The endocrinological control of metabolism in the upstream migrating phase of the life cycle of the river lamprey, L. fluviatilis.

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THE ENDOCRINOLOGICAL CONTROL OF METABOLISM IN THE UPSTREAM MIGRATING PHASE OF THE LIFE CYCLE OF THE RIVER LAMPREY, L. *FLUVIATILIS*

Submitted by

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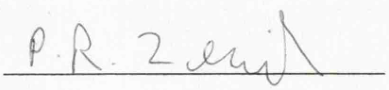

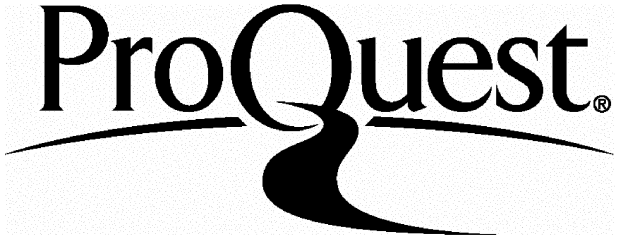

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SUMMARY

1. These studies were aimed at elucidating some of the endocrinological factors controlling metabolism in the upstream migrating stage of the life cycle of the river lamprey, *L. fluviatilis*.

2. Both subtotal and total extirpation of the islet tissue has been carried out. Blood glucose levels remain substantially unaffected in subtotal isletectomy, but in total extirpation of the islet tissue these rose to a mean level of 283 mg / 100 ml compared with a control value of 52 mg / 100 ml.

3. Insulin values for totally isletectomised animals 6 days after operation were about half those of controls.

4. Comparisons of the glucose tolerance curves for intact and totally isletectomised animals indicate that the operated animals have completely lost the ability to regulate blood glucose levels and that the return toward baseline values after 24 hr can probably be accounted for by urine losses.

5. The time course of the circulating levels of insulin after a single injection of glucose indicated significantly elevated values 8 and 24 hr after injection but not 2 hr after injection. Significant elevations of insulin levels were also observed after twice daily glucose loading for 12 days.

6. Hyperglycaemia has been demonstrated in lampreys subjected to a reduced oxygen tension of 20% air saturation for periods of up to 14 days. Maximum blood sugar values were recorded after 7 days hypoxia (mean 92.1 $\pm$ 30.9 mg/100 ml) compared with a mean value of 44.5 $\pm$ 2.3 mg/100 ml for controls. Similar tests over 7 days on hypophysectomised animals failed to show significant changes in glycaemia.

7. Circulating insulin values were reduced after adrenalin administration. Marked hyperglycaemia was also observed after injections of adrenalin.

8. After 7 days hypoxia, slight vacuolisation occurred in the islet tissue of only a few of the experimental animals, but at 14 days all showed extensive hydropic degeneration affecting the light cells. Equally severe lesions were also seen in all the hypophysectomised animals after only 7 days hypoxia, but did not appear in any of the control series that had been hypophysectomised 1-2 months previously and maintained under normal oxygen tensions.

9. Measurements of nuclear diameters of islet cells showed marked increases in nuclear volumes after 24 hr of glucose loading or after adrenalin injections, but not after hypoxia treatment.

10. Increased nuclear volumes were recorded in chromaffin tissue in the earlier stages of hypoxia and after glucose loading, but were not observed in hypophysectomised animals. No nuclear

enlargement was seen in the interrenal tissue under hypoxic conditions, but a slight increase in mean volumes occurred after glucose loading. In adrenalin injected animals, nuclear hypertrophy was very pronounced, probably accompanied by hyperplasia.

11. Glucagon-like immunoreactivity in the intestine has been confirmed by radioimmunoassay.

PUBLICATIONS

Some of the work described in this thesis has already been published and copies of the papers, whose titles are shown below, are included in the thesis.

HARDISTY, M.W., ZELNIK, P.R. and MOORE, I.A. (1975). The Effects of Subtotal and Total Isletectomy in the River Lamprey, *Lampetra fluviatilis*. *Gen. Comp. Endocrin.* **27**, 179-192.

HARDISTY, M.W., ZELNIK, P.R. and WRIGHT, V.C. (1976). The Effects of Hypoxia on Blood Sugar Levels and on the Endocrine Pancreas, Interrenal, and Chromaffin Tissues of the Lamprey, *Lampetra fluviatilis* (L.) *Gen. Comp. Endocrin.* **28**, 184-204.

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SECTION 1

INTRODUCTION

1. INTRODUCTION

The cyclostomes, the lampreys and the hagfishes, have affinities with the fossil ostracoderms from the Silurian and Devonian strata, one of the most primitive vertebrate groups. For this reason they are of great importance to comparative endocrinologists concerned with the origin and evolution of vertebrate endocrine systems. Much of the basic physiology of this class is still poorly understood.

Morphological and physiological differences between the two groups of cyclostomes, the myxinoids and the lampreys are so marked that Jarvik (1968), Hubbs and Potter (1971) and Stensiö (1968) have considered the class to be diphyletic and it may therefore be difficult to draw general conclusions applicable to both groups. Furthermore, since the cyclostomes have evolved along completely separate lines and are not the direct ancestors of the gnathostomes, general conclusions on the evolution of vertebrate endocrine systems drawn from information gained from the cyclostomes must be treated with some caution. In spite of this, the cyclostomes, as nearest extant relatives of the primitive ostracoderms, may provide some valuable clues to the condition of the endocrine system in the earliest vertebrates.

These studies are primarily aimed at elucidating some of the endocrine factors that control metabolism in the river lamprey, *Lampetra fluviatilis* in the upstream migrating stage of its life cycle.

1.1 ISLET TISSUE OF LAMPREYS

a) Historical

The existence of a swelling at the anterior end of the intestine of the adult river lamprey was first noted by Bojanus (1821). He was not certain whether this tissue was muscular or "glandular like the pancreas". The presence of this structure was confirmed by Rathke (1826). The first observations of larval islet tissue were made by Langerhans (1873) who noted groups of cells in the intestinal submucosa at the junction of the oesophagus and intestine. Because of their position close to the bile duct he considered that they might represent pancreatic tissue.

These cells, subsequently referred to as "follicles of Langerhans", were also considered by Brachet (1897) to correspond to islet tissue. This suggestion was supported by the investigations of the 1920's (Cotronei 1923; Boenig 1927, 1928, 1929) which showed that these cells had a histological appearance which implied an endocrine function. Barrington (1942) demonstrated that the cells stained rather feebly with Heidenhain's Azan, but in a way which showed that these cells had broadly similar tinctorial properties to the B cells of the islet tissue of higher vertebrates. In this study it was demonstrated that the follicle cells exhibited vacuolization subsequent to glucose injections, and hyperglycaeamia followed cauterization of the follicles. This provided the first experimental evidence that the follicles of Langerhans were involved in the control of carbohydrate metabolism.

Keibel (1927) and Boenig (1929) have demonstrated that the gland-like structure of the lamprey is partially derived from the larval follicles of Langerhans. During the 1920's there was controversy as to whether this tissue was homologous with the exocrine pancreas (Boenig,1929) or the endocrine islet tissue (Cotronei, 1927). This controversy was resolved by Barrington's work (1945) which showed that the cells of this tissue were histologically similar to the B cells of the islet tissue of higher vertebrates and cells homologous with the exocrine zymogen cells of the pancreas of higher vertebrates occurred in the intestinal epithelium.

Boenig (1929) in his studies on *L. planeri*, a non-parasitic species which does not feed at all in the adult phase of its life cycle, held that this tissue was functionless and degenerate in the adult. In the same species, Sterba (1955) also noted an indication of degeneration in the islet tissue of this species from the onset of metamorphosis. Ermisch (1966) was not, however, able to confirm these findings and showed that the structure of the islet tissue was broadly similar in the adult phases of *L. planeri* and *L. fluviatilis*. This author could find no evidence of functional degeneration in the adult brook lamprey *L. planeri*.

b) Development

The islet tissue in the ammocoete originates from cells at the junction of the oesophagus and the intestine. Follicles develop firstly within the mucosal epithelium which then penetrate the

FIGURE 1.1

Micrograph illustrating relationship of islet tissue (I) to intestinal diverticula (D) and oesophagus (O)

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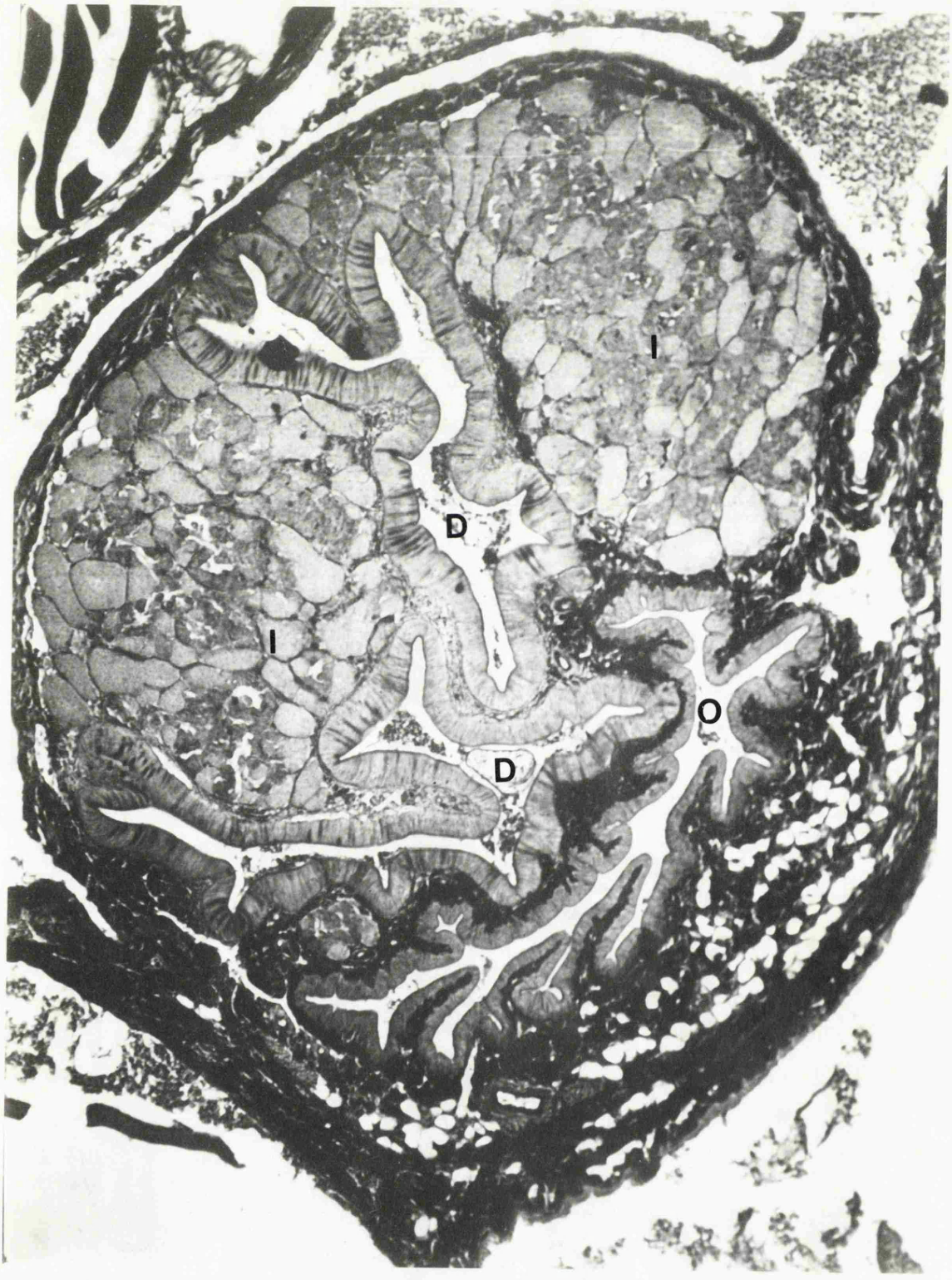
I
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submucosa. At metamorphosis this tissue forms solid clumps of cells which proliferate to form a distinct glandular nodule, the cranial pancreas, lying dorsal to the anterior intestine immediately adjacent to the pericardial wall (Barrington, 1972). At this point the intestine continues forwards for some distance above the caudal end of the oesophagus, where it breaks up into a series of diverticula lying amongst, but generally below, the cranial islet tissue (Figure 1.1). The bile duct, which degenerates at metamorphosis, gives rise to the caudal pancreas, which is embedded in the liver over the region where the intestine is attached to this organ and also to a few intermediate islets in the ventral part of the intestinal submucosa.

The most extensive study of the histogenesis of islet tissue has been that of Ermisch (1966) who distinguished four stages:

Stage I : Follicles maintain contact with alimentary epithelium and are not bound by a connective tissue capsule.

Stage II : Follicles are situated in the loose connective tissue of the submucosa. A follicular lumen is absent.

Stage III : As Stage II but follicles are large and have a lumen.

Stage IV : Adult follicles without lumen.

Van Noorden *et al* (1972) and Van Noorden and Pearse (1974) have shown by both *in vivo* and *in vitro* techniques that the islet cells of lampreys take up and decarboxylate the biogenic amines L DOPA and 5HT. These cells are thus possessed of some of the characteristics of the APUD - Amine Precursor Uptake and

Decarboxylation series (Pearse, 1968). It has been found by Pearse and his co-workers that many polypeptide secretory endocrine cells have APUD characteristics and this school has proposed that all such cells should be considered to belong to the APUD series. Pearse and Polak (1971) have reported that APUD-like cells were present in chick neural crest and in the mouse embryo. APUD cells appeared to migrate ventrally from the neural crest to invade the gut and its derivatives, including the pancreas. These authors have therefore, proposed a neural crest origin for all APUD-like cells in the gastro-intestinal tract and pancreas. However, since Phelps (1975) has found that pancreatic endocrine cells in the embryonic rat develop, even after the removal by trypsin of the ectoderm including the neural groove, prior to fusion of the neural folds, there is some doubt whether the hypothesis of Pearse and Polak (1971) applies to the B cells of the pancreas.

c) Histology, Cytochemistry and Electron Microscopy

Cotronei (1927) described "light" and "dark" cell cords, "cordoni chiari" and "cordoni oscuri" in the islet tissue of the sea lamprey *Petromyzon marinus*. He suggested that the light and dark cells might respectively represent the A and B cells of the islet tissue of higher vertebrates. Barrington (1945) failed to find any evidence for A cells and this was confirmed by Windbladh (1966) who was unable to demonstrate A cells with the silver

impregnation technique of Grimelius (1964) or Manochio's (1964) special method for A cells. These negative results were confirmed by Ermisch (1966) and Morris and Islam (1969a). Applying ultrastructural techniques no cells homologous with A cells were found by Windbladh (1966) and Van Noorden *et al* (1972) and Van Noorden and Pearse (1974), using immunofluorescent studies, failed to observe glucagon-like immunoreactivity in the pancreas, although such activity was found in the intestinal epithelium of adult lampreys.

