidney samples. Among the latter is one tetraploid value (40 units). Diploid values range from 11 to 23 units for control kidney and 16 to 30 units for test adrenal specimens. The difference between mean DNA values for adrenal and kidney nuclei is highly statistically significant ($P < 0.01$). Nuclei were isolated in a citric acid medium.

Fig. 26.

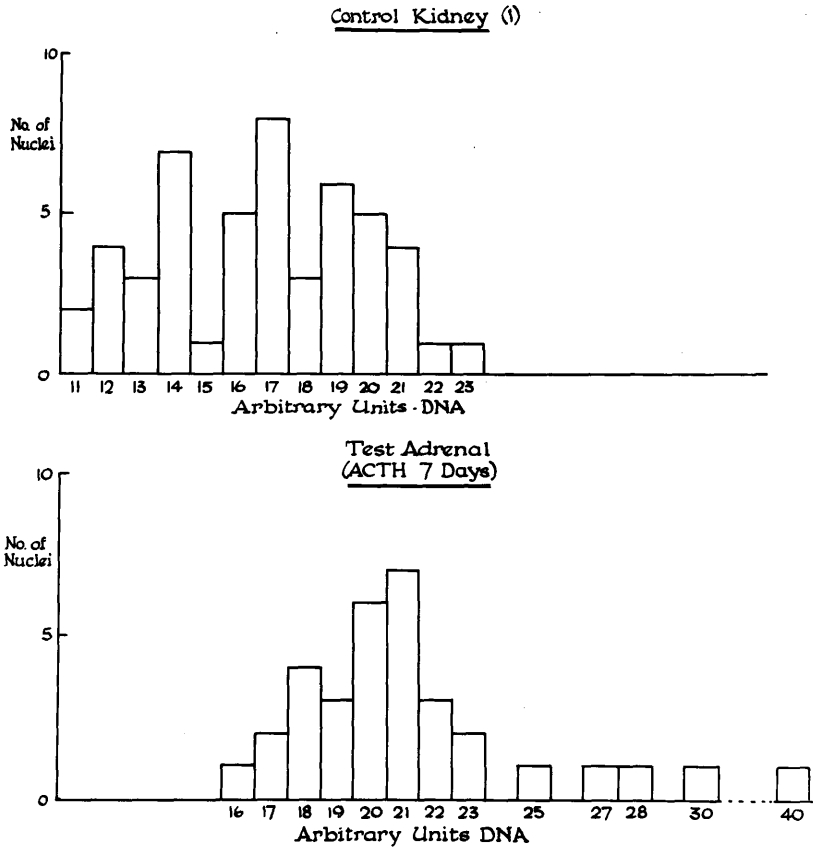


Fig. 27. Histograms showing the patterns of distribution of nuclear DNA values for 3 day test kidney and corresponding control kidney specimens. All values except one lie in the range 5 to 10 arbitrary units. No tetraploid value was found. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 27.

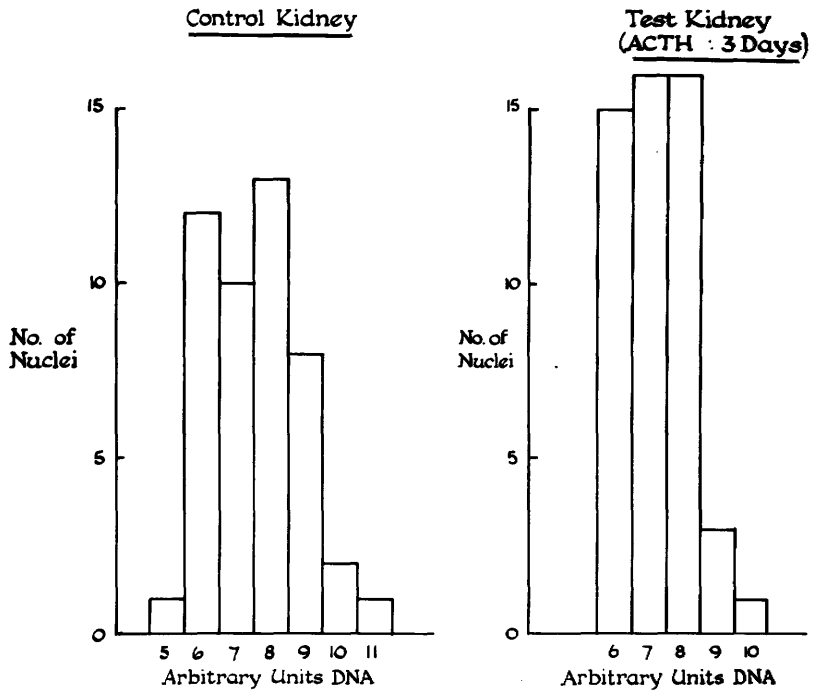


Fig. 28. Histograms showing patterns of distribution of nuclear DNA values for 3 day test adrenals and corresponding control kidneys. All diploid values except one range from 6 to 11 arbitrary units. One tetraploid DNA value was found for a control kidney nucleus. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 28

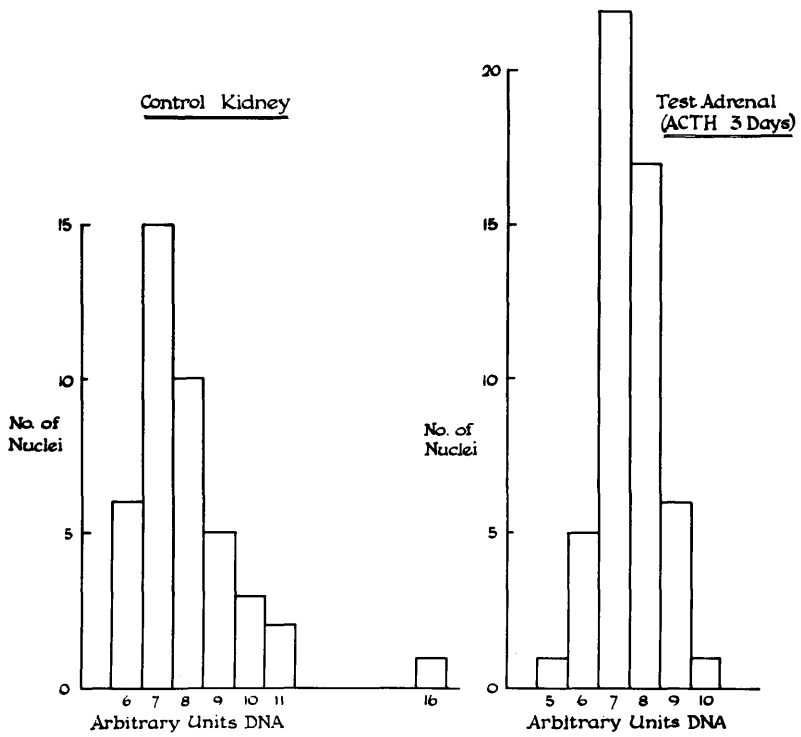


Fig. 29. Histograms showing patterns of distribution of DNA values found for 10 day test kidneys and corresponding control kidneys. All values are diploid and range from 5 to 10 arbitrary units DNA. The difference between mean nuclear DNA values found for test adrenals and for corresponding control kidneys is statistically significant at the 5% level. Nuclei were isolated in a sucrose-calcium chloride.

Fig. 29.

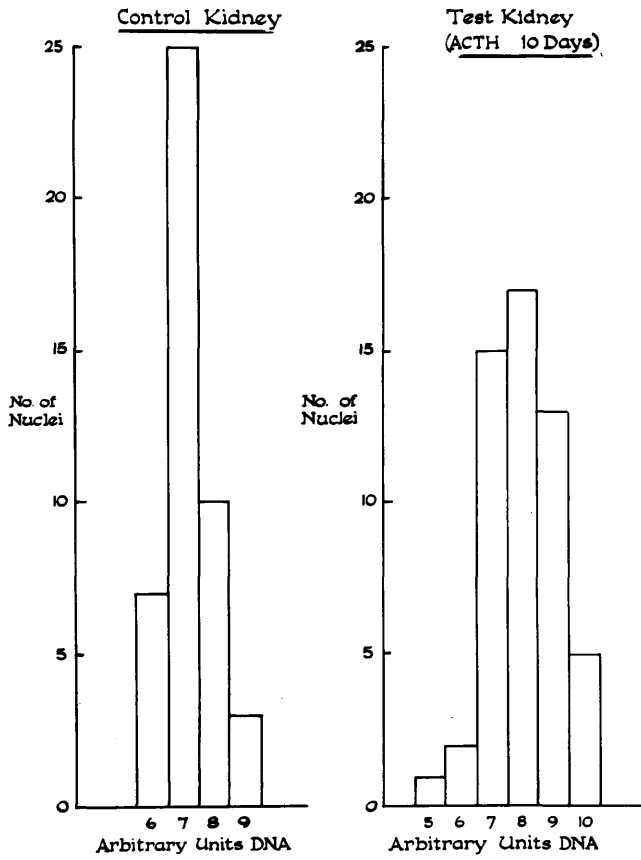


Fig. 30. Histograms showing patterns of distribution of nuclear DNA values for 10 day test adrenal and corresponding control kidney specimens. Almost all values range from 5 to 10 arbitrary units DNA. No tetraploid value was found. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 30.

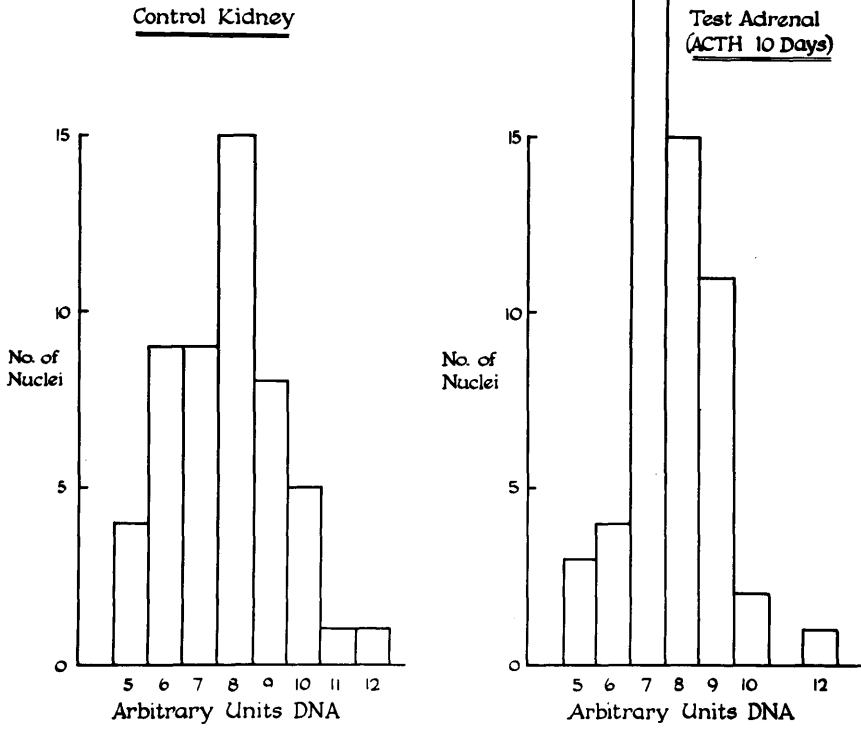


Fig. 31. Histograms showing patterns of distribution of nuclear DNA values for 14 day test kidneys and corresponding control kidneys. All values range from 5 to 11 arbitrary units DNA. No tetraploid value was found. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 31.

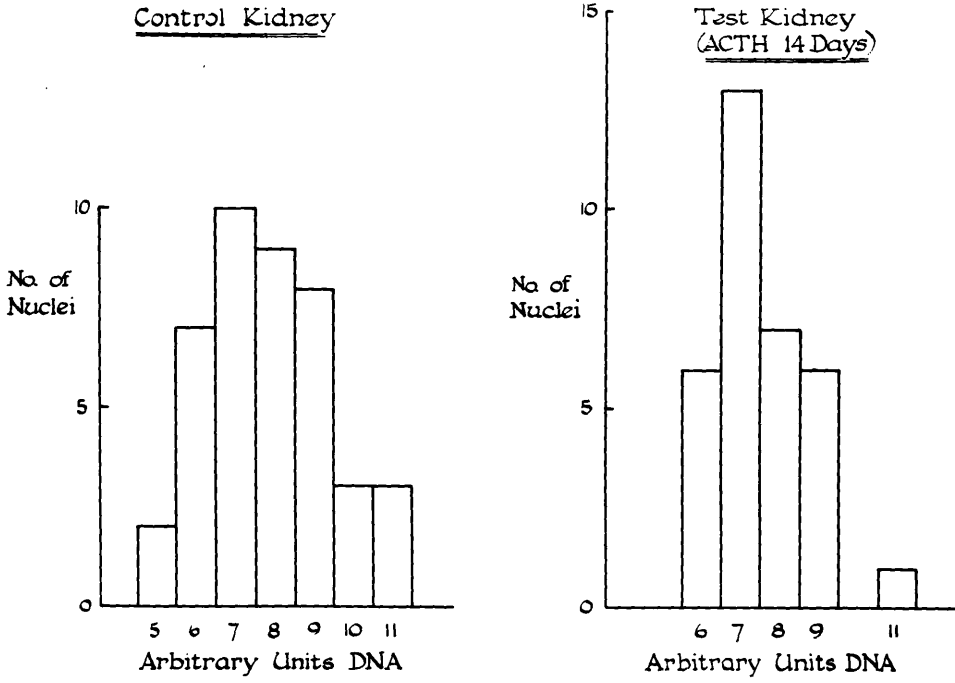


Fig. 32. Histograms showing patterns of distribution of nuclear DNA values for 14 day test adrenal and corresponding control kidney. All diploid values range from 5 to 11 units DNA. One tetraploid DNA value was found for an adrenal nucleus. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 32.

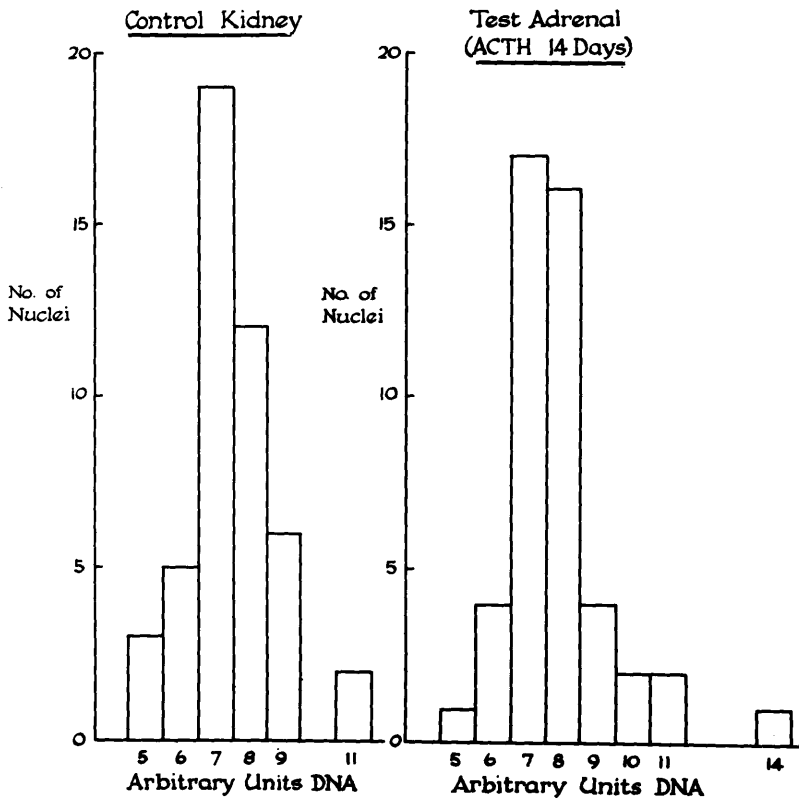


Fig. 33. Histograms showing patterns of distribution of nuclear DNA values for 21 day test kidneys and corresponding control kidneys. All diploid values range from 6 to 11 units. One kidney nucleus was found to have a tetraploid amount of DNA. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 33

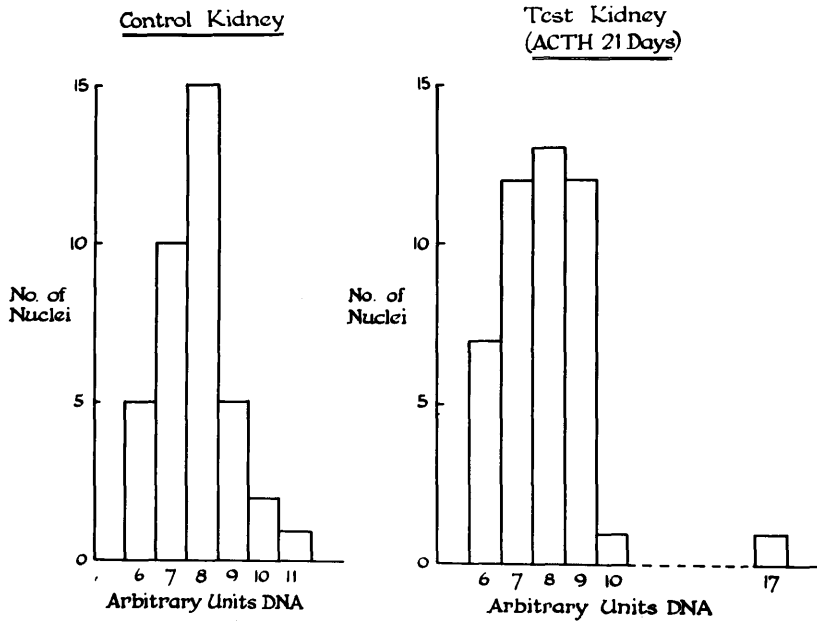


Fig. 34. Histograms showing patterns of distribution of nuclear DNA values for 21 day test adrenals and corresponding control kidneys. All diploid values range from 6 to 12 units DNA. One control kidney nucleus was found to have a tetraploid amount of DNA. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 34.