The status of the light and dark cells is still a subject of some confusion. This is partly due to a doubt as to which cells should be regarded as "dark" cells. Ermisch (1966) considers the cells, which he calls granular aldehyde-fuchsin negative as equivalent to the "cordoni oscuri" of Cotronei. Windbladh (1966) and Morris and Islam (1969a), regard those cells which stain especially heavily with aldehyde-fuchsin and pseudo-isocyanin as the representatives of the "dark" cells. In this study the latter convention will be adopted. The "light" cells are distinguished from those cells by weaker staining reaction with aldehyde-fuchsin and pseudo-isocyanin. The ratio of light to dark cells is about 2 or 3 to 1 in the adult of *L. fluviatilis* (Windbladh, 1966). These two different cell types probably represent different development stages of the B cell (Barrington, 1972). Confusion of the nomenclature is compounded by the fact that there appear to be "dark" cells in the follicles of the *L. planeri* ammocoete. These are not aldehyde-fuchsin or pseudo-isocyanin positive but are derived from the connective tissue capsules (Morris and Islam, 1969a).

During the 1960's extensive histological and histochemical studies on lamprey islet tissue have been undertaken. Ermisch (1966) examined the larval and adult stages of *L. fluviatilis* and *L. planeri*; Windbladh has studied the adult stage of *L. fluviatilis*, whereas Morris and Islam (1969a) investigated the ammocoete of *L. planeri*. These studies have shown that the islet tissue of lampreys exhibit the general staining reaction of B cells with Heidenheim's Azan (Pantin, 1946) and chrome alum haematoxylin (Gomori, 1941). The more specific histochemical stains for insulin, such as dihydroxy-dinaphthyl disulphide (DDD) method for S-S bridges (Barnett and Seligmann, 1952) and the pseudoisocyanin role method for SS and SH groups (Schiebler and Schiessler, 1959) are also positive for lamprey islet tissue. The B cells of ammocoetes of *L. planeri* stain weakly with these stains (Morris and Islam, 1969a). This may be interpreted as indicating a low level of insulin storage in the B cell, which presumably is adequate to the continuous requirements of a filter feeder.

Immunofluorescent techniques have demonstrated an insulin-like immnoreactivity in the ammocoete (Van Noorden *et al*, 1972) and in the adult of *L. fluviatilis* (Van Noorden and Pearse, 1974).

Windbladh (1966) in her electron microscopic examination of the islet tissue of *L. fluviatilis* has distinguished three types of cell: granular cells, vesicular cells, and agranular cells. The granular cells have electron dense granules enclosed by a single membrane. The diameter (170 mm) and origin of these granules within a well developed ergastoplasm corresponds well with the B cell structure of higher vertebrates. Ergastoplasmic whorls are

found in this cell type and in the vesicular cells, which differ from the granular cells by having empty membranous vesicules instead of electron dense granules. These cells may represent immature B cells in which the granules are yet to be synthesized, or depleted B cells after the electron dense material has been extruded. Less probably, the vesicular cells may represent a distinct cell type. Occasionally agranular cells, devoid of granules or vesicles and with a poorly developed ergastoplasm, but rich in mitochondria are found. These cells may be undifferentiated reserve cells.

d) Experimental

Barrington's (1942) observations that glucose loading caused degranulation and vacuolization of the B cell in ammocoetes has been confirmed (Ermisch, 1966; Morris and Islam, 1969b). Vacuolization occurred in some adult *L. planeri* and *L. fluviatilis* after eleven daily glucose injections (up to 8 g / kg). In both ammocoetes and adults of *L. planeri* the terminal phase of serial glucose injections was necrosis, but this was not observed in adults of *L. fluviatilis*.

Bentley and Follett (1965a) and Morris and Islam (1969b) found that both adult *L. fluviatilis* and ammocoetes were capable of regulating their blood glucose concentrations when injected with glucose.

The effects of the diabetic agent alloxan was not specific to B cells but produced general toxic effects in larvae and adults.

Bentley and Follett (1965a) observed hyperglycaemia following alloxan administration, but reported that their animals were in poor condition and felt that their results may have been due to non-specific stress. Morris and Islam (1969b) found hyperglycaemia in the ammocoete after alloxan administration, together with histological evidence of hypersecretion and necrosis of the cells of the follicles of Langerhans. Windbladh Biuw (1970) in her studies on *L. fluviatilis* observed hyperglycaemia accompanied by degenerative changes in the islet tissue after alloxan administration. This study was unable to show any correlation between the magnitude of the alloxan dose and the degree of hyperglycaemia or severity of B cell lesions. The variability and non-reproducibility of the alloxan response renders data obtained from the use of this agent unsatisfactory and both the hyperglycaemia and the histological degeneration of islet tissue could just as well be secondary to general "stress" reactions caused by this agent, as a specific cytotoxical effect on the B cells.

Mammalian insulin is hypoglycaemic in adult *L. fluviatilis* (Bentley and Follett, 1965a). Liver glycogen was found to be elevated by these authors but the content of muscle glycogen remained constant. Ermisch (1966) produced convulsions, presumably hypoglycaemic, in ammocoetes and in the adults of both *L. fluviatilis* and *L. planeri* by insulin injection. The effects of mammalian insulin on the histology of the lamprey B cell in Ermisch's study were however, variable. Some animals showed an increase in B cell granulation accompanied by partial cell atrophy and reduction in

nuclear size, whereas others exhibited B cell degranulation with vacuolization and nuclear enlargement. Morris and Islam (1969b) similarly found no clear cut effect of insulin on ammocoete follicles but was able to show a fall in blood sugar levels. Leibson and Plisetskaya (1968) have carried out extensive studies on the biochemical effect of insulin but unlike Ermisch (1966) were unable to produce hypoglycaemic convulsions. This discrepancy may well be related to the dose of injected insulin. Ermisch (1966) injected up to 300 IU / kg, whereas Leibson and Plisetskaya (1968) injected up to 60 IU / kg. Leibson and Plisetskaya (1968) followed the time course of the insulin response. In animals injected with 30-60 IU / kg the hypoglycaemic response persisted for eleven days but when the dose was lowered to 15-20 IU / kg the duration of hypoglycaemia was reduced to seven days. These authors found that insulin had a variable effect on liver and muscle glycogen. Statistically significant results were obtained in one group of experiments but these were not reproduced in numerous subsequent trials. Such discrepancies may well be accounted for by the fact that these authors failed to take into account the sex of the experimental animals when evaluating their results in these studies. *In vitro* studies (Plisetskaya and Leibson, 1973) have shown that insulin significantly stimulated glycogen synthetase activity in skeletal muscle and liver of *L. fluviatilis*. This is difficult to reconcile with the apparently small effects of insulin on tissue glycogen *in vitro* but insulin may well play a large part in lipid as well as carbohydrate metabolism *in vivo* and lipogenesis may well explain this apparent inconsistency.

In extracts of islet tissue Rothwell and Fielding (1970) detected "insulin-like activity" capable of producing hypoglycaemia in both lampreys and rabbits.

Ermisch (1966) using a haemaglutination technique was able to demonstrate the presence of insulin-like immunoreactivity in the lamprey. Plisetskaya and Leibush (1972) found that bio-assay techniques (isolated rat diaphragm and epididymal fat pads in rats) were not sensitive enough to measure circulating levels of insulin-like activity but that radioimmunoassay was capable of detecting insulin-like immunoreactivity in lamprey blood. This level was lower in winter and spring than in the autumn. These results are open to criticism, since, unless it can be shown that mammalian insulin standards and the lamprey insulin unknowns have parallel dose-response curves, quantification of lamprey insulin is difficult, and the values of unknowns can only be compared with each other if such unknowns are determined in the same assay run. Injection of anti-insulin serum raised to mammalian insulin caused hyperglycaemia in the lamprey.

All these studies are generally consistent with the view that the B cells in both adult and larval lampreys produce a substance which is pharmacologically and immunologically similar to mammalian insulin. This substance has a definite hypoglycaemic effect in the lamprey and, it would seem, a physiological role in the regulation of carbohydrate and possibly lipid metabolism.

e) Comparison with Hagfishes

The islet tissue of *Myxine* forms a discrete organ at the point of entry of the bile duct into the intestine. This tissue, like the islet tissue of lampreys has cells homologous with the B cells of gnathostomes and similarly has no A or D cells (Falkmer and Windbladh, 1964). However, recent electron microscopical studies (Thomas *et al*, 1973) have demonstrated a rare second granular cell, whose granules are spherical in shape in contrast to the elipsoid granules characteristic of the B cells.

The islet tissue of the two groups of Agnatha have tumour-like cysts (Falkmer *et al*, 1973; Hardisty, 1976). Immunofluorescent studies (Ostberg *et al*, 1975) have shown that most of the cells of the hagfish islet tissue contain an insulin-like immunoreactivity. Hagfishes,like lampreys, exhibit hypoglycaemia after injection with mammalian insulin or extracts of islet tissue from their own species (Falkmer and Matty, 1966).

Neither isletectomy (Schirner *et al*, 1963; Falkmer and Matty, 1966) nor injection of antisera to mammalian insulin (Falkmer and Matty, 1966) produced hyperglycaemia. The failure of isletectomy to produce hyperglycaemia has until recently been thought to indicate the presence of extra-insular producing cells capable of maintaining normal glycaemia levels. These have been demonstrated (Ostberg *et al*, 1975) in the bile duct glucosa, using immunofluorescent techniques but the number of these cells was probably insufficient to maintain normal glycaemia levels after the removal of the islet organ.

The study of the chemical structure of hagfish insulin is far more advanced than that for lamprey insulin. Preliminary results of amino acid sequence studies show that A and B chains differ from mammalian insulins at about half the positions but those residues, which as a result of comparative biochemical studies are considered to be invariant throughout vertebrate evolution, are also conserved in the hagfish (Peterson *et al*, 1973; Steiner *et al*, 1973).

It has also been shown that, as in the higher vertebrates, insulin biosynthesis in the hagfish proceeds via proinsulin (Steiner *et al*, 1973).

f) Evolutionary Perspectives

Insulin-like activity is present in the gut of many protostomian and deutrostomian invertebrates and in the hepatopancreas of *Carcinus* (Falkmer and Patent, 1972) but invertebrate "insulins" have not been sufficiently characterised to determine whether they are more similar to insulin or proinsulin. The gnathostomes have a pancreas consisting of both exocrine and endocrine tissue, although in many teleosts the islet tissue forms discrete "Brockman bodies". Thus the cyclostomes represent an intermediate stage between the invertebrates where insulin producing cells do not apparently form discrete tissue and the gnathostomes where the endocrine islets are associated with exocrine pancreatic zymogen cells.

On reflection there seems to be no obvious teleological reason for the incorporation of islet tissue into the exocrine pancreas in higher vertebrates and Henderson (1969) has asked the provocative question "Why are the islets of Langerhans ?". Janowitz and Ruddick (1969) have found that glucagon has a significant inhibitory effect on secretin and pancreozymin-stimulated pancreatic juice in the dog. They have therefore, suggested that the distribution of the islets of Langerhans containing glucagon secreting A cells amongst the exocrine pancreatic tissue might facilitate the regulation of exocrine function by inhibition of pancreatic exocrine secretion. Evolutionary considerations appear to be consistent with this explanation. The agnatha do not have A cells in their islet tissues, nor are the islet tissues intimately associated with exocrine pancreatic tissues. The gnathostomes have A cells in the islet tissue which has also become associated with the exocrine pancreatic tissue. Thus the formation of true islets of Langerhans and the appearance of A cells in the islet tissue appears to be linked.

Steiner *et al* (1973) have proposed that the primitive mucosal cell of the digestive tract may have liberated a proinsulin-like protein, "protoinsulin", into the digestive tract. "Protoinsulin" was, according to this model, then hydrolysed in the digestive tract and reabsorbed into the bloodstream from the gut. In the course of time specialised cells (ancestral B cells) developed, in which both the biosynthesis of protoinsulin and the hydrolysis to insulin took place. If this model is correct, this must have happened at a stage of evolution before that of the ancestral cyclostomes since a fully functional B cell appears to be present in both the lamprey and the hagfishes.

1.2 INTERRENAL AND CHROMAFFIN TISSUES

(a) Interrenal

Giacomini (1902) first described interrenal tissue in the lamprey and this was confirmed by Gaskell (1912).

In ammocoetes and adults, larger groups of interrenal cells are found in the pronephric region on the dorsal side of the pericardium, although in smaller numbers, they may extend posteriorly in the mesonephric kidney tissue (Youson, 1972). In the ammocoete the pronephric interrenal tissue is present along the ventro-medial surface of the cardinal veins and on the ventral surface of the aorta, where they are often closely associated with chromaffin and pigment cells.

At metamorphosis the pronephric tubules degenerate, although the funnels remain intact. This degenerating tissue gives rise to adipose and vascularised sinusoidal tissue in which most of the interrenal cells are situated, but some small cell groups persist along the medial walls of the cardinal veins.

(i) Development

The embryological development of the interrenal of the lamprey is broadly similar to that of higher vertebrates (Poll, 1904; Sterba, 1955). Sterba (1955) recognised the presumptive interrenal tissue as a thickening of the coelomic epithelium in the angle

between the mesentary and the somatopleur in the pronephric region. This situation was contrasted by Sterba with that in elasmobranchs, where the interrenal has receded to the caudal mesonephros. Although the majority of the interrenal cells are of pronephric origin, the origin of the scattered groups of interrenal cells in the posterior region is still uncertain (Hardisty, 1972a).

(ii) Cytology, Histochemistry and Electron Microscopy

The most conspicuous feature of interrenal cells is the presence of lipid filled membrane bound vesicles of similar size to the nucleus which cause them, in Bouin fixed paraffin embedded sections, to present a "honeycomb" appearance. The nuclei have a pronounced chromatin pattern with a centrally placed nucleus.

Cholesterol, phospholipids and unsaturated lipids have been demonstrated histochemically, but attempts to localise Δ^{5}-3β hydroxysteroid dehydrogenase have given negative results (Seiler *et al*, 1970; Hardisty, 1972a).

More recently small amounts of 17α hydroxylase and 21 hydroxylase and 20 desmolase have been demonstrated biochemically and this tissue appears to be able to synthesise 11-deoxycortisol 17α-hydroxyprogesterone and an androstenedione, but not able to synthesise cortisol, cortisone, and corticosterone (Weisbart, 1975; Weisbart and Youson, 1976). The interrenal tissue seems therefore, to be steroidogenic but the spectrum of steroids produced are quite different from those of the adrenocortical tissues of higher vertebrates.

The electron microscope structure of the interrenal in *L. fluviatilis* is suggestive of steroidogenic tissue (Hardisty and Baines, 1971). The lipid vacuoles of light microscopy correspond to liposomes of 8μm diameter which are only faintly electron opaque. In addition to these major liposomes, are smaller lipid droplets of greater electron density. It is probable that the major liposomes contain precursor material and the smaller droplets secretory material.

The endoplasmic reticulum is agranular and there are free ribosomes scattered throughout the cytoplasm. The Golgi apparatus is rare and is found in cells with a relatively undeveloped vesicular structure. This is also true of the mammalian adrenal cortex: the cells of the zona glomerulosa, whose cells similarly have an undeveloped vesicular formation; have more developed Golgi bodies than those of other zones (Sabatini and de Robertis, 1961; Nishikawa, Murone and Sato, 1963; Idleman (1970).

The mitochondria are elongated and cylindrical, and of varied internal structure. At one end of the spectrum they have parallel arrays of narrow tubular cristae, while at the other extreme the cristae are of an irregular tubular or tubulo-vesicular type and the mitochondria are elongated with a dense matrix and intramitochondrial granules.

Thus, the lamprey interrenal presents all the ultrastructural characteristics typical of steroidogenic cells, namely an abundant endoplasmic reticulum, free ribosomes scattered throughout the cytoplasm, membrane bound lipid vesicles with tubular cristae and an electron dense matrix. The relationships between the mitochondria,

liposomes and the endoplasmic reticulum appear to be those of steroidogenic tissues of higher vertebrates.

Seiler *et al* (1970) thought that, since there is no evidence of steroidogenesis in the interrenal of lampreys, this tissue might be more akin to the corpuscles of Stannius than the adrenocortical tissues of higher vertebrates, but the corpuscles of Stannius present a completely different ultrastructural picture (Oguri, 1966; Fujta and Honma, 1967). They have a well developed rough endoplasmic reticulum and a prominent Golgi body; the corpuscles of Stannius therefore, present a picture which is characteristic of protein secreting tissues, rather than steroidogenic tissues.

(iii) Distribution and Numbers of Interrenal Cells

Sterba (1955) in his classical study, counted interrenal cells in serial sections on one side of the body of *L. planeri*. He noted marked increases in numbers during transformation through adult life until the spawning stage. For this reason Sterba concluded that the interrenal, under pituitary influence, was involved in the initiation of metamorphosis.

Hardisty (1972a) failed to confirm these results in *L. fluviatilis*. He found no evidence for an increase in cell numbers between the pre-metamorphic stage and the macrophthalmia stages and the increase in cell numbers between the macrophthalmia stage and mature upstream migrating phase was not marked. There was a great variability in individual cell counts.

The relative volume of the interrenal tissue of *L. planeri* (calculated from Sterba's (1955) data) is very small, 0.04 mg % body weight. Corresponding values in mouse, rat, and man are in the range of 10 mg % and 50 mg % and in the guinea pig the value is about 120 mg %.

(iv) Physiological Role

Chester Jones's (1963) report of relatively high levels of cortisol and cortisone in lamprey plasma has not been confirmed by the double isotope technique (Weisbert and Idler, 1970). Cortisol and aldosterone decreased sodium losses in the river lamprey, whereas sodium losses were increased by aldactone, an aldosterone inhibitor (Bentley and Follett, 1962, 1963). These authors have also found evidence for a pituitary-adrenal axis, since mammalian ACTH also decreases the rate of sodium loss. Cortisol, as the water soluble sodium succinate salt, increased the levels of blood glucose and liver glycogen. Single injections of ACTH did not alter blood glucose or liver glycogen but after daily administration in gelatine for three days, a decrease in liver glycogen was observed (Bentley and Follett, 1965a).

Sterba (1955) found interrenal hyperplasia following ACTH injection in *L. planeri* and this has been confirmed in the ammocoetes of *P. marinus* after repeated injections for seven days (Youson, 1973).

Youson (1973) found that daily ACTH injections (1 or 2 IU/animal) in ammocoetes of *P. marinus* initiated ultrastructural changes after two injections. These ultrastructural changes, reduced the number of lipid droplets and increased the number of mitochondria, and are strongly suggestive of stimulation of secretion.

Strahan (1969) has demonstrated that lamprey pituitary extracts have corticotrophic activity when tested in mice. Such extracts also cause moulting in the hypophysectomised toad (Larsen and Rothwell, 1972).

Hardisty, (1972b) has carried out quantitative cytological investigations on the response of the interrenal tissue to hypophysectomy and various forms of stress in adult *L. fluviatilis*. He found some evidence of hyperplasia in sham operation, hypophysectomy, light stress, osmotic stress, saline injection and ACTH injection. In osmotically stressed animals, saline injected animals and ACTH injected animals, hyperplasia was accompanied by nuclear enlargement, whereas a slight decrease in nuclear size (not observed in sham operated animals) was noted in hypophysectomised animals. Light stress caused a very highly significant reduction in nuclear size.

These experiments have not determined conclusively the extent of hypophysial control of the interrenal. Although nuclear diameters were increased after ACTH injection and decreased after hypophysectomy, an even larger increase was shown after injection with isotonic Ringer, and the decrease in nuclear size could be

attributed to post-operative hyperplasia. Unlike the ammocoetes of *L. planeri* (Sterba, 1955) or *P. marinus* (Youson, 1973), there is no evidence of hyperplasia in *L. fluviatilis* following ACTH injection.

The interrenal in lampreys appears to be involved in the response to "stress" but the nature of its secretion and the degree of hypophysial control over its function are problems that remain to be resolved.

(b) Chromaffin Tissue

The chromaffin cells follow a similar distribution pattern to the interrenal cells with which they are often closely associated (Giacomini, 1902; Gaskell, 1912; Sterba, 1955). These cells are very abundant in the heart and large blood vessels. The report (Johnels, 1956) that the chromaffin cells of the heart are innervated has not been confirmed by electron microscope studies (Caravita and Coscia, 1966). Dahl *et al* (1971) have demonstrated histochemically, by the Hillarp-Falk fluorescent technique, that biogenic amines are present in these cells.

The cytoplasm of the chromaffin cells contains membrane bound granules of a size (10 μm - 30 μm) and shape similar to those of the adrenal-medullary cells of higher vertebrates (Ostlund *et al*, 1960; Caravita and Coscia, 1966).

Noradrenaline seems to be the prominent circulating catecholamine in the plasma of *P. marinus* (Mazeaud, 1969).

Determination of catecholamine levels in lampreys under normal and stress conditions (Bloom *et al*, 1963; Stabrowsky, 1967; Dahl *et al*, 1971; Mazeaud, 1969, 1972) have shown increased adrenalin levels after asphyxia and forced swimming.

Hardisty (1972b) found indications of a proliferation of the chromaffin cells in response to "stress" which appeared concomittantly with interrenal hyperplasia. Intense cytoplasmic basophilia was also observed in light stressed and, to a lesser degree, in saline injected or osmotically stressed animals.

(c) "Stress" and Associated Endocrine Interactions

It appears that both interrenal and chromaffin cells are involved in the response to "stress" (Hardisty, 1972b). Moreover, "stress" induces hyperglycaemia in lampreys (Bentley and Follet, 1965a; Morris and Islam, 1969b; Leibson and Plisetskaya, 1968). There is, therefore, an interaction between interrenal and chromaffin tissues, whose secretions are hyperglycaemic, and the islet tissue whose product, insulin, reduces blood sugar levels. Such an interaction is confirmed by Hardisty's (1972b) observation of cyst formation and fatty degeneration in the islet tissues of stressed animals.

Plisetskaya and Pozorovskaya (1971) reported increases in plasma catecholamine levels as a result of insulin induced

hypoglycaemia. It therefore appears that the islet, chromaffin and interrenal tissues are all involved in the regulation of carbohydrate metabolism and that any modulation in carbohydrate metabolism caused by variation in one of these endocrine tissues induces a compensatory response in the others. The extent to which other endocrine organs may be involved in the control of metabolism is discussed in the next section of this Introduction.

1.3 OTHER ENDOCRINE TISSUES

(a) The Hypophysis

This section is confined to the hypophysial control of metabolism and does not include a detailed description of the embryology, morphology and cytology of the pituitary.

Larsen (1969, 1973) has shown that the development of the gonads and of secondary sexual characteristics are inhibited by hypophysectomy in *L. fluviatilis*, indicating a gonadotrophic function. Biochemical and physiological events in the upstream migrating phase of *L. fluviatilis* which could well be secondary to sexual maturation also appear to be under hypophysial control. Thus, the mobilisation of tissue from the body wall, the degeneration of the intestine, reduction in length, and the development of a green, bileverdin - loaded liver are inhibited by hypophysectomy. It remains to be determined whether such hypophysial control is exerted directly by gonadotrophins, indirectly by gonadotrophins through gonadal steroid release, or by a hypophysial hormone(s) other than gonadotrophin. Glycaemia levels, which Larsen found to be constant throughout the spawning run, was not affected by hypophysectomy.

Hardisty (1972b) has reported that degenerative changes in the islet tissue in sham operated animals appear to be considerably greater than in hypophysectomised animals. This could be explained by a hyperglycaemic tendency in sham operated animals,

due to catecholamine and interrenal secretion, consequent to surgical stress leading to degenerative changes in the islet tissue. This hyperglycaemic tendency may be reduced in hypophysectomised animals, hence the more normal appearance of the islet tissues of this group. Now it is well known that, in higher vertebrates the hyperglycaemia of pancreatectomised animals may be alleviated by hypophysectomy (because of the removal of the hyperglycaemic influence of ACTH and growth hormone); this is called the Houssay phenomenon. (Houssay and Biasotti, 1931).

Militating, to some extent, against this explanation is Larsen's (1976) report that stress hyperglycaemia was only slightly reduced in hypophysectomised animals as against intact controls. Similarly the hypoglycaemic response to insulin injection was only slightly greater in hypophysectomised animals than in intact control. Thus, Larsen (1976) concludes that the hypophysis exerts only a slight anti-insulinary effect, but her studies are open to the criticism that she compared her hypophysectomised animals with intact, rather than hypophysectomised controls, her hypophysectomised animals were subject to surgical stress as well as the removal of pituitary influences. It therefore, still remains to be resolved whether or not stress hyperglycaemia is under significant hypophysial control.

b) Glucagon-Like Immunoreactivity

Van Noorden and Pearse (1974), using immunofluorescent techniques, have shown glucagon-like immunoreactivity in the cells of the intestinal epithelium of the lamprey.

Assan *et al* (1969) have found glucagon-like immunoreactivity in mammals, birds, reptiles, amphibians, in the mesenteric extracts from the goldfish and the eel, from the ascidian tunicate *Cynthia papillosa*, in echinoderms and in hepato-pancreatic extracts of molluscs and the crab. Van Noorden and Pearse (1976) have found such immunoreactivity in the gut of *Amphioxus*.

The presence of glucagon-like immunoreactivity in the lamprey gut would therefore be consistent with the wide distribution of this activity throughout the animal kingdom.

1.4 THE AIMS OF THE PRESENT INVESTIGATION

These investigations are aimed at determining some of the endocrine factors which influence metabolism in the upstream migrating phase of the river lamprey. More particularly:

(a) to investigate the effects of cranial, caudal and total isletectomy on glycaemia levels, glucose tolerance characteristics, and interrenal and chromaffin cytology.

(b) to investigate the effects of hypoxia, and adrenalin administration on glycaemia levels, on interrenal and chromaffin cytology in intact and hypophysectomised lampreys.

(c) to compare the dose - c.p.m. curves for mammalian standard insulin, with those for lamprey islet extracts in the radioimmunoassay of insulin and to investigate the effects of experimental treatment (isletectomy, glucose loading and adrenaline treatment) on circulating levels of this insulin-like immunoreactivity.

(d) to confirm by radioimmunoassay the presence of a glucagon-like immunoreactivity in the gut.

SECTION 2

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 CAPTURE AND STORAGE OF EXPERIMENTAL ANIMALS

River lampreys were caught below the weir at Tewkesbury on the River Severn, or were obtained from the screens of the Power Stations at Berkeley or Oldbury-on-Severn in the course of their upstream spawning migration, which generally reaches its peak in the period between October and December (Hardisty and Potter, 1971). After transport to the laboratory, they were kept in large wire mesh live boxes, either in a lake or in a rectangular concrete basin, provided with a recirculation and filtration unit. Experimental animals were transferred to fibre glass tanks at least ten days before experimentation and these were held in a cold room at 9-10^{o}C. All tap water used was dechlorinated by continuous aeration and the tanks with surgically treated animals were provided with Eheim pump filters.