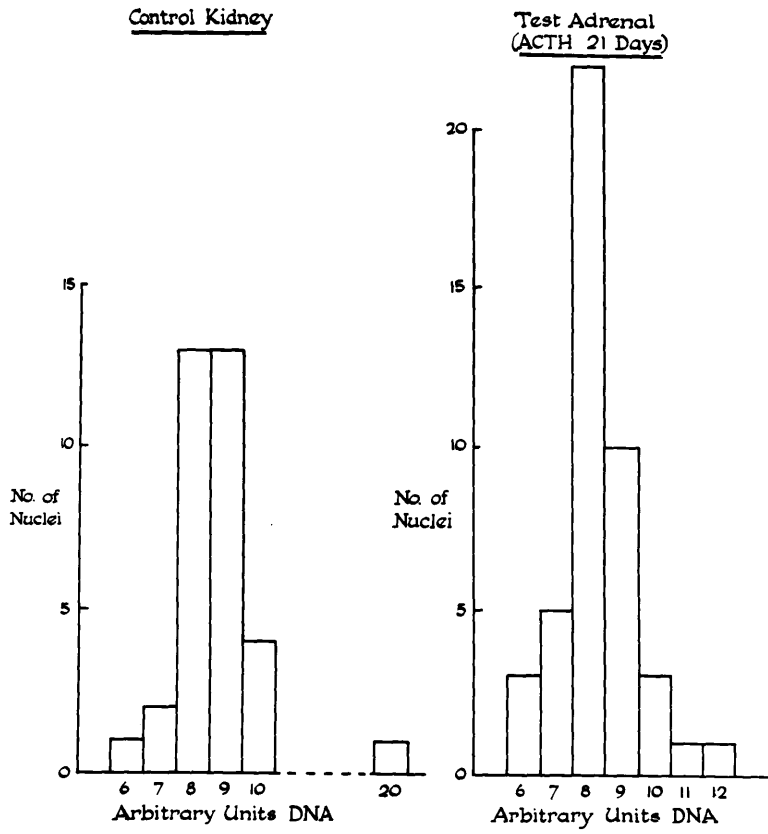


Fig. 35. Histograms showing patterns of distribution of nuclear DNA values for control adrenals and corresponding control kidneys. Almost all values range from 5 to 10 units for the adrenals and from 6 to 12 units for the kidneys. Two tetraploid values were found for normal adrenal nuclei. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 35.

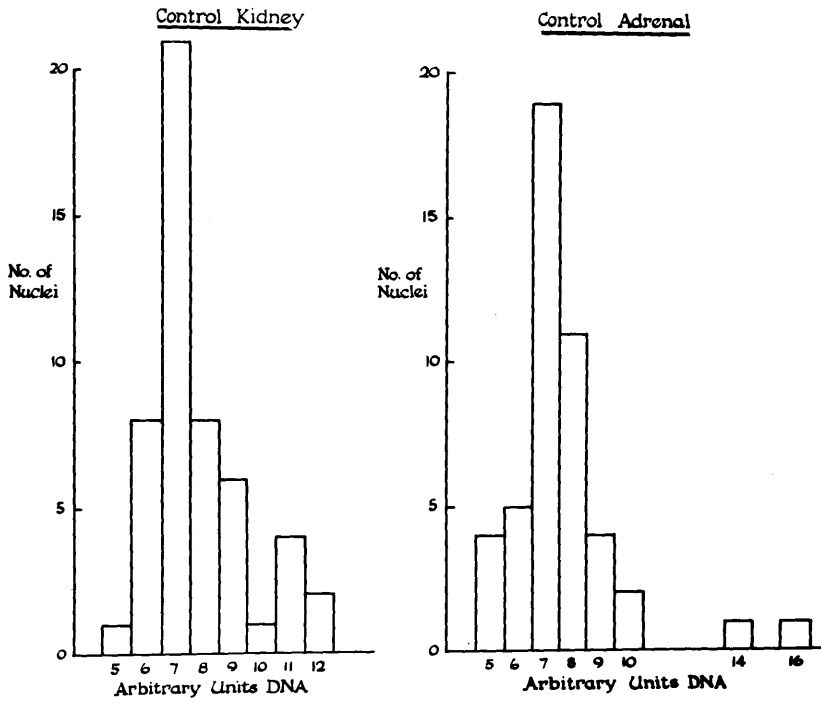


Fig. 36. Histograms showing patterns of distribution of nuclear DNA values for 3 day test kidney and corresponding control kidney nuclei. All values except one from 5 to 11 units DNA. No tetraploid value was found. Test kidney nuclei are from guinea-pigs which received a high dosage of ACTH (50 mg per 500 g body weight per day). Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 36.

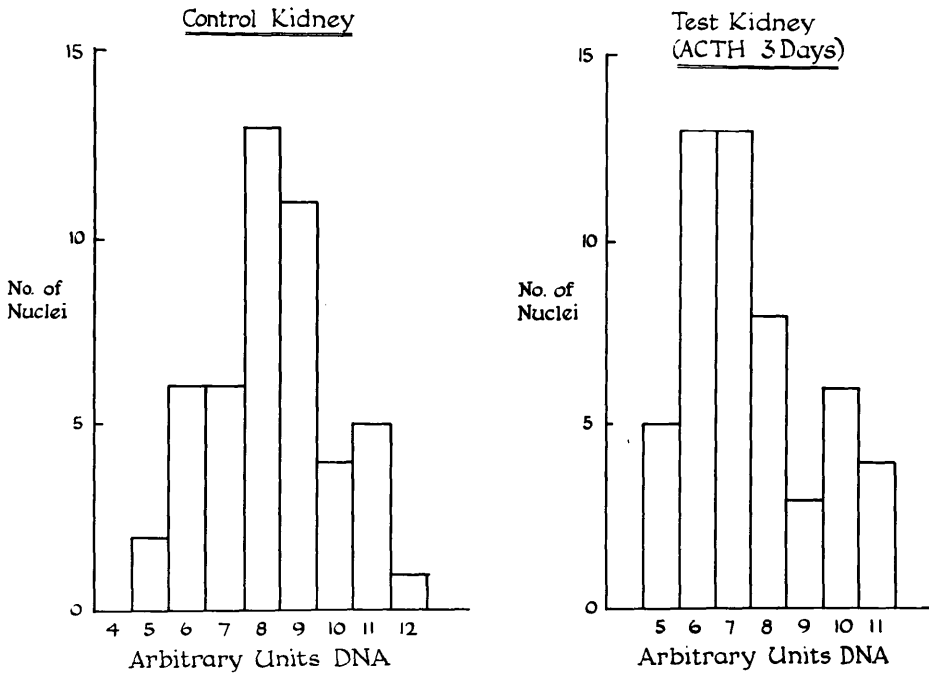
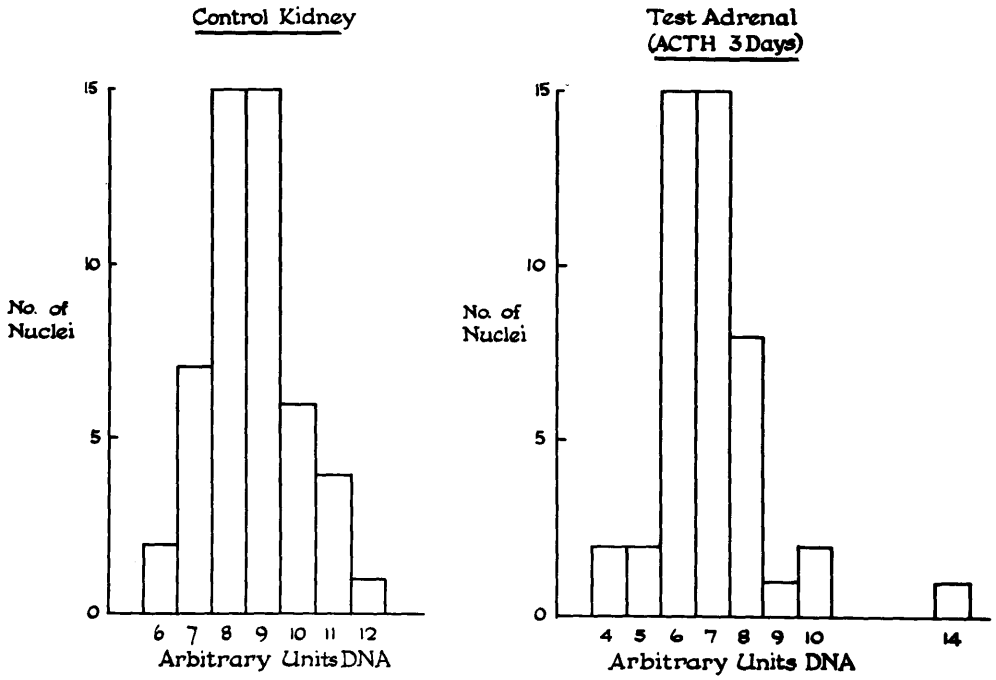


Fig. 37. Histograms showing patterns of distribution of nuclear DNA values for 3 day test adrenal and corresponding control kidney nuclei. Control kidney DNA values range from 6 to 12 units and almost all test adrenal DNA values range from 5 to 10 arbitrary units DNA. One tetraploid DNA value was found for an adrenal nucleus. High dosage of ACTH, 50 mg/500g body weight per day, was administered to experimental animals. Nuclei were isolated in a sucrose-calcium chloride medium.

Fig. 37.



RUBONUCLEIC ACID

Fig. 38. Control guinea-pig adrenal stained for ribonucleic acid (RNA). RNA is present in greatest concentration in the zona reticularis and zona glomerulosa. The former zone is formed almost entirely of 'compact cells'. Stained Methyl-Green Pyronin. X 100.

Fig. 39. Guinea-pig adrenal from 3 hr test animal showing RNA distribution. Scattered cells occurring singly and in small groups in the zona reticularis and inner zona fasciculata showed increased RNA content. Other cells generally showed a content of RNA similar to normal.

Stained Methyl-Green Pyronin. X 100.

Fig. 40. Guinea-pig adrenal from 5 day test animal. All zones showed an increase in RNA content. This was least evident in the outer zona fasciculata

Stained Methyl-Green Pyronin. X 100.

Fig.38.

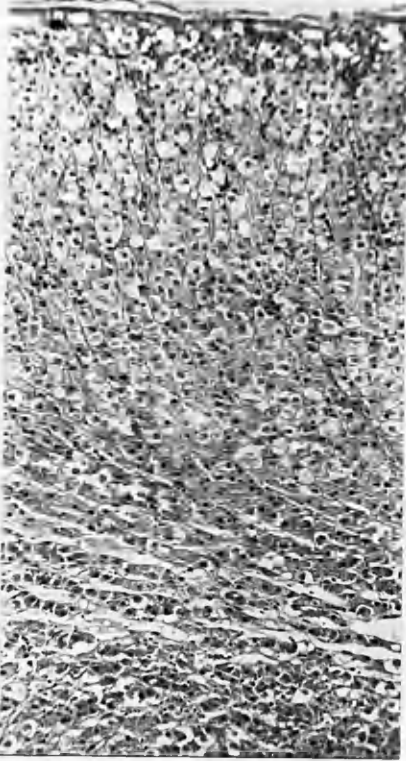


Fig.39.

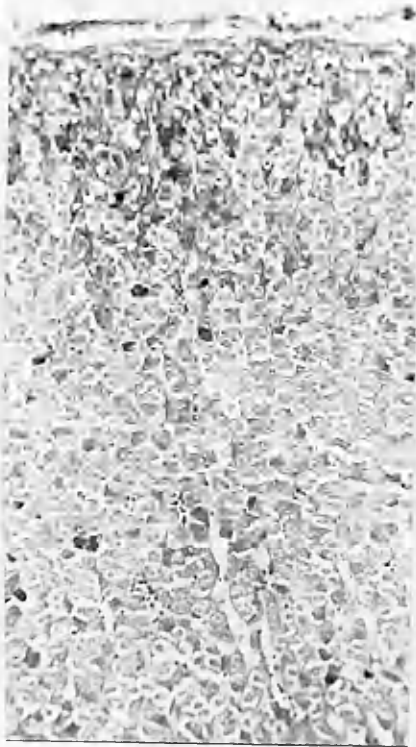


Fig.40



RIBONUCLEIC ACID

Fig. 41. Guinea-pig adrenal from 7 day test animal. Most of this field is occupied by the zona reticularis which contained the maximum observed concentration of RNA.

Stained Methyl-Green Pyronin. X 100.

Fig. 42. High power photomicrograph of part of centre of field from Fig. 41, showing compact cells rich in RNA granules.

Stained Methyl-Green Pyronin. X 1,000.

Fig. 41

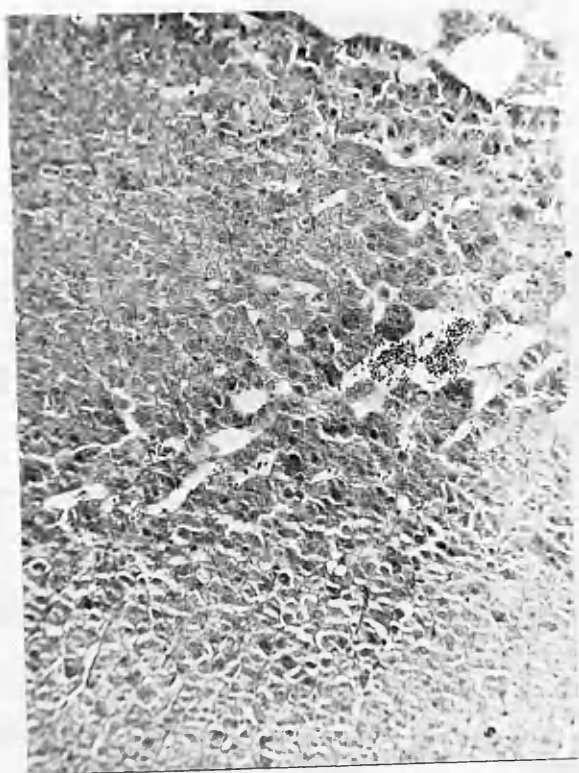
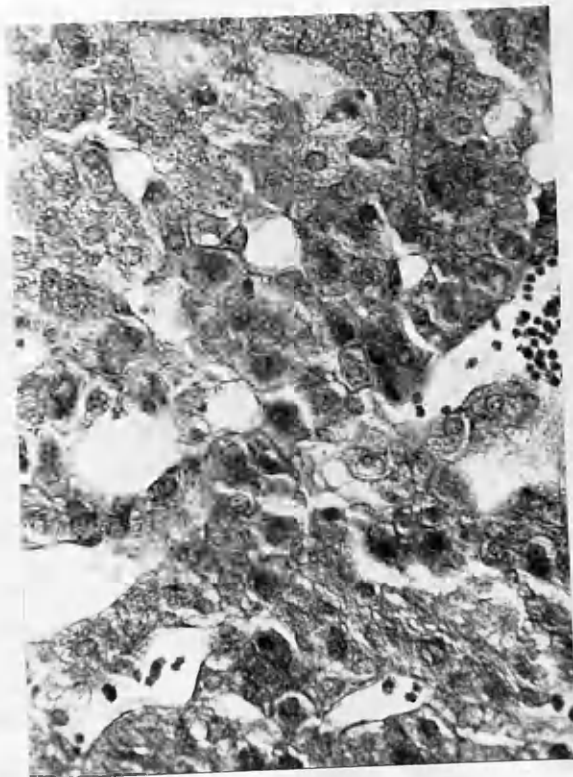


Fig. 42



RIBONUCLEIC ACID

Fig. 43. Adrenal from 21 day test animal. An area extending from the capsule to the inner zona fasciculata was found to be composed entirely of 'compact cells' rich in RNA in one animal. 'Clear cells', almost devoid of RNA are evident to the left and right of this area. Stained Methyl-Green Pyronin. X 100.

Fig. 44. Guinea-pig adrenal from 28 day test animal. RNA is still increased in all zones and is uniformly distributed. Deepest staining is evident in the zona glomerulosa. Stained Methyl-Green Pyronin. X 100.

Fig. 43.

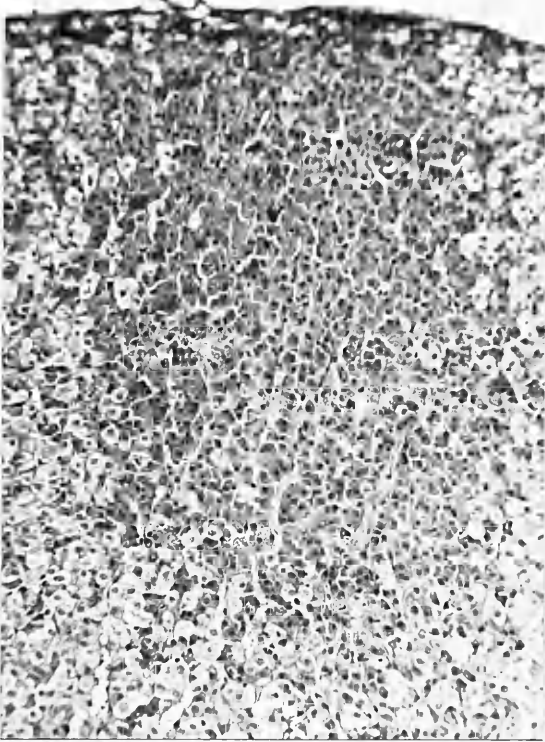
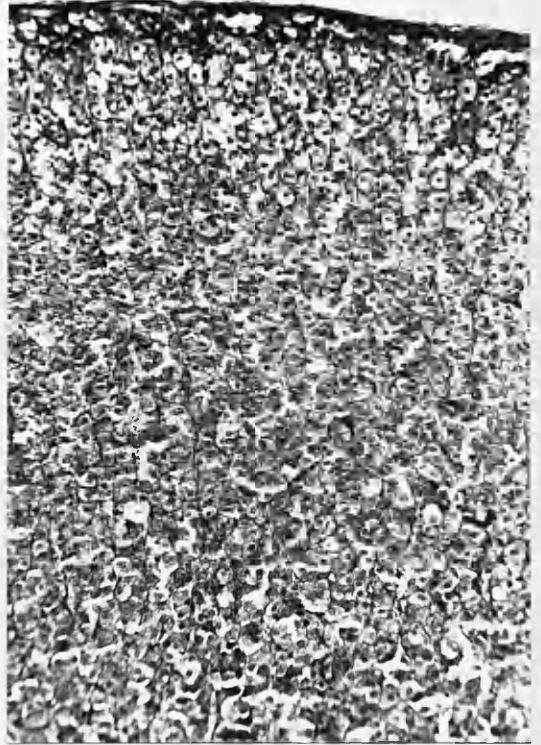


Fig. 44.