2.2 HYPOPHYSECTOMY

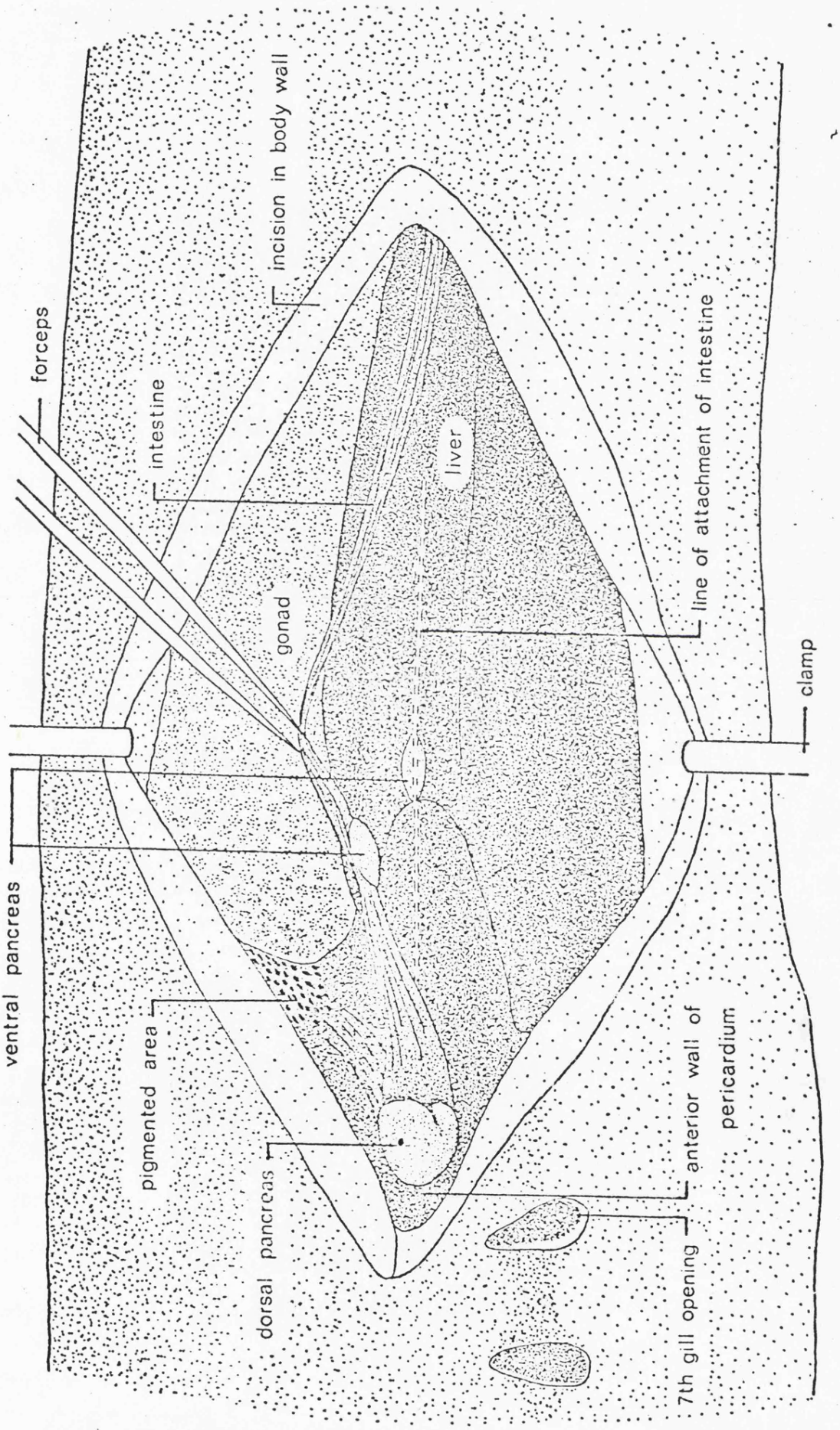
After prior anaesthetisation, hypophysectomy was carried out, following substantially the procedure described by Larsen (1965) and the extirpation of the adenohypophysis was completed by electro-cautery. During the autumn and winter, few post-operative mortalities occurred, but owing to a greater incidence of fungal infection, the survival rate was reduced after operations conducted during the spring.

2.3 ISLETECTOMY

Prior to operation the lampreys were immersed for five minutes in a 0.1% solution of MS 222 and where necessary, anaesthesia was maintained in the course of operation, by pipetting the solution on to the gill openings. After the animal had been laid in the operating dish on its right side, covered with a damp cloth, kept cool by ice, an incision was made on the left side of the body starting directly in front of, and some 3-6 mm above, the last gill opening and extending horizontally backwards for a distance of about 2 cm in the liver region (Fig. 2.1). The skin and muscle of the body wall were then cut through to expose the body cavity. On the anterior wall of the pericardium, the cranial pancreas may be identified as a slight swelling on the gut, at the point where the latter passes through the pericardial wall. This was then held in the forceps and trimmed away from the anterior end of the intestine and adjacent tissues using a fine pair of irridectomy scissors. To expose the caudal pancreas, the intestine was separated from the liver by cutting through its point of attachment to the dorsal surface. The caudal pancreas, partially embedded in the surface of the liver, could then be identified by its paler colour and was destroyed by electrocautery. The incision in the body was closed with a continuous silk suture and the animal returned to the tank. In the winter, mortality from the operations was very low and the animals were killed after periods varying from two to three weeks, but there is no reason to doubt that they would survive for longer periods, especially if fungus infection is successfully controlled. There were indications that, as with hypophysectomy, mortality is higher in late winter or early spring

FIGURE 2.1

Exposure of the islet tissue in the course of isletectomy as seen from the left side of the lamprey.


forceps
incision in body wall
intestine
liver
gonad
line of attachment of intestine
clamp
ventral pancreas
pigmented area
dorsal pancreas
anterior wall of pericardium
7th gill opening

and at these periods the majority of isletectomised animals were killed a week after operation.

2.4 GLUCOSE LOADING

In the author's experience, the hyperglycaemic response of lampreys to injection techniques is less marked when they have been previously anaethetised with MS 222 (Sandoz) immediately before injection. Other investigations have shown that this reagent may safely be used in experiments on carbohydrate metabolism in fish, where concentrations are controlled and anaethetisation is carried out within a brief and standardised time before sampling (Black and Connor, 1964; Crowley and Berinati, 1972).

In glucose loading experiments, the lampreys, after prior anaesthetisation for 5 min. in a 0.1% solution of MS22, were injected intraperitoneally with 100 mg of glucose as 0.2 ml of a 50% solution. For a 50 g lamprey this approximates to a dosage of 2 g per kg of body weight. Groups of animals were killed and blood samples were taken at various intervals between 2 and 24 h. after injection. Throughout the course of the experiments, control samples were taken from animals receiving an injection of 0.2 ml of 8.125% sodium chloride solution, which was equi-osmotic with the 50% glucose solution. In long term glucose loading, animals were injected twice daily with 100 mg of glucose for periods of 8 to 11 days.

2.5 ADRENALINE INJECTIONS

In experiments involving exogenous adrenaline administration, the animals were given twice daily, intraperitoneal injections of 0.1 mg adrenaline hydrogen tartrate as 0.1 ml of a 1 mg/ml solution. Loss of pigmentation generally occurred within a few hours of injection and became more pronounced with the passage of time. Beyond three days, the animals were in poor condition and the majority were killed after 4 days. Control animals were given the same number of injections of lamprey Ringer of the same volume as the adrenaline injection.

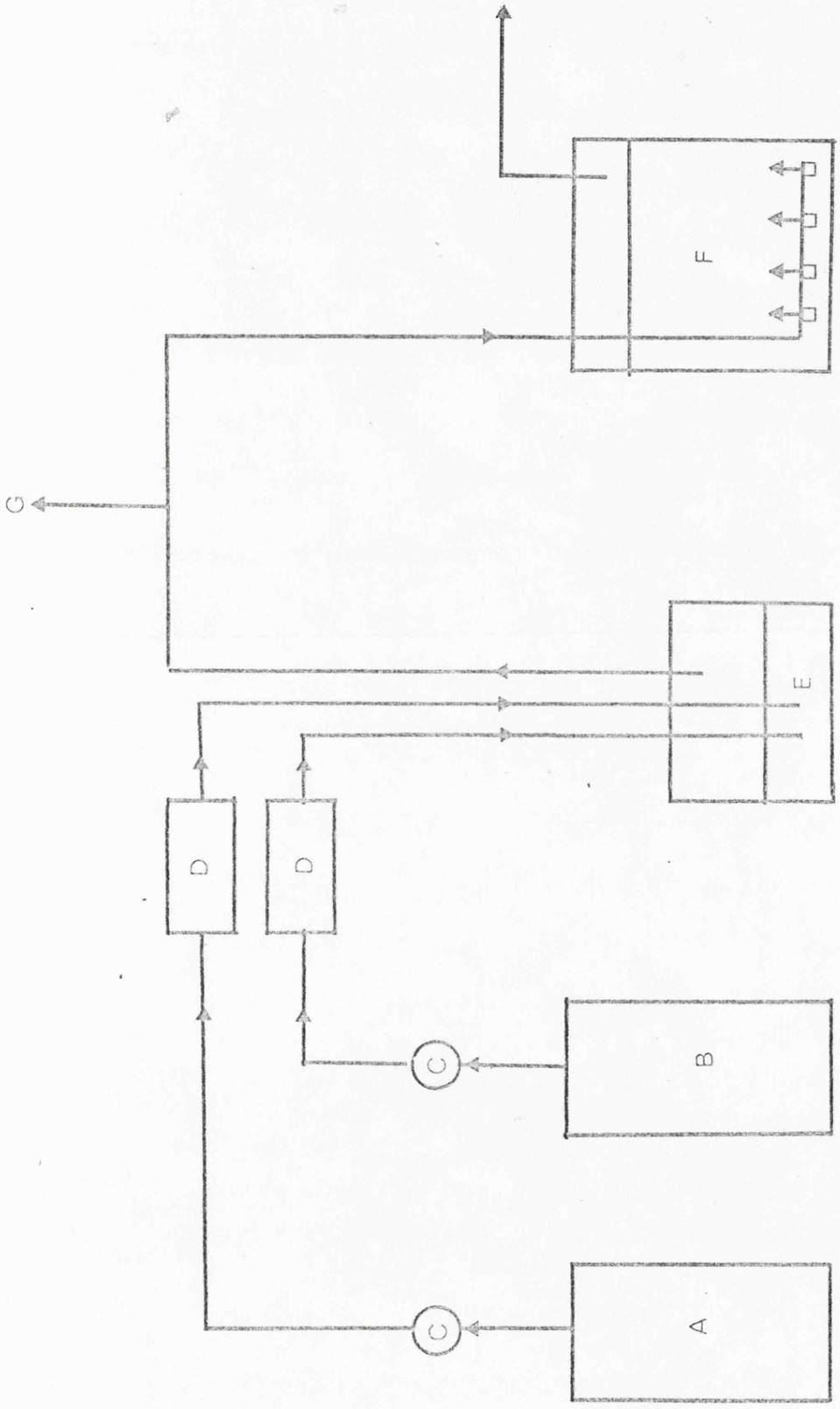
2.6 TECHNIQUE FOR MAINTAINING HYPOXIA

During the period of the experiment, lampreys were maintained at an oxygen concentration equivalent to a 20% saturation with air by employing appropriate volumes of compressed air and nitrogen. The compressed air and nitrogen were passed through a series of valves, before being bubbled through a mixing jar, containing water (Fig. 2.2). The valves were then adjusted until the required ratio of flowmeter readings was achieved. The mixture was bubbled through dechlorinated water in a large chromatography jar containing the experimental animals. To reduce the pressure required to maintain an adequate flow rate, the air/nitrogen mixture was bubbled through several diffuser stones in series, and the pressure was monitored with a mercury manometer. The concentration of oxygen in the water was checked during the experiments by the Winkler method.

FIGURE 2.2

Apparatus used in the maintenance of hypoxia.

A. Nitrogen cylinder.
B. Compressed air.
C. Valves.
D. Flow meters.
E. Mixing jar.
F. Chromatography jar containing experimental animals.
G. Mercury manometer.


A
B
C
C
D
D
E
F
G

2.7 GLUCOSE ANALYSIS

Blood samples were taken by cutting of the tip of the tail after prior anaesthetisation in 0.1% MS 222 for 5 minutes. Glucose analyses were carried out by the Glucose Oxidase-Perid method (Boehringer). Blood samples, collected in a centrifuge tube, were spun at 800 g, and 50 µl of the supernatant was deproteinised by the addition of uranyl acetate solution to make up the volume to 500 µl. This was then centrifuged at 800 g for 5 minutes and 100 µl of the supernatant assayed in accordance with the manufacturer's instructions. The method is capable of an accuracy of $\pm$ 3%. Since aliquots of the plasma sample were required for insulin radioimmuno-assays, duplicate glucose analyses were not routinely carried out. At the end of the 24 h glucose loading experiments, samples of the aquarium water were assayed for glucose and the total volume of water measured, to enable estimates to be made of urinary glucose excretion.

2.8 LIVER GLYCOGEN

Glycogen was determined by a modification of the method of Krebs *et al.* (1963). Approximately 500 mg of the liver tissue was washed for 3 min. in ice cold lamprey Ringer and then homogenised in sufficient acetate buffer (pH 4.5) to give a homogenate of 0.11 mg per ml. 0.5 ml standard glucose solution was added to 4 ml of acetate buffer. The following procedure was employed in duplicate for both standards and homogenates.

0.5 ml of the solution was incubated with 0.5 ml amyloglucosidase (1 mg / ml in acetate buffer, pH 4.5) at 55°C for 2 h. 0.2 ml of 0.3 M perchloric acid was added, which was neutralised after 5 min by adding 1 ml of 0.6 M potassium dihydrogen phosphate (pH 7.0). The neutralised solution was then centrifuged at 700 g for 4 min before removal of the supernatant for assay by the glucose oxidase method.

2.9 HISTOLOGICAL TECHNIQUES

For routine examination, the whole of the body containing the islet tissue was fixed for 24 hr in Bouin's solution and after paraffin embedding, sections were stained by the aldehyde-fuchsin technique (Gomori, 1950) in the modification of Cameron and Steele (1959) or by Pollak's (1944) trichrome technique. In early stages of the work, the effectiveness of the surgical techniques was checked by examining serial sections of partially or totally isletectomised animals.