LIPID

Fig. 45. Control guinea-pig adrenal stained to show lipid. This is present in all zones but abundant in the zona fasciculata only.
Stained Sudan III. X 100.

Fig. 46. Guinea-pig adrenal from 18 hr test animal showing lipid distribution. Moderate lipid depletion had occurred in the zona fasciculata and zona reticularis. The latter zone shows an early reversion pattern, deposition of lipid having occurred in cells distributed singly and in small groups.
Stained Sudan III. X 100.

Fig. 45.

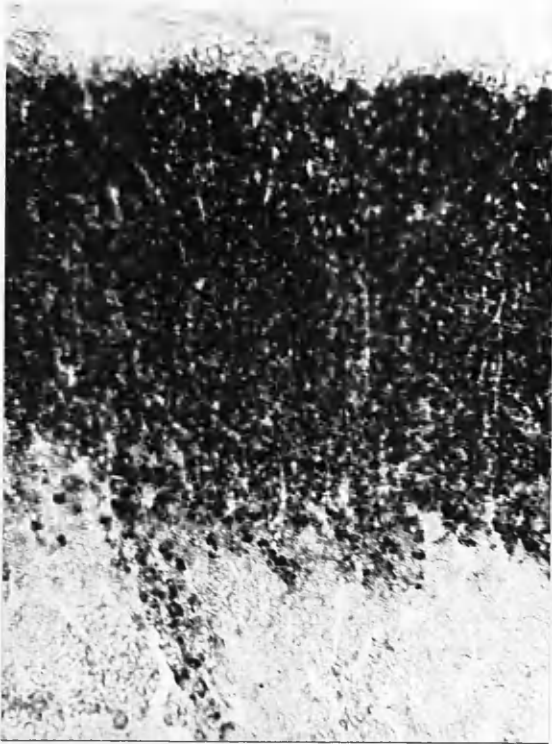
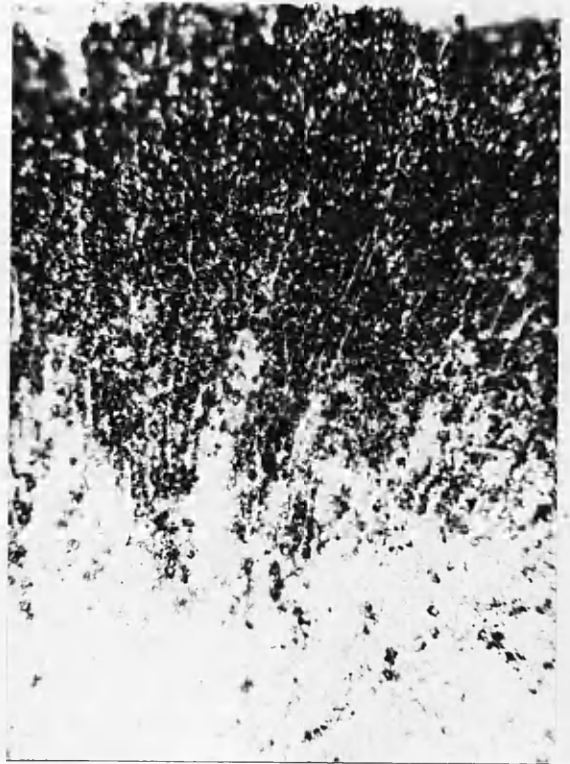


Fig. 46.



LIPID

Fig. 47. Guinea-pig adrenal from 1 day test animal. Lipid is present in normal amount in the zona fasciculata, and in greater than normal amount in the zona reticularis.

Stained Sudan III. X 100.

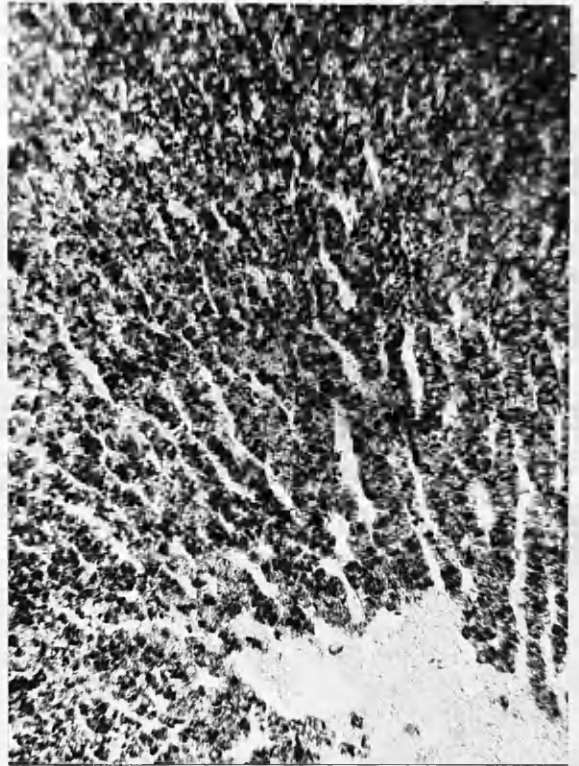
Fig. 48. Guinea-pig adrenal from 28 day test animal. The zona reticularis shows a patchy distribution of lipid which was present in this zone in greatest amounts in the adrenals of the 21 day test animals. The width of the zona reticularis is markedly increased.

Stained Sudan III. X 100.

Fig. 47.



Fig. 48.



PLASMALOGENS

Fig. 49. Control guinea-pig adrenal stained for 'plasmalogens'. These substances show a distribution which is essentially similar to that of lipids (Fig. 44). They are present in greatest amount in the zona fasciculata, and present in moderate amount in the zona glomerulosa and zona reticularis.
Plasmal technique. X 100.

Fig. 50. Adrenal from 18 hr test guinea-pig. Depletion of plasmalogens is evident in the zona fasciculata. They are present in greater than normal amount in the zona reticularis.
Plasmal technique. X 100.

Fig. 49.

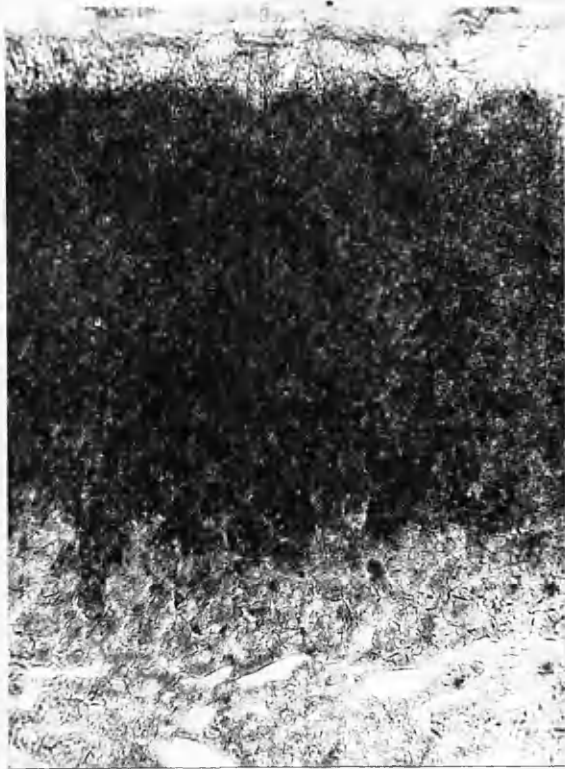
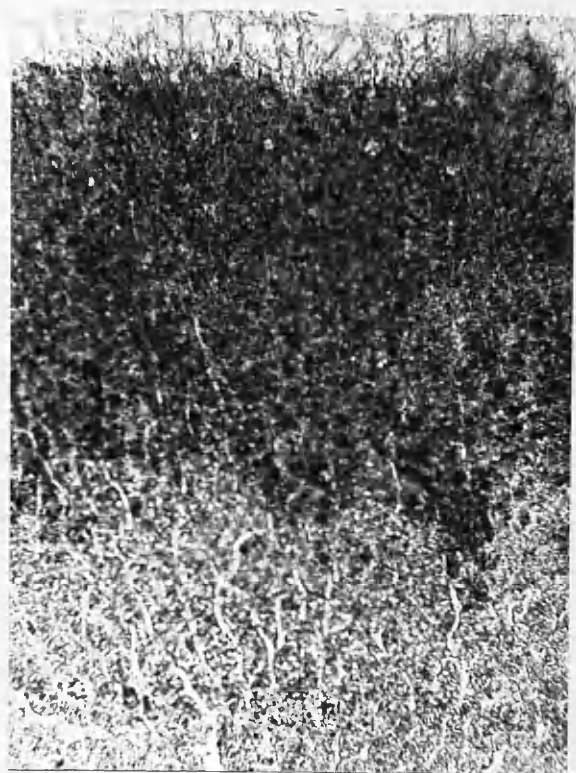


Fig. 50.



PLASMALOGENS

Fig. 51. Adrenal from 21 day test animal. Plasmalogens are present in moderate amount in the zona fasciculata and outer zona reticularis, but in small amount in the zona glomerulosa. The diminution in concentration of these substances was accompanied by a marked increase in adrenal weights. Plasmal technique. X 100.

Fig. 52. Inner zona reticularis from 28 day test guinea-pig. Plasmalogens are depleted in most cells of the inner parts of this zone. Plasmal technique. X 100.

Fig. 51.

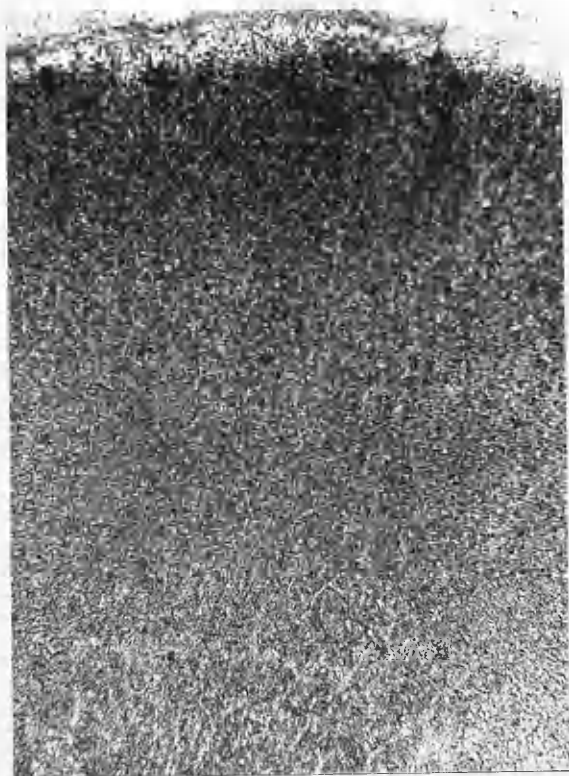
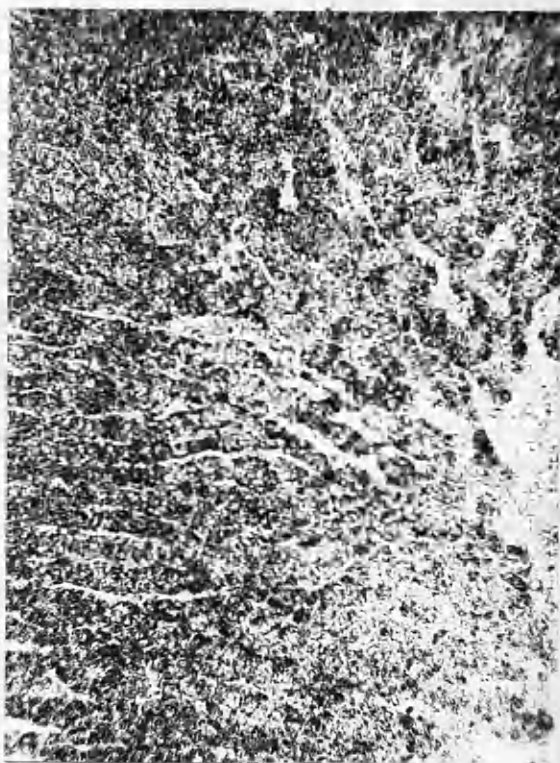


Fig. 52.



ALKALINE PHOSPHATASE

Fig. 53. Control guinea-pig adrenal stained for alkaline phosphatase. This enzyme is present in maximal concentration in the zona glomerulosa, and in small amounts in the zona fasciculata and zona reticularis. Azo dye coupling method (Brentamine Fast Red TR salt, I.C.I.). X 100.

Fig. 54. Adrenal from 6 hr test animal. Alkaline phosphatase is increased in the zona glomerulosa and outer zona fasciculata. Azo coupling method as for Fig. 53. X 100.

Fig. 53.

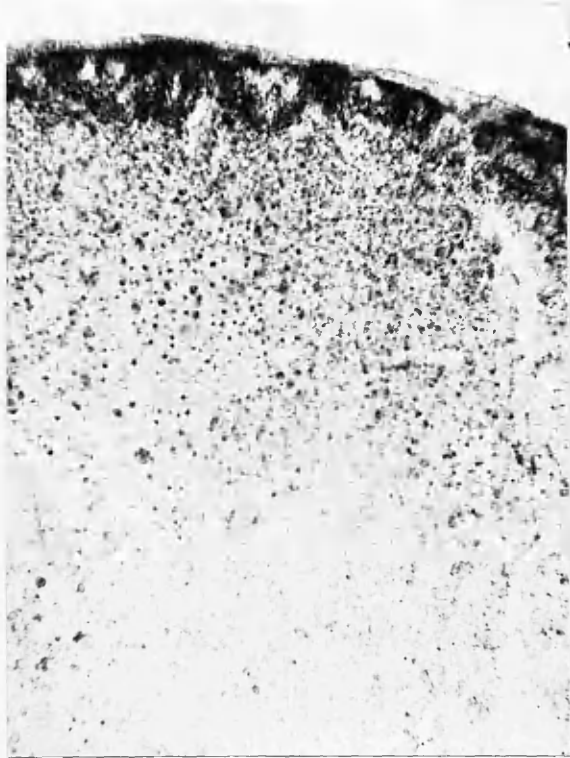
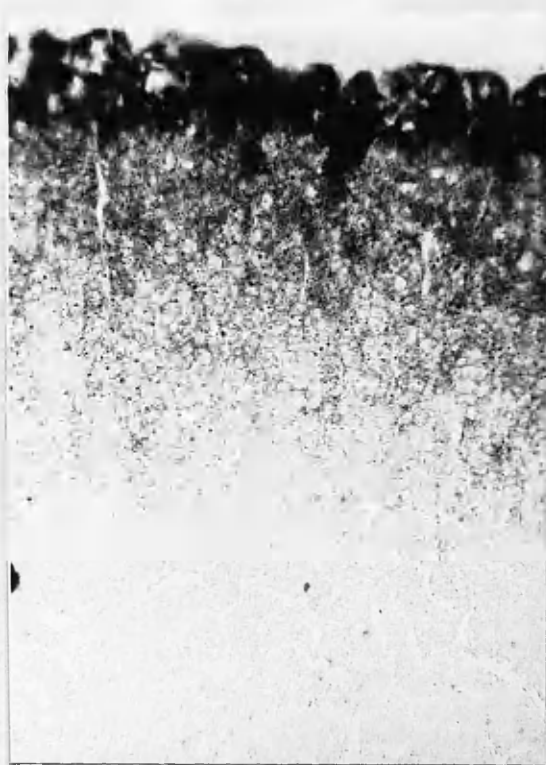


Fig. 54.



ALKALINE PHOSPHATASE

Fig. 55. Adrenal from 5 day test guinea-pig. Abundant alkaline phosphatase is present in the zona glomerulosa and most of the zona fasciculata. Azo dye coupling method as for Fig. 53. X 100.

Fig. 56. Adrenal from 7 day test guinea-pig. A further increase in alkaline phosphatase content of the zona fasciculata is evident. An increase in enzyme content of the zona reticularis has also occurred. Azo dye coupling method as for Fig. 53. X 100.

Fig. 57. Adrenal from 28 day test animal. Alkaline phosphatase concentration has diminished in all zones, but is still greater than normal in the zona fasciculata and zona reticularis. Azo dye coupling method as for Fig. 53. X 100.

Fig. 55.

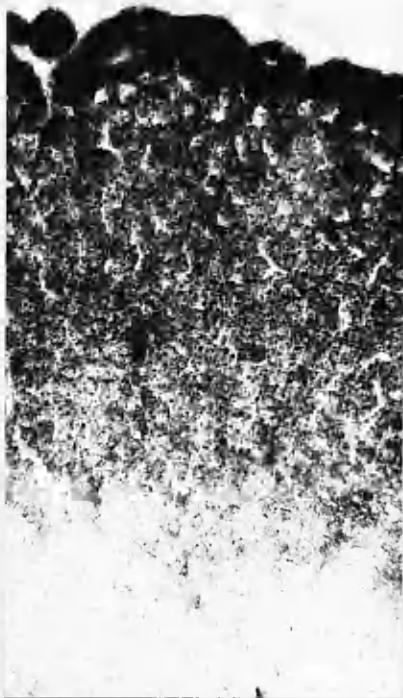


Fig. 56.