2.10 ESTIMATES OF ISLET TISSUE VOLUMES

These were made by serially sectioning the pericardial and liver region, subsequently tracing the outline of the islet tissue at intervals of 5 or 10 sections, depending on the size of the animal. Cut-outs of these areas on drawing paper were then measured on an automatic area meter (Hayashi Denko, model AAM-5*) which is estimated to have an accuracy of $\pm$ 1%. The total volume was then estimated, taking into account the magnification factors and the appropriate intervals between successive tracings.

2.11 MEASUREMENT OF NUCLEAR DIAMETERS

Measurements of islet cell nuclei were made with a Filar ocular micrometer**. The nuclei were measured at random over the area enclosed by grid lines superimposed on the microscope field. Fifty nuclei were measured in each animal and the results expressed as the mean of the shorter and longer diameters. No attempt was made to differentiate between the cells of the 'light' and 'dark' cords.

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*U.K. Agents: Scientific Dimensions Ltd., Ford House, 54 High St., Fordingbridge, Hampshire SP6 1AX.

** American Optical Company, Buffalo, New York, U.S.A.

2.12 RADIOIMMUNOASSAY OF INSULIN

The kit (IM 78) supplied by the Radiochemical Centre was used, which contained:

^{125}I Insulin (not more than 10 μCu/l).

Freeze-dried insulin binding reagent (double antibody).

Human insulin for standards.

Freeze-dried buffer.

The binding reagent, buffer and human insulin were reconstituted according to manufacturer's instructions. The human insulin standard was diluted to 160, 80, 40, 20, 10 and 5 μM/ml.

100 μl aliquots of the standard or unknown were pipetted into polydystyrene tubes and then 100 μl of binding reagent was added. The tubes were vortex mixed and then incubated for 45 minutes at 2-4°C. 100 μl iodinated insulin was then added and the tubes were again mixed and incubated in the refrigerator for 2 hrs 15 mins.

700 μl of buffer was then added to each tube and then each tube was centrifuged at 1500 g for 25 minutes. The tubes were carefully removed and the supernatant drained off. The tubes containing the precipitate of bound insulin and bound iodinated insulin and bound iodinated insulin were counted in a gamma counter.

2.13 RADIOIMMUNOASSAY OF GLUCAGON

The kit, supplied by the Novo Research Institute, Copenhagen, contained the following:

Porcine glucagon for standard

^{125}I Porcine glucagon

Anti porcine glucagon rabbit serum (K44) non-specific.

Serial dilutions of the standard glucagon and the unknown samples wereprepared. 100 µl of the standard or unknown sample was added to each tube and then 100 µl of antiserum. The tubes were vortex mixed then incubated in the refrigerator for 20-24 hrs. The ^{125}I glucagon was then added and the tube was vortex mixed and incubated for 20-24 hrs. 1.6 ml of 96% ethanol was then added. The tubes were vortex mixed and then centrifuged for 10 minutes at 2000 g. The supernatant was carefully poured into a scintillation vial and 10 ml of Unisolve scintillant was added. The radioactivity was counted in a liquid scintillation counter.

SECTION 3

RESULTS

3. RESULTS

3.1 NORMAL ANIMALS

a) Islet Tissue Volumes

In both estimates for islet tissue volumes of adult *L. fluviatilis*, the caudal component was considerably larger than the cranial region, although the ratio of the two volumes varied from 1.9 in one animal to 6.1 in the other (Table 3.1). In the case of the individual with the high caudal/cranial ratio, the cranial tissue was necrotic and the larger volume of the caudal region may therefore be the result of compensatory hypertrophy. The single estimate for the macrophthalmia stage (a period in the life cycle after completion of the external metamorphic changes, but before the onset of downstream migration and feeding) shows that, even at this period, the caudal islet tissue is larger than the cranial component. On the other hand, in the two adult brook lampreys *L. planeri*, (a species which does not feed after metamorphosis), there is little, if any difference in the volumes of the two regions. This may reflect the general atrophy which overtakes the gut of the non-parasitic species during the onset of sexual maturation following almost immediately upon metamorphosis.

TABLE 3.1

VOLUMES OF CRANIAL AND CAUDAL ISLET TISSUE IN *Lampetra fluviatilis* AND *L. planeri*

SPECIES AND STAGE	BODY WT (g)	VOLUMES OF ISLET TISSUE mm^3			RATIO Caudal / Cranial	VOLUME OF ISLET TISSUE mm^3 / 100 g body wt.
		Cranial	Caudal	Total		
Lampetra fluviatilis						
Macrophthalmia	1.4	0.02	0.03	0.05	1.5	3.6
Adults	50.0	0.32	1.96	2.28	6.1	4.6
	33.5	0.48	0.09	1.38	1.9	4.1
Lampetra planeri						
Adults	2.7	0.07	0.07	0.14	1.0	5.2
	5.5	0.09	0.11	0.20	1.2	3.6

b) Blood Glucose

Records for the 1973/74 season, based on 127 animals of both sexes, gave a mean value of 51.9 $\pm$ 1.5 mg/100 ml glucose over the autumn and winter periods, with no detectable upward or downward trends throughout this time. The mean value for 76 males (49.8 $\pm$ 1.9 mg/100 ml) was below that of the 51 females (55.2 $\pm$ 2.5 mg/100 ml) but the difference is of only a low order of statistical significance ($t = 1.719$, $0.1 > P > 0.05$).

c) Liver Glycogen

Apart from a single report by Plisetskaya and Zheludkova (1971), there do not appear to be references to sex differences in liver glycogen levels. In the data for the 1973/74 season the mean value for 21 females was 141 $\pm$ 16.1 mg/100 g compared with a value of 261 $\pm$ 20.8 mg/100 g for 17 males, which is statistically highly significant ($t = 4.54$, $P < 0.001$).

3.2 COMPARISON OF HUMAN INSULIN STANDARD CURVE WITH LAMPREY ISLET TISSUE DILUTION CURVE

Lamprey islet tissue extracts (in .065%) saline) were serially diluted down to $^{1}/32$, and the c.p.m. for each dilution determined by radioimmunoassay. This dilution curve may be compared with the standard curve, (Table 3.2, Fig. 3.1). The slope of the standard curve is -0.009139, whereas that for the islet tissue dilution curve is -0.003943.

The variance of the slope of the standard curve is 4.42×10^{-7} and the variance of the slope of the dilution curve is 5.52×10^{-7}. The slopes of these curves are significantly different ($t = 17.3$ $P < 0.001$).

FIGURE 3.1

Comparison of human insulin calibration curve and lamprey islet tissue dilution curve.

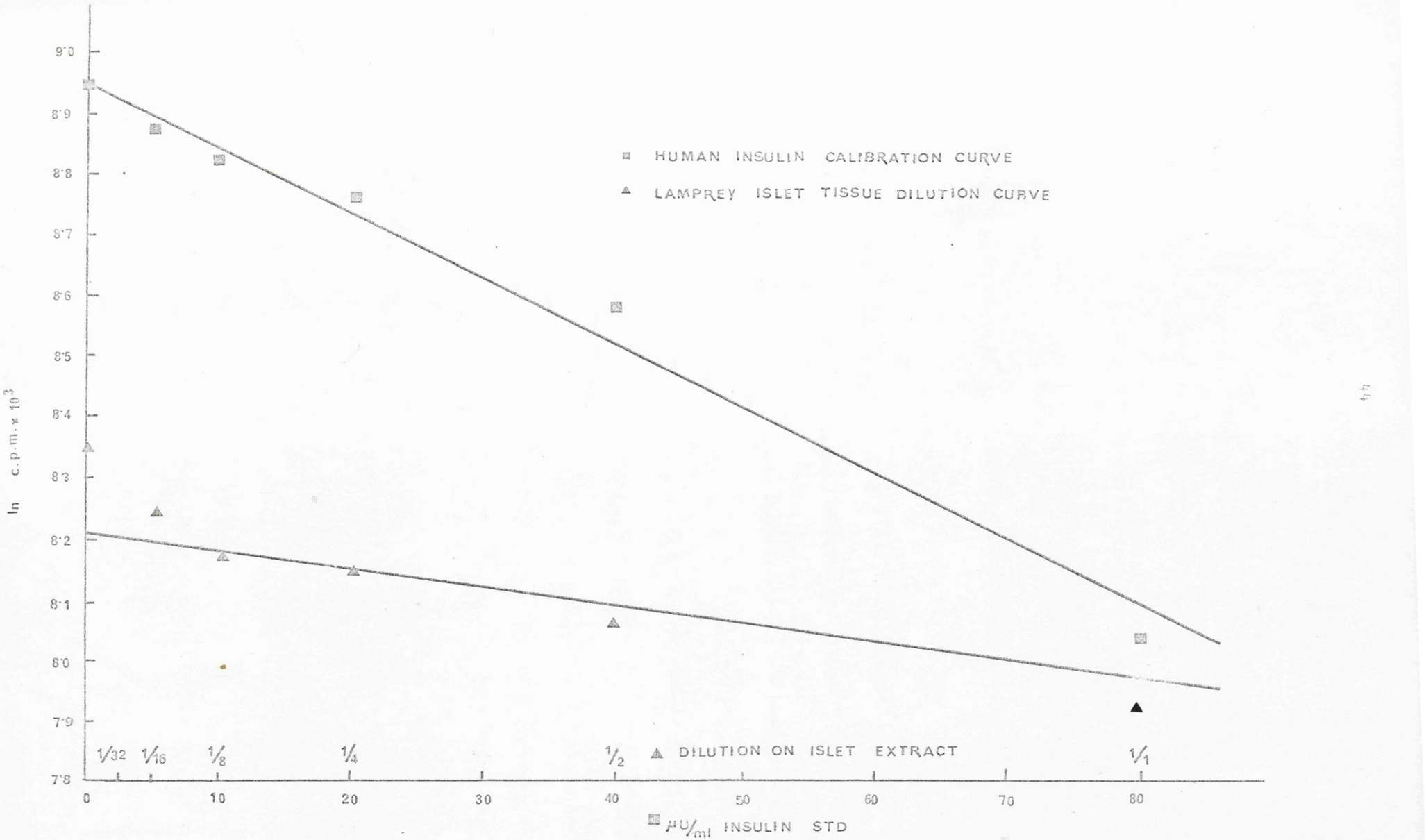

HUMAN INSULIN CALIBRATION CURVE
LAMPREY ISLET TISSUE DILUTION CURVE
ln c.p.m. × 10³
9·0
8·9
8·8
8·7
8·6
8·5
8·4
8·3
8·2
8·1
8·0
7·9
7·8
1/32
1/16
1/8
1/4
1/2
1/1
DILUTION ON ISLET EXTRACT
0
10
20
30
40
50
60
70
80
μU/ml INSULIN STD

TABLE 3.2 (A) INSULIN RIA STANDARD CURVE

CONC. μU INSULIN	0.0	5.0	10.0	20.0	40.0	80.0
cpm	7813.0 7575.0 7753.0	7299.0 7092.0 7134.0	6623.0 6757.0 6944.0	6536.0 6410.0 6289.0	5495.0 5291.0 5434.0	3745.0 3509.0 3624.0
ln cpm	8.9635 8.9326 8.9558	8.8955 8.6667 8.8726	8.7983 8.8183 8.8456	8.7851 8.7656 8.7466	8.6116 8.5738 8.6004	8.2282 8.1631 8.1953
Mean ln cpm	8.9507	8.8783	8.8207	8.7657	8.593	8.1955

Slope = -0.00913

TABLE 3.2 (B) LAMPREY PANCREAS DILUTION CURVE

DILUTION	1/32	1/16	1/8	1/4	1/2	1/1
cpm	4310.0 4115.0 4386.0	3953.0 3846.0 3636.0	3521.0 3584.0 -	3448.0 3389.0 -	3226.0 3125.0 2941.0	2976.0 2933.0 -
ln cpm	8.3687 8.3224 8.3362	8.2822 8.2548 8.1986	8.1665 8.1842 -	8.1455 8.1283 -	8.0790 8.0472 -	7.9983 7.9838 7.9865
Mean ln cpm	8.3591	8.2452	8.1754	8.1369	8.0631	7.9865

Slope = - 0.003943

3.3 PARTIAL AND TOTAL ISLETECTOMY

a) Blood Glucose

Blood glucose values are available for 22 lampreys of both sexes which had undergone total isletectomy and were sampled at periods from 1-3 weeks after operation (Table 3.3). Although there was a wide range of blood sugar levels, there were no indications that values increased or decreased with the lapse of time between operation and blood sampling. In over half the experimental animals, the blood glucose concentration exceeded 300 mg/100 ml and the mean was 283 $\pm$ 17.1 mg/100 ml with a range from 72-383 mg/100 ml.

In subtotal isletectomy, removal of cranial islet tissue was attempted in 14 animals and in this group (Table 3.3), values varied from 21-69 mg/100 ml with a mean of 42.9 $\pm$ 4.2 mg/100 ml. Although this is lower than the control value, the difference is not statistically significant. After caudal isletectomy, the range and mean values were rather higher than in the cranially isletectomised lampreys (32-85 mg/100 ml and 51.8 $\pm$ 5.4 mg/100 ml), but neither the differences between the means of the two operated groups nor the comparison with the control group is significant.

TABLE 3.3

BLOOD GLUCOSE AND LIVER GLYCOGEN CONCENTRATIONS AFTER SUB-TOTAL AND TOTAL ISLETECTOMY

	BLOOD GLUCOSE mg / 100 ml ± S.E.		LIVER GLYCOGEN mg / 100 g ± S.E. (Males only)	
	N		N	
Intact Controls	127	52.0 ± 1.5	17	261.1 ± 20.8
Cranial Isletectomy	14	42.9 ± 4.2	9	274.9 ± 27.8
Caudal Isletectomy	12	51.8 ± 5.4	7	59.1 ± 12.8
Total Isletectomy	22	283.0 ± 17.1	12	260.2 ± 40.3

b) Glucose Tolerance in Isletectomised Animals

Glucose tolerance experiments were carried out on 24 lampreys that had been completely isletectomised a week previously. Groups were sampled at intervals of 2, 8 and 24 h after the initial injection. At 2 h individual values varied from 762-1650 mg/100 ml and in four cases exceeded 1000 mg/100 ml. Over the remaining period the decline in glycaemia levels appear to have been approximately linear, approaching initial baseline levels and becoming more uniform at 24 h (Fig. 3.2). Analyses of the water at the end of the 24 h period indicated losses of about 60% of the injected glucose, but because of the possibility of bacterial breakdown, this is likely to be a conservative figure.

c) Insulin Values

In Table 3.4 the insulin and blood glucose levels for completely isletectomised and sham operated controls 6 days after operation are compared.

The insulin determinations for all samples for both treatment groups were performed in the same assay run but this was a different run than that for the glucose tolerance and long term glucose loading data. The mean value of the isletectomised group was 5.7 μ/ml as compared with 11.4 for controls (Table 3.4). The analysis of variance table (Table 3.5) shows that the difference between the two treatment groups is significant. It is interesting to note that the animal (504) in the isletectomised groups with the lowest blood sugar has the highest insulin value.

FIGURE 3.2

Changes over 24 hr in blood sugar levels of totally isletectomised river lampreys (- □ - □ -) after glucose loading compared with intact glucose loaded animals (-● - ● -) and saline (8.125%) injected controls (- ■ - ■ -). The vertical bars represent standard errors of the means.

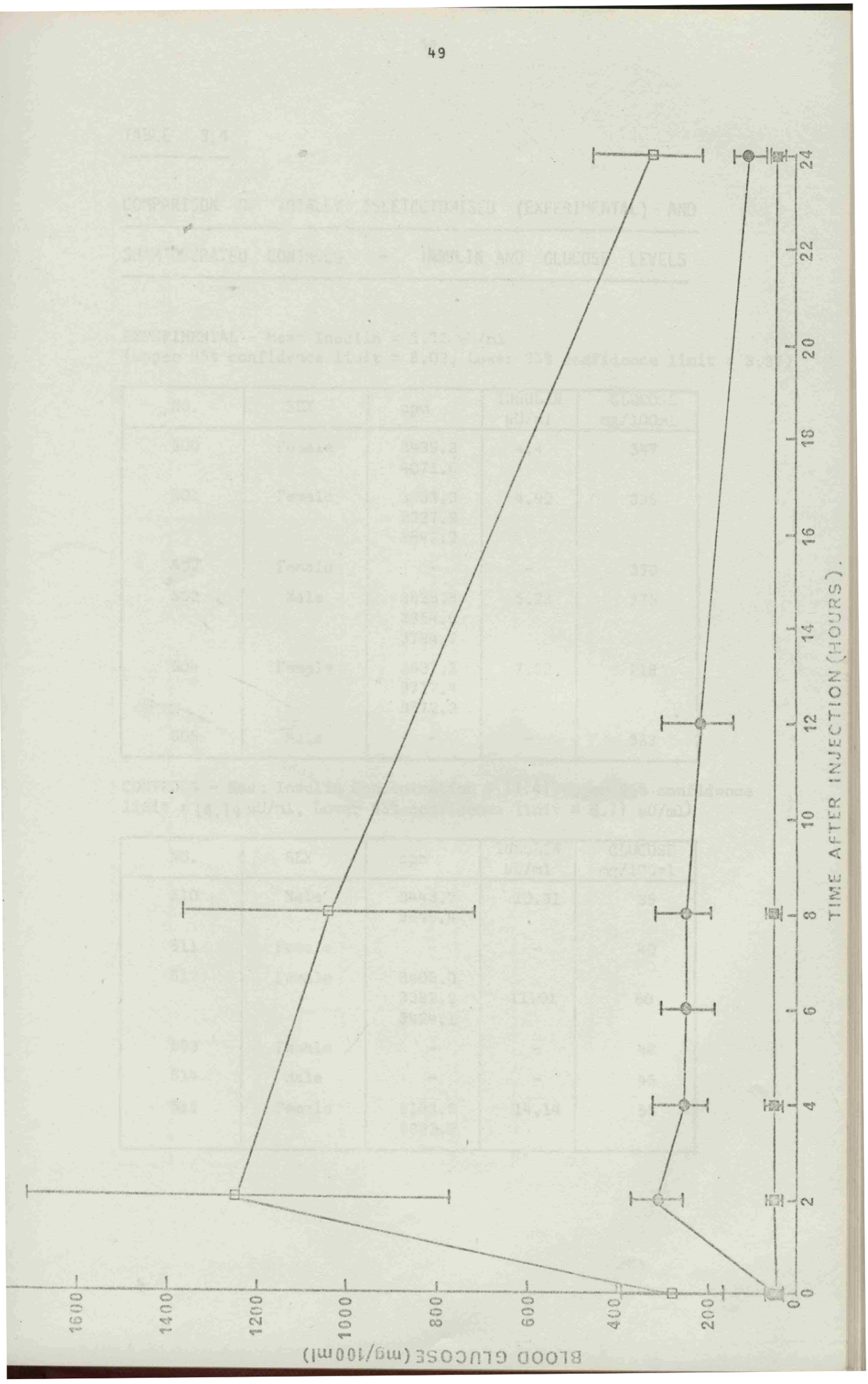

BLOOD GLUCOSE (mg/100ml)
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME AFTER INJECTION (HOURS).

TABLE 3.4

COMPARISON OF TOTALLY ISLETECTOMISED (EXPERIMENTAL) AND SHAM OPERATED CONTROLS - INSULIN AND GLUCOSE LEVELS

EXPERIMENTAL - Mean Insulin = 5.72 µU/ml
(Upper 95% confidence limit = 8.08, Lower 95% confidence limit = 3.37)

NO.	SEX	cpm	INSULIN µU/ml	GLUCOSE mg/100ml
500	Female	3499.3 4071.6	4.4	347
501	Female	3853.3 3827.9 3642.2	4.92	335
502	Female	-	-	350
502	Male	3625.8 3854.6 3784.7	5.23	375
504	Female	3537.1 3717.4 3572.2	7.52	218
505	Male	-	-	383

CONTROLS - Mean Insulin Concentration = 11.41 (Upper 95% confidence limit = 14.14 µU/ml, Lower 95% confidence limit = 8.71 µU/ml)

NO.	SEX	cpm	INSULIN µU/ml	GLUCOSE mg/100ml
510	Male	3443.7 3446.4	10.31	38
511	Female	-	-	40
512	Female	3406.0 3382.2 3424.1	11.01	60
563	Female	-	-	48
514	Male	-	-	45
515	Female	3183.6 3322.2	14.14	55

TABLE 3.5

ANALYSIS OF VARIANCE - INSULIN ASSAY - ISLETECTOMISED ANIMALS AND CONTROLS

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	0.043457	0.0434570	22.933	0.00056
Between Animals (Within Treatments)	5	0.008545	0.0017090	0.902	0.51326
Between Animals	6	0.052002	0.0086670	4.574	0.01440
Residual	11	0.020264	0.0018422		
Residual (Reg. + Unknown)	21		0.0018950		
Total	17	0.072266			

d) Liver Glycogen

In the isletectomised group, only males were present in adequate and consistent number in the various groups and for this reason the data on liver glycogen in Table 3.3 refers only to male lampreys. Mean values for all groups, except the caudally isletectomised animals are very similar, but in these the mean is significantly low compared with either the controls or the cranial and total islectomised groups.

e) Measurements of Nuclear Diameters

In lampreys subjected to cranial isletectomy, a relatively small but significant increase ($P < .02$) in nuclear diameters was observed in the remaining caudal tissue (Table 3.7). A much larger increase however, occurred in animals subjected to removal of the caudal pancreas when compared with the controls ($P < 0.001$), and the functional demand imposed by partial isletectomy appear to be parallelled by other cytological changes. Thus, in section stained by the aldehyde-fuchsin technique, degranulation of the aldehydefuchsinophil cords appeared to be more marked in the cranial tissue than in the caudal islets after partial isletectomy. In addition, vacuolisation has been observed in two cases in the cranial islets of caudally isletectomised animals, but has not been seen in the caudal tissue after removal of the cranial component.

3.4 HYPOXIA

a) Blood Sugar Changes

At or below 20% air saturation at 10°C, the lampreys became extremely excitable, displaying restless and vigorous swimming movements accompanied by hyperventilation (see Claridge and Potter, 1975). Even at saturation values down to 10% there was little or no mortality during a 7 day period and at 20% saturation the majority of the experimental animals survived a test period of 14 days. Over the first 2-3 days blood sugar analyses showed a small but significant rise compared with the controls but the increase in glycaemia levels became most marked between 4-7 days, when mean values increased from 75.5 $\pm$ 8.0 mg/100 ml to 92.1 $\pm$ 6.9 mg/100 ml (Fig.3.3). In general, blood sugar levels for males tended to be lower than those of the females at all time intervals, but because of the small numbers and the variability in individual values, these differences were not statistically significant. A similar (but not significant) sex difference was also present in the control group, where the mean value for 10 females was 47.7 $\pm$ 11.9 mg/100 ml compared with 41.5 $\pm$ 7.5 mg/100 ml for the same number of males. Since the 14 day hypoxia group consisted entirely of males, the apparent decrease in the mean blood sugar level (Fig.3,3)may therefore be more apparent than real.

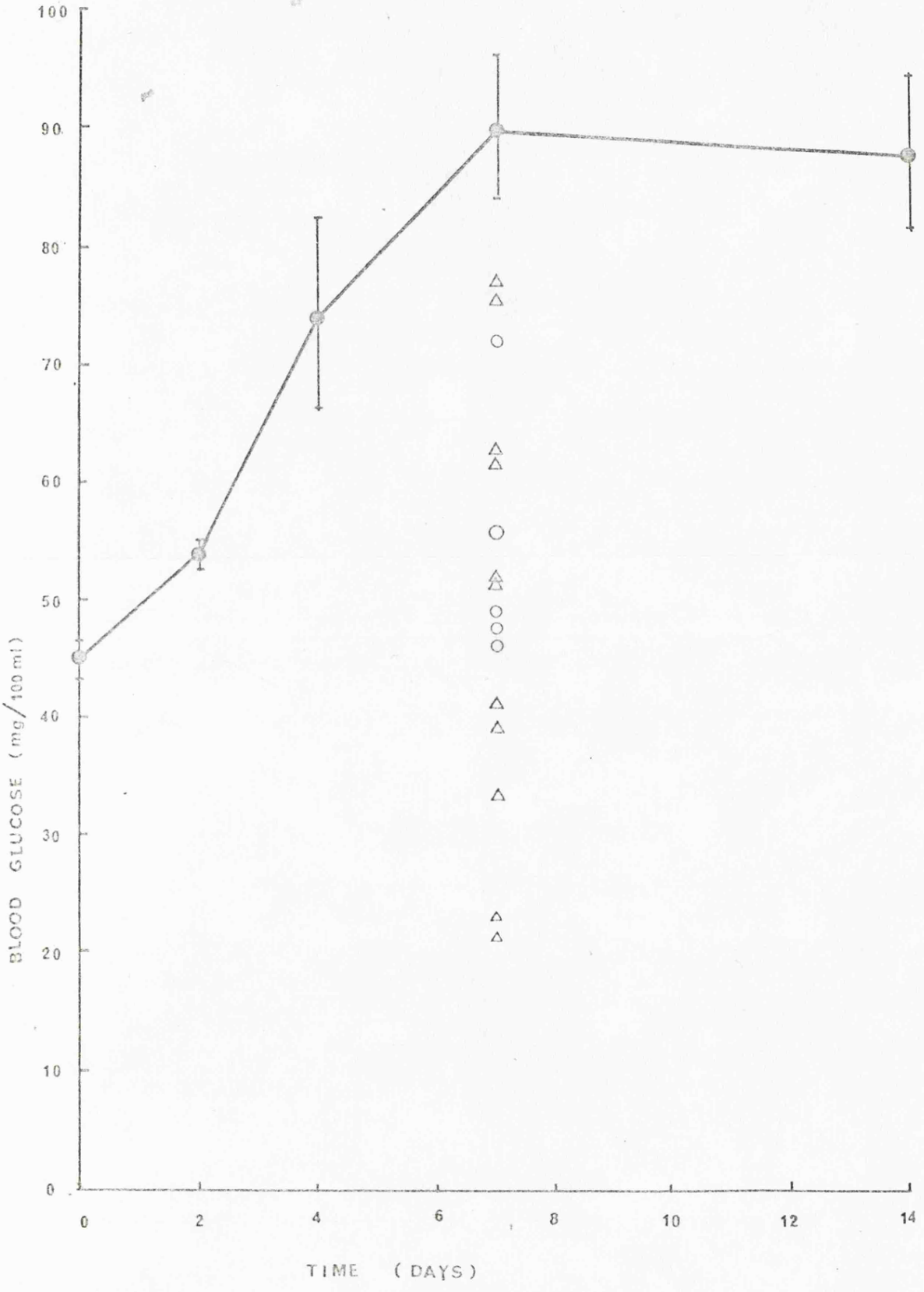
Similar tests were also carried out on five lampreys hypophysectomised 2-3 weeks previously. In their reaction to hypoxia these animals behaved in a manner characteristic of

FIGURE 3.3

Blood sugar levels in hypophysectomised and intact lampreys after periods of hypoxia. —●—●, Intact animals. Vertical lines indicate standard errors.

○, Hypophysectomised animals subjected to 7 days hypoxia.

△, Hypophysectomised animals maintained under normal oxygen tensions.