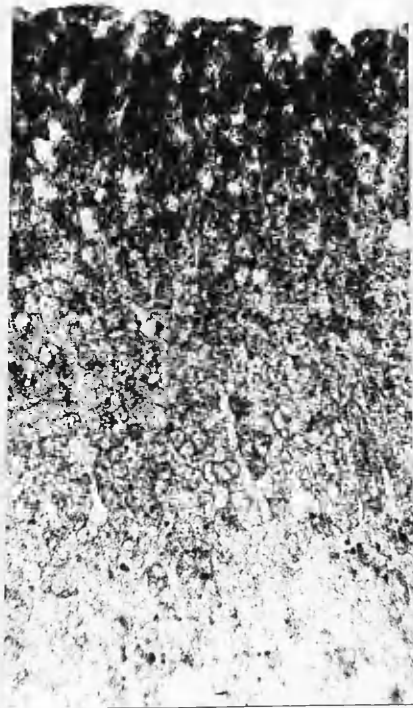
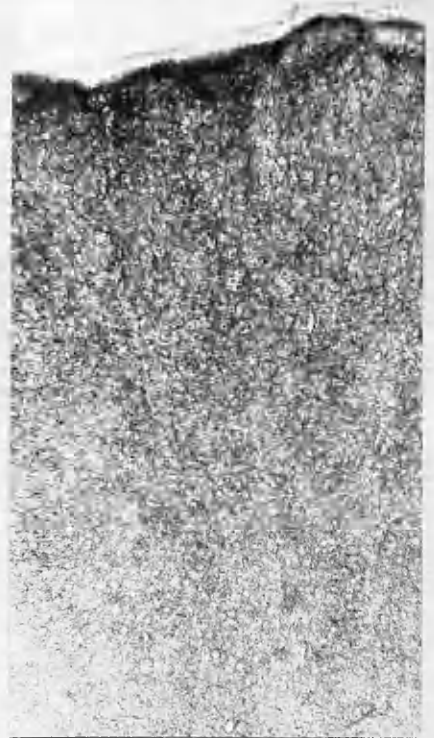


Fig. 57.



ALKALINE PHOSPHATASE

Fig. 58. Alkaline phosphatase in control guinea-pig adrenal. Azo dye coupling using Brentamine Fast Blue B salt (I.C.I.). The enzyme is abundant in the zona glomerulosa and the outer zona fasciculata. Staining of cell walls and crystal formation occurred not infrequently. The density of staining did not facilitate observations of variations in concentration of the enzyme.
X 100.

Fig. 59. Adrenal from 5 day test guinea-pig. Alkaline phosphatase content of the zona fasciculata is increased. Azo coupling method as for Fig. 58. X 100.

Fig. 58

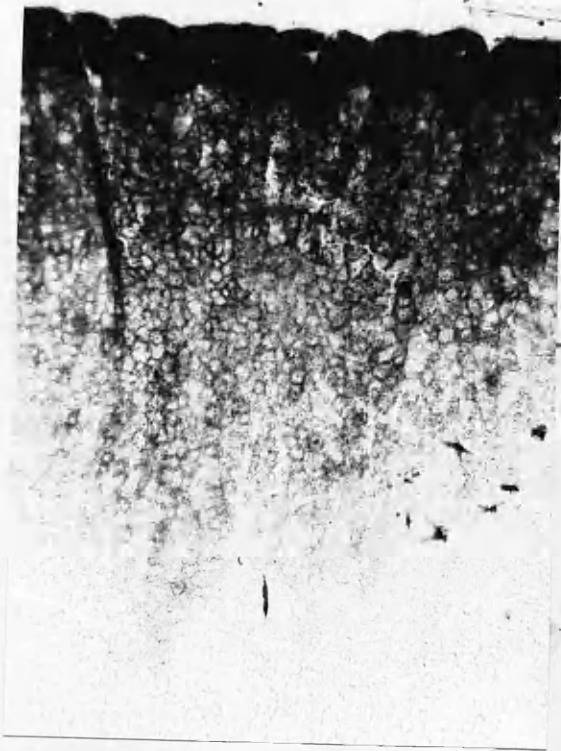
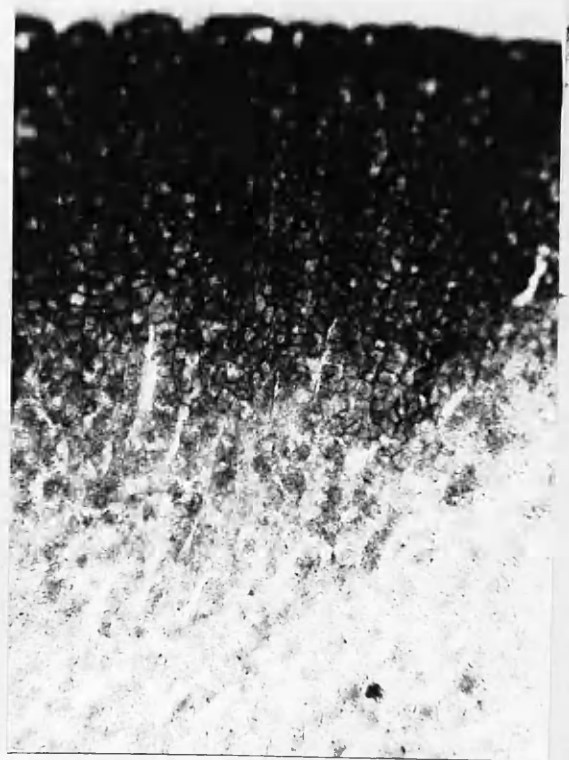


Fig 59.



ACID PHOSPHATASE

Fig. 60. Adrenal from 3 day test guinea-pig showing acid phosphatase content and distribution. The enzyme is present in greatest amount in the zona glomerulosa, and in moderate amount in the zona fasciculata. Control adrenals showed very little acid phosphatase and sections were unsuitable for photomicrography.

Azo dye coupling method using Brentamine Fast Red RC salt. X 100.

Fig. 61. Adrenal from 7 day test guinea-pig. Acid phosphatase is present in high concentration in the zona glomerulosa and in moderate concentration in the zona fasciculata and zona reticularis. Method as for Fig. 60. X 100.

Fig. 62. Adrenal from 14 day test guinea-pig. Acid phosphatase present in moderate concentration in all zones. Method as for Fig. 60. X 100.

At 21 and 28 days concentration had diminished and was similar to normal.

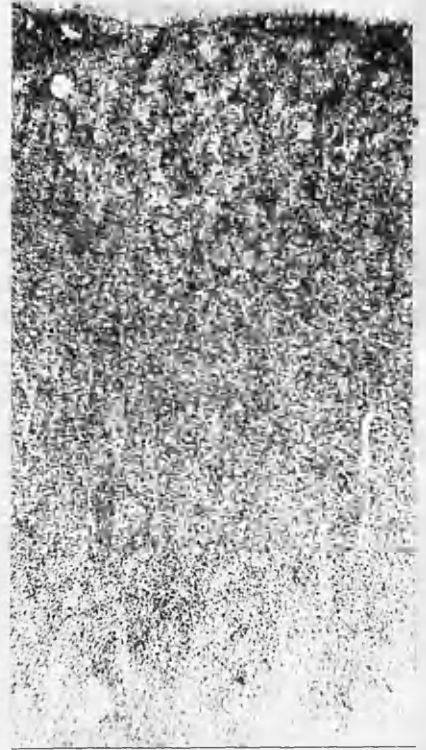
Fig. 60.



Fig. 61.



Fig. 62.



ASCORBIC ACID

Fig. 63. Control guinea-pig adrenal stained for ascorbic acid. Granules are present in moderate amount in the zona glomerulosa and zona fasciculata.

Acid silver nitrate method. X 1,000.

Fig. 64. Zona reticularis of control guinea-pig adrenal stained for ascorbic acid.

Maximum concentration of granules is normally found in this zone. Aggregations of granules sometimes occurred in the walls of sinusoids.

It is considered likely that some brown granules found are composed of "pigment" which occurs normally in the zona reticularis of the guinea-pig adrenal.

Acid silver nitrate method. X 1,000.

Fig. 63.