100
90
80
70
60
50
40
30
20
10
0
BLOOD GLUCOSE (mg/100 ml)
0
2
4
6
8
10
12
14
TIME (DAYS)

hypophysectomised lampreys, lying for the most part motionless on the bottom of the aquarium and without the rapid increase in ventilatory frequency seen in the intact animals under hypoxia. In this group, the mean blood sugar level after 7 days was 55.0 $\pm$ 10.4 mg/100 ml, with individual values from 48-73 mg/100 ml (Fig.3.3). Compared with intact lampreys subjected to hypoxia for the same period, the difference in the means is highly significant ($P<0.001$), but the value for the hypophysectomised group is not significantly different from that of the intact control animals. Hypophysectomy itself has not been shown to result in alterations in glycaemia levels (Larsen, 1973) and the mean value for 12 hypophysectomised lampreys that had been maintained in this laboratory for periods of 1-2 months after operation was 49.8 $\pm$ 5.4 mg/100 ml, which is very similar to that of the operated animals after 7 days hypoxia.

b) Liver Glycogen

Because of the relatively small numbers of each sex in some of the experimental groups, liver glycogen values are given as totals for all periods of hypoxia from 2-14 days, and there were no indications of any trends towards changing values with the duration of the experimental period. The values for the two sexes are presented separately (Table 3.6). Clearly there is no evidence that prolonged hyperglycaemia results in changes in liver glycogen values. For hypophysectomised lampreys subjected to hypoxia, numbers were small and invidual variations too large, to make meaningful comparisons, but the range of values fell well within those of intact animals.

TABLE 3.6

LIVER GLYCOGEN LEVELS IN MALE AND FEMALE LAMPREYS AFTER PERIODS OF HYPOXIA

	N	FEMALES	N	MALES
Hypoxia	18	123.2 ± 13.8	12	275.1 ± 40.3
Controls	18	140.9 ± 12.7	19	262.6 ± 20.7

Mean Liver Glycogen mg/100 g + S.E.

c) Histological Changes in Islet Tissue

After 2-3 days hypoxia, some increase was noted in the vascularity of the tissue and blood cells appeared in the inter-lobular areas and within the lumina of the cell cords and follicles. Mitoses were frequent within the light cell lobules.

More obvious changes were seen after 7 days hypoxia and vascularisation became more pronounced (Fig.3.4). In many cases, the dark cells tended to be arranged in follicles or cords; the lumina of which were packed with erythrocytes. In the majority of this group there was considerable irregularity, particularly of the dark cords, due to separation between the luminal surfaces of adjacent cells, in some cases leading to necrosis (Fig. 3.5). Crevices also tended to develop towards the centre of light cell lobules. The nuclear configuration resembled that seen in hyperplastic islet tissue (Hardisty, 1976) with a very distinct pattern of chromatin granules and a large, deeply staining central nucleolus. As in the earlier stages of hypoxia, mitoses were frequent but confined to the light cells. Vacuolisation was present in only 3 of the 12 animals examined and was relatively slight, affecting only a small proportion of the light cells cords (Fig.3.6). Within this group there appeared to be some correlation between the incidence of vacuolisation and hyperglycaemia; in the three animals showing these lesions, blood sugar values were 175, 150 and 129 mg/100 ml compared with a mean value of 80 ± 4.0 mg/100 ml for 16 animals in which vacuolisation did not develop after 7 days hypoxia.

FIGURE 3.4

Seven days hypoxia. Vascularisation and follicle development.
Trichrome. X 80.

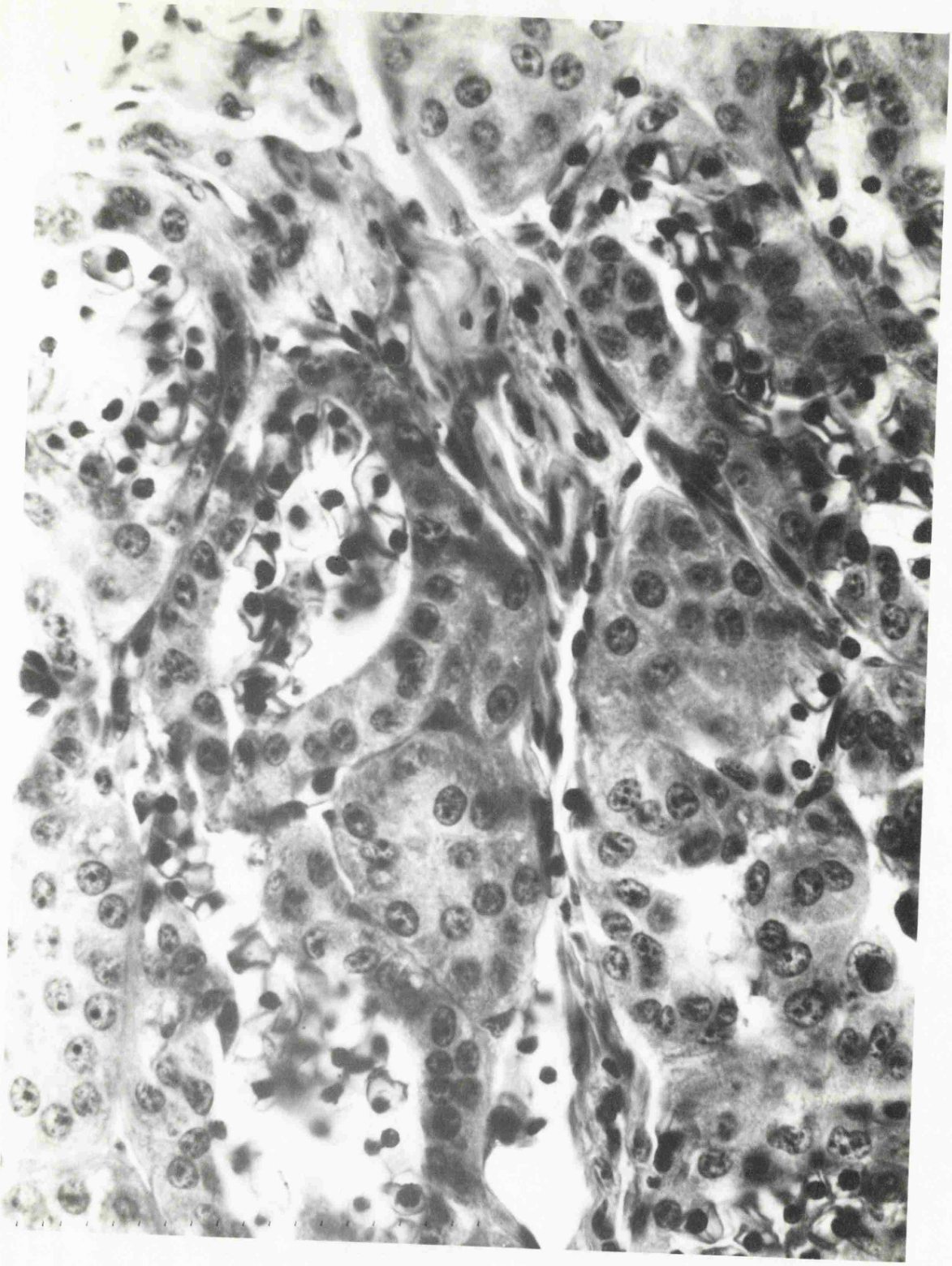


FIGURE 3.5

Seven days hypoxia. Necrotic changes in dark cells.
Trichrome X 30.

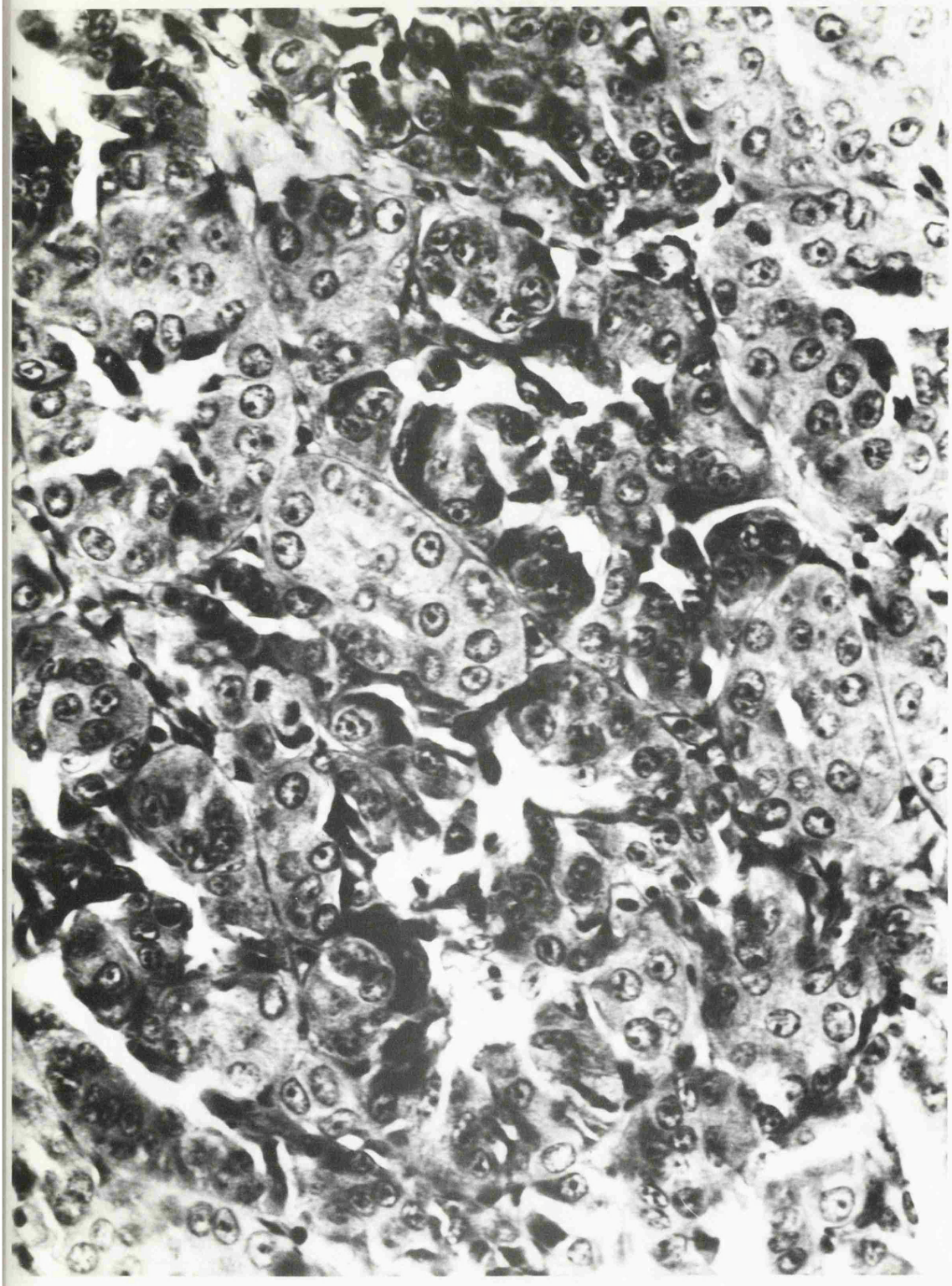
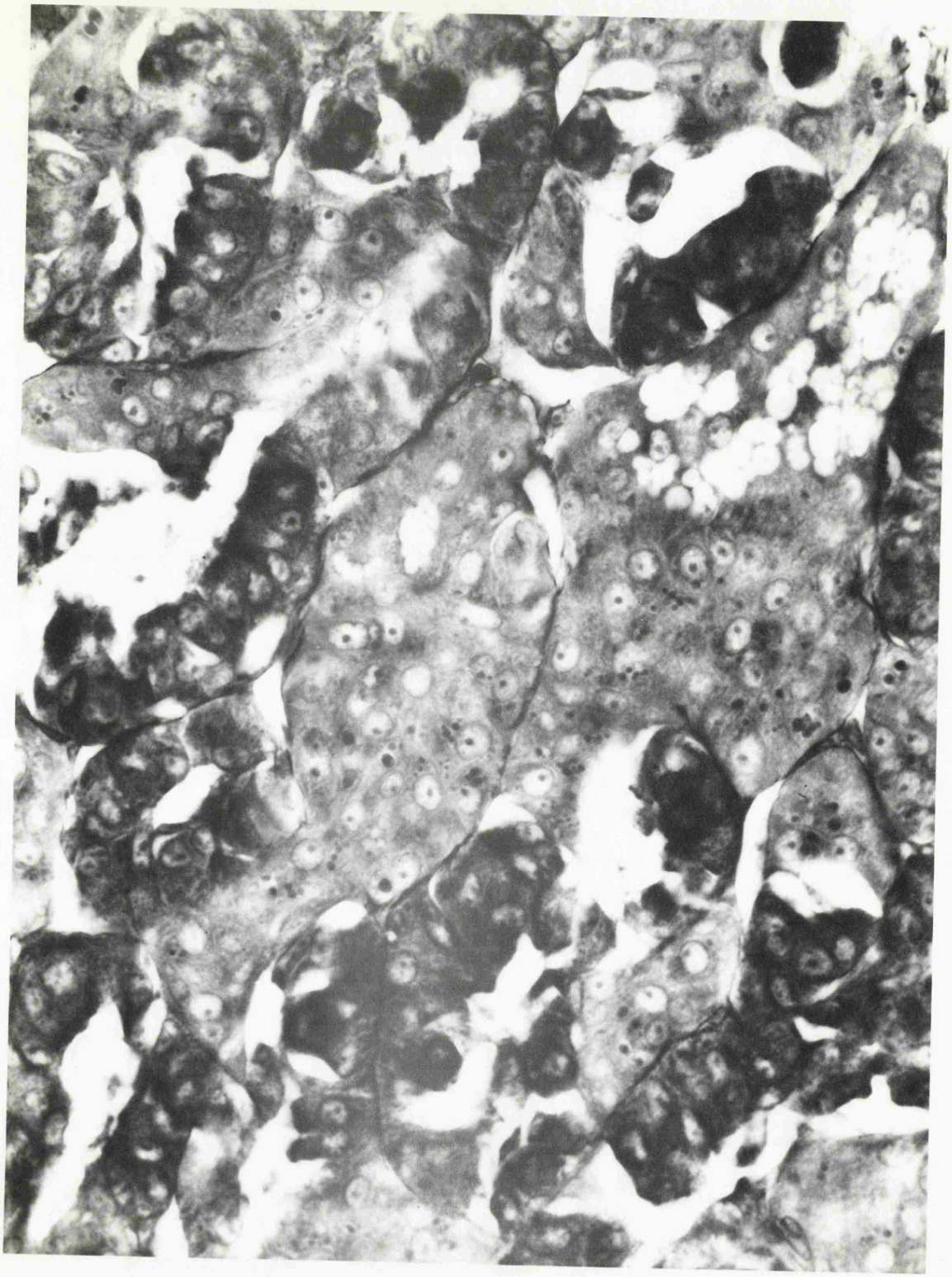


FIGURE 3.6

Seven days hypoxia. Slight vacuolisation of light cells.