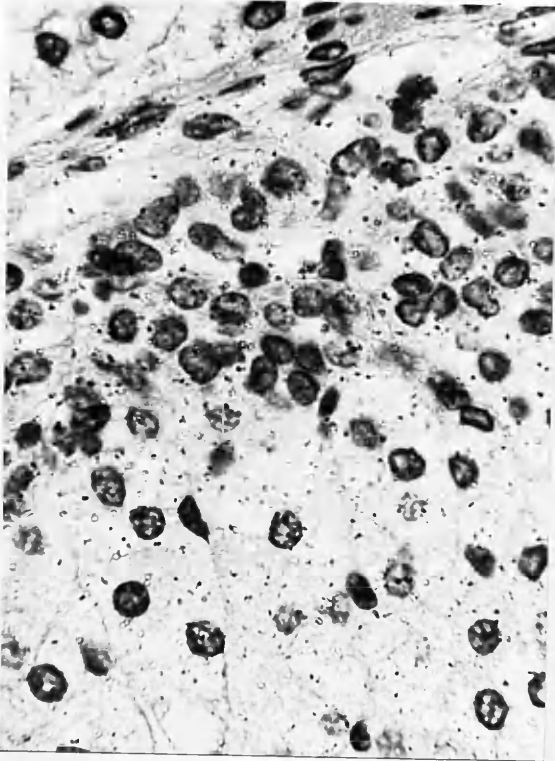
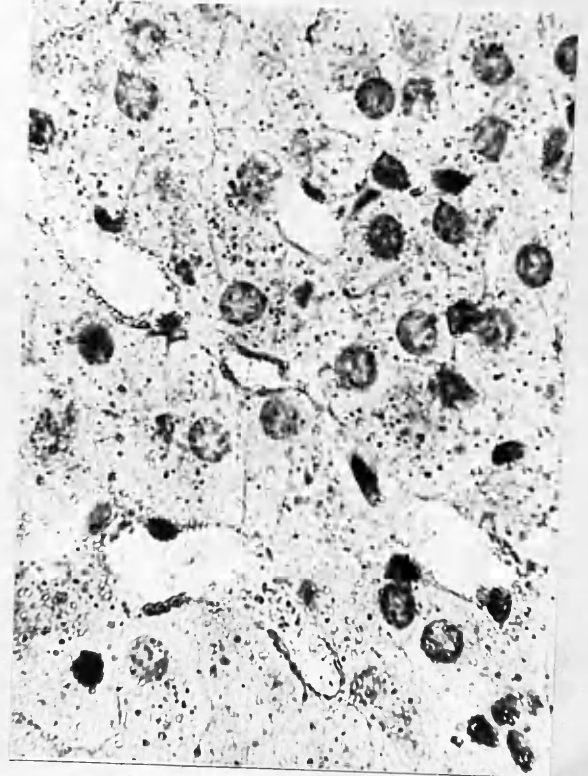


Fig. 64.



ASCORBIC ACID

Fig. 65. The zona glomerulosa and zona fasciculata of 3 hr test guinea-pigs stained to show ascorbic acid. Diffuse depletion of stainable material had occurred. A light diffuse staining of cell cytoplasm was sometimes observed as in the left half of this photomicrograph. Acid silver nitrate method. X 1,000.

Fig. 66. Zona reticularis of adrenal shown in Fig. 65. Diffuse depletion of ascorbic acid had occurred. Acid silver method. X 1,000.

Fig. 65.

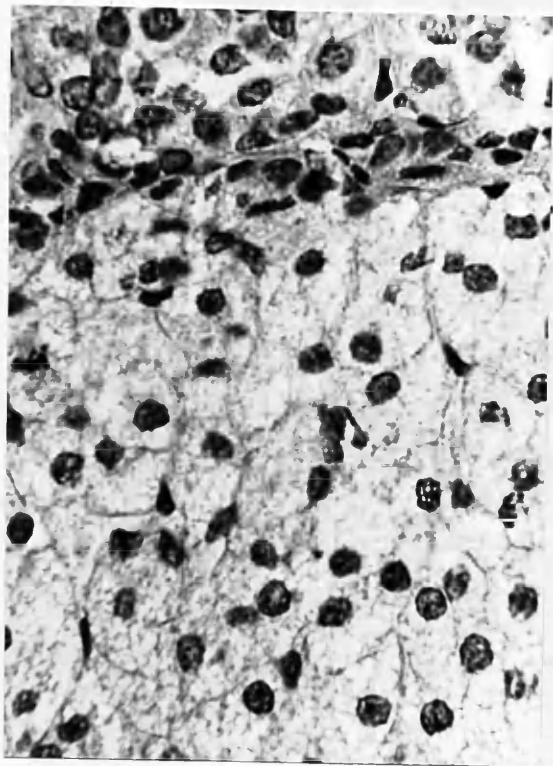
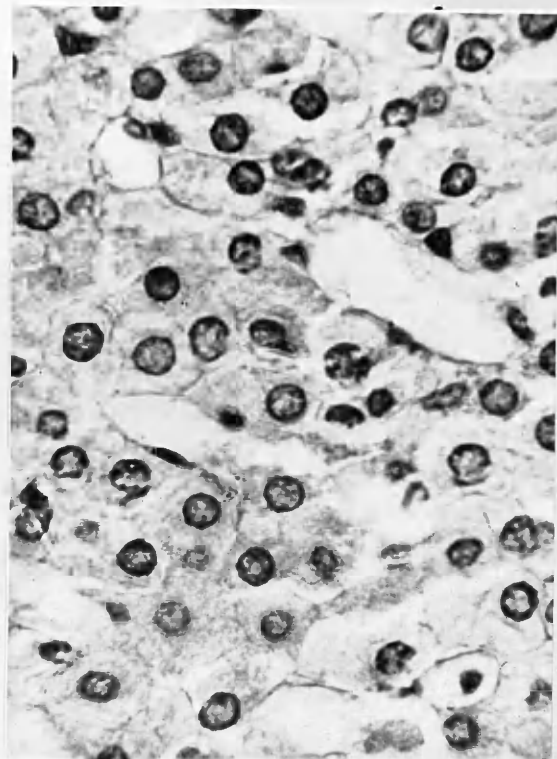


Fig. 66



ASCORBIC ACID.

Fig. 67. Zona glomerulosa and zona fasciculata of 3 day test guinea-pig. Ascorbic acid is present in moderate amounts in these zones, but still in subnormal concentration. In animals treated with ACTH for longer periods ascorbic acid was present in normal amounts.
Acid silver nitrate method. X 1,000.

Fig. 68. Zona reticularis of adrenal shown in Fig. 67. Ascorbic acid content is similar to normal. Increased concentration of "pigment" which occurs normally in this adrenocortical zone is evident.
Acid silver nitrate method. X 1,000.

Fig. 67.

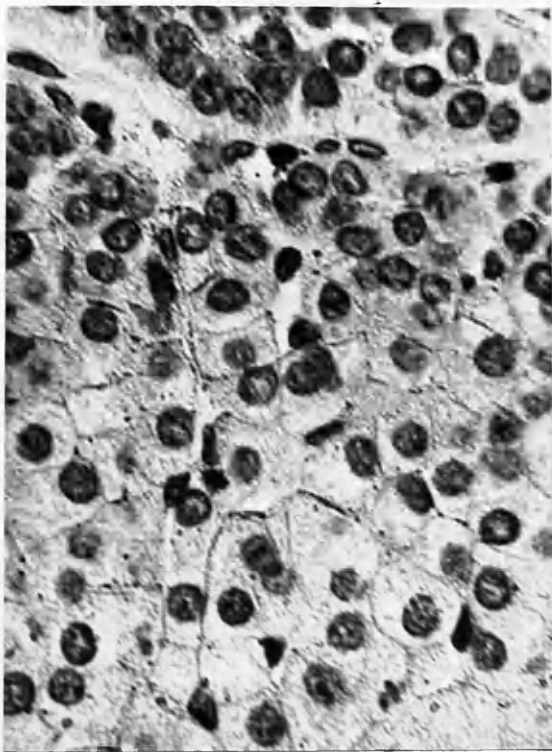
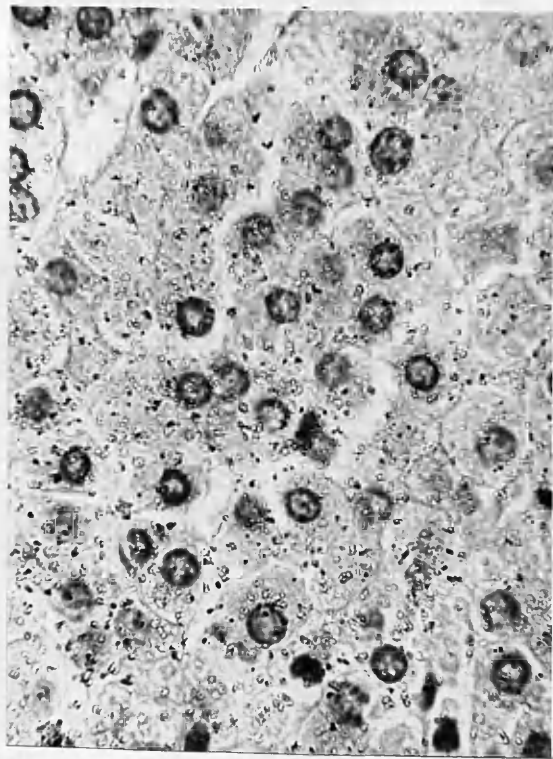


Fig. 68



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