Aldehyde fuchsin X 80.



After 14 days hypoxia, every animal showed vacuolisation and within one exception, this was so severe that almost all the light cells were affected and in these areas only the lobular envelope, cell membranes and nuclei remained intact (Figs. 3.7, 3.8) The surviving dark cells retained a highly granular cytoplasm which was intensely aldehyde-fuchsinophil. No mitoses have been observed in this experimental group.

In spite of their failure to develop hyperglycaemia, every one of the hypophysectomised lampreys subjected to only 7 days hypoxia showed 'hydropic degeneration', which was even more severe and extensive than in the intact animals after 14 days under similar conditions (Fig. 3.9). In this group also the surviving dark cells retained a conspicuously coarse and dense aldehyde-fuchsinophil granulation and few if any, of the light cells escaped vacuolisation. The possibility that these lesions might be attributable to the operation of hypophysectomy itself may be discounted, since no vacuolisation was observed in a control group of hypophysectomised animals maintained under normal laboratory conditions for periods of 1-2 months.

d) Interrenal Tissue (Fig. 3.11)

No evidence was found for the involvement of this tissue in the hyperglycaemic response to hypoxia and in every group from 18 h to 14 days, the mean diameters of the interrenal nuclei was almost identical to that of the control group (Table 3.7). However, in comparison with either intact or hypophysectomised controls,

FIGURE 3.7

Fourteen days hypoxia. Extensive hydropic degeneration.

Trichrome X 80.

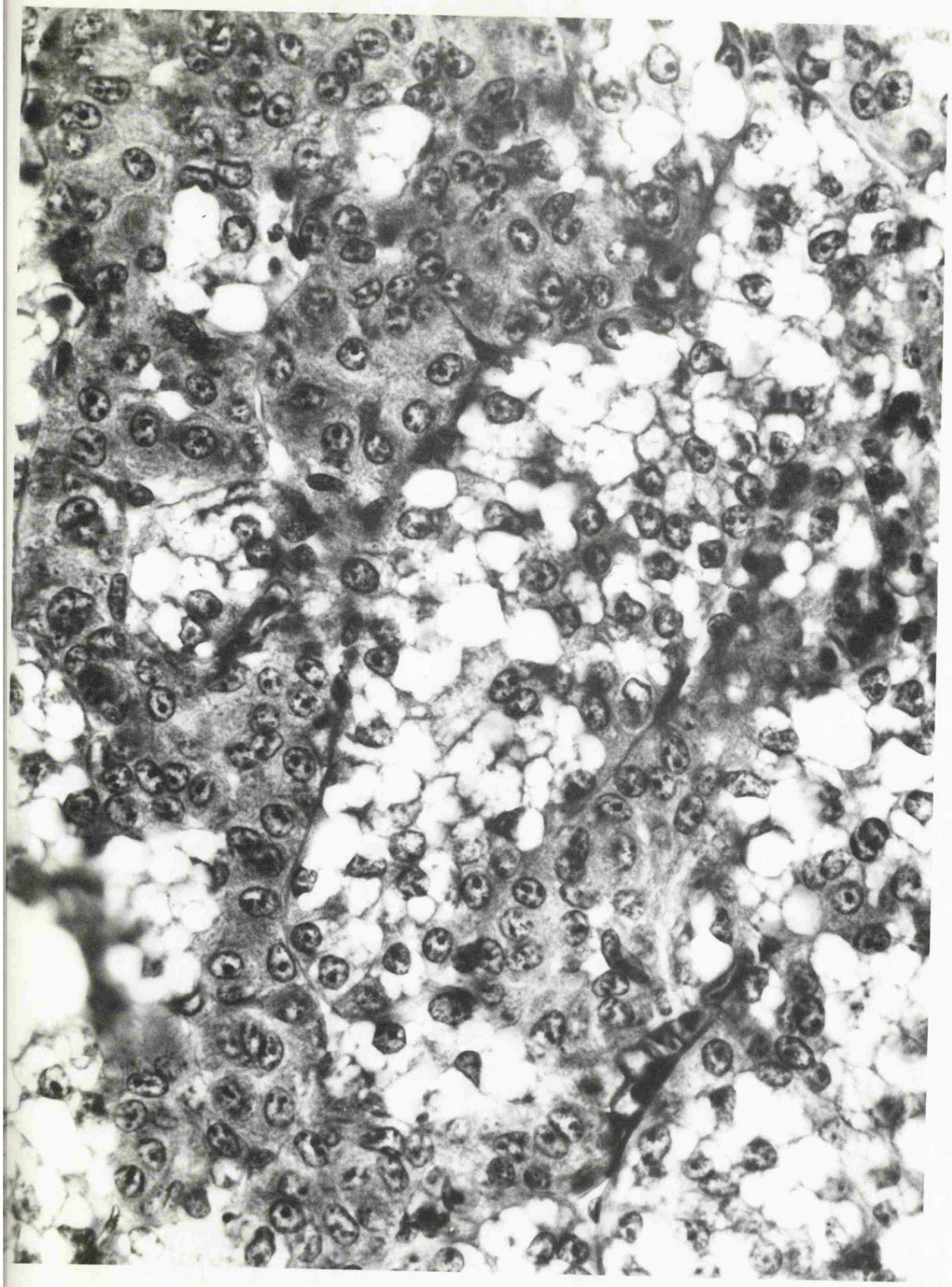


FIGURE 3.8

Fourteen days hypoxia. Vacuolisation affecting light cell lobules.

Trichrome X 120.

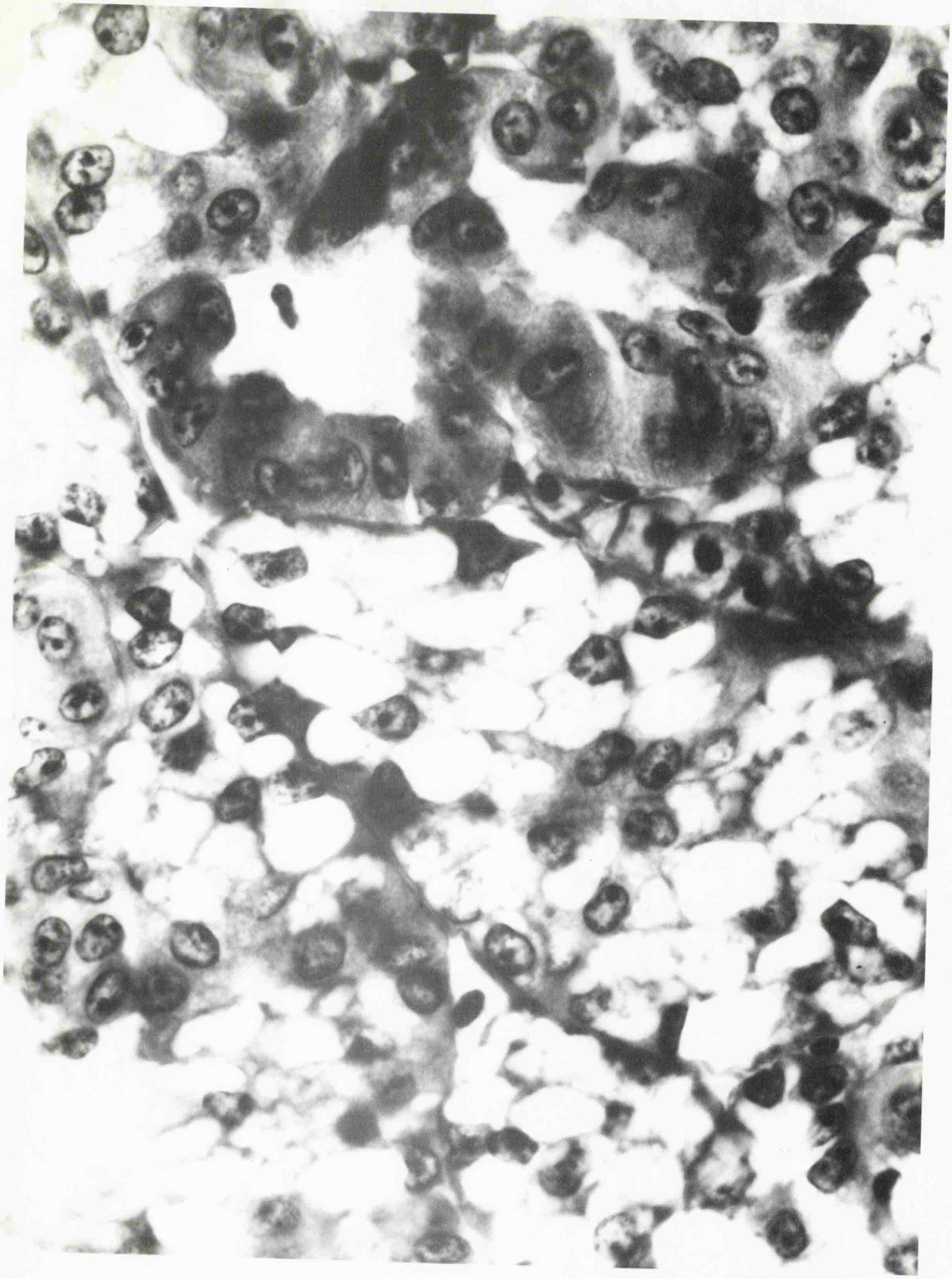
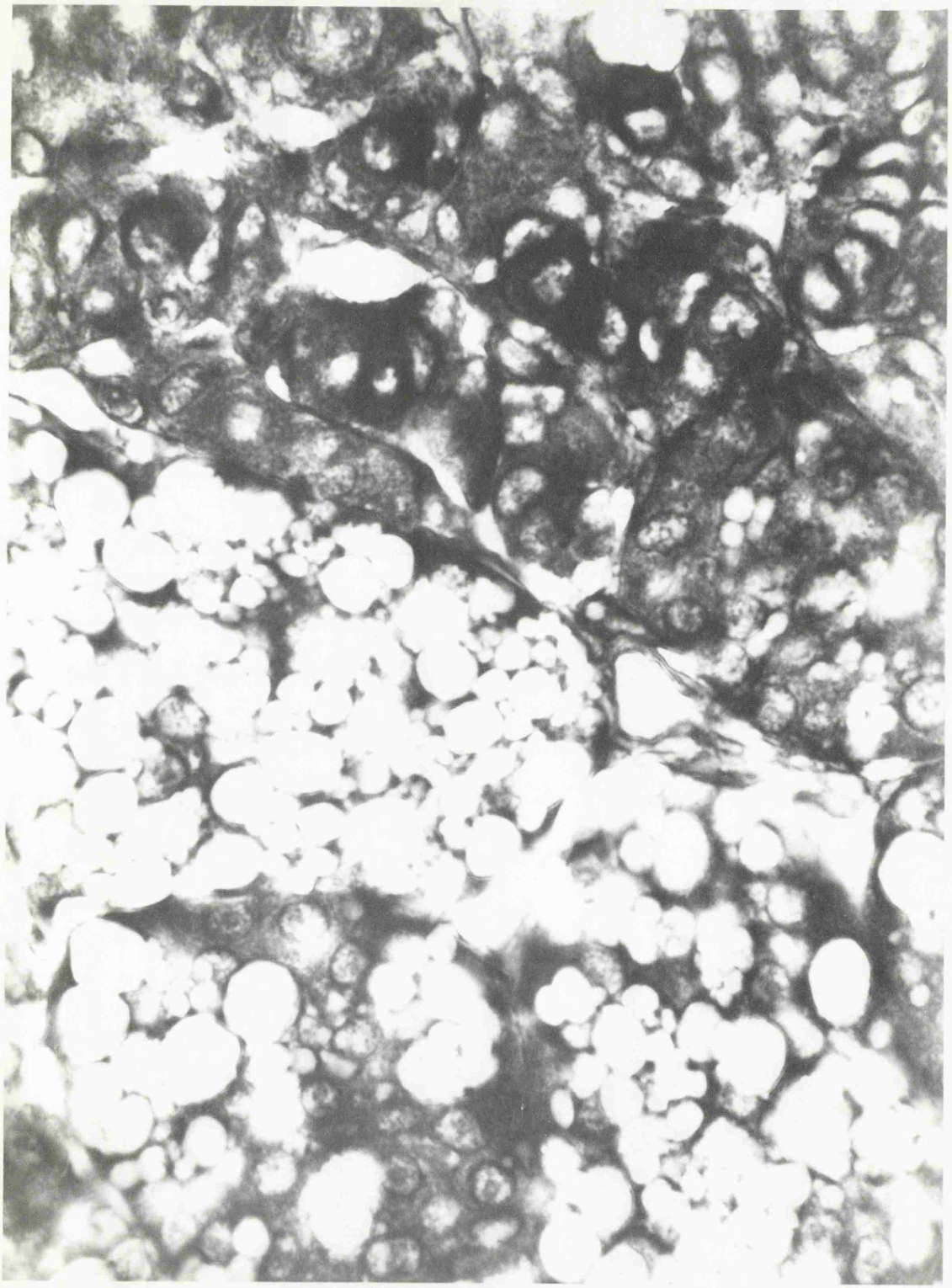


FIGURE 3.9

Extensive hydropic degeneration in light cells of a hypophysectomised animal subjected to 7 days hypoxia.

Trichrome X 80.



significantly smaller nuclei were observed in the hypophysectomised animals subjected to 7 days hypoxia.

e) Chromaffin Tissue (Fig. 3.11)

After 18 hr hypoxia treatment, the mean diameters of the chromaffin cells showed a very considerable increase compared with the control group (Table 3.7) and at 3 days, the difference, although reduced, was still significant ($P<0.001$). On the other hand, after 14 days hypoxia treatment, values were almost identical to those of the control group. In both the hypophysectomy control group and also in those subject to hypoxia, mean chromaffin nuclear diameters were significantly smaller than those of intact controls ($P > .001$).

FIGURE 3.10

Vacuolisation in light cell of an animal subject to light stress for 7 days.

Aldehyde fuchsin X 80.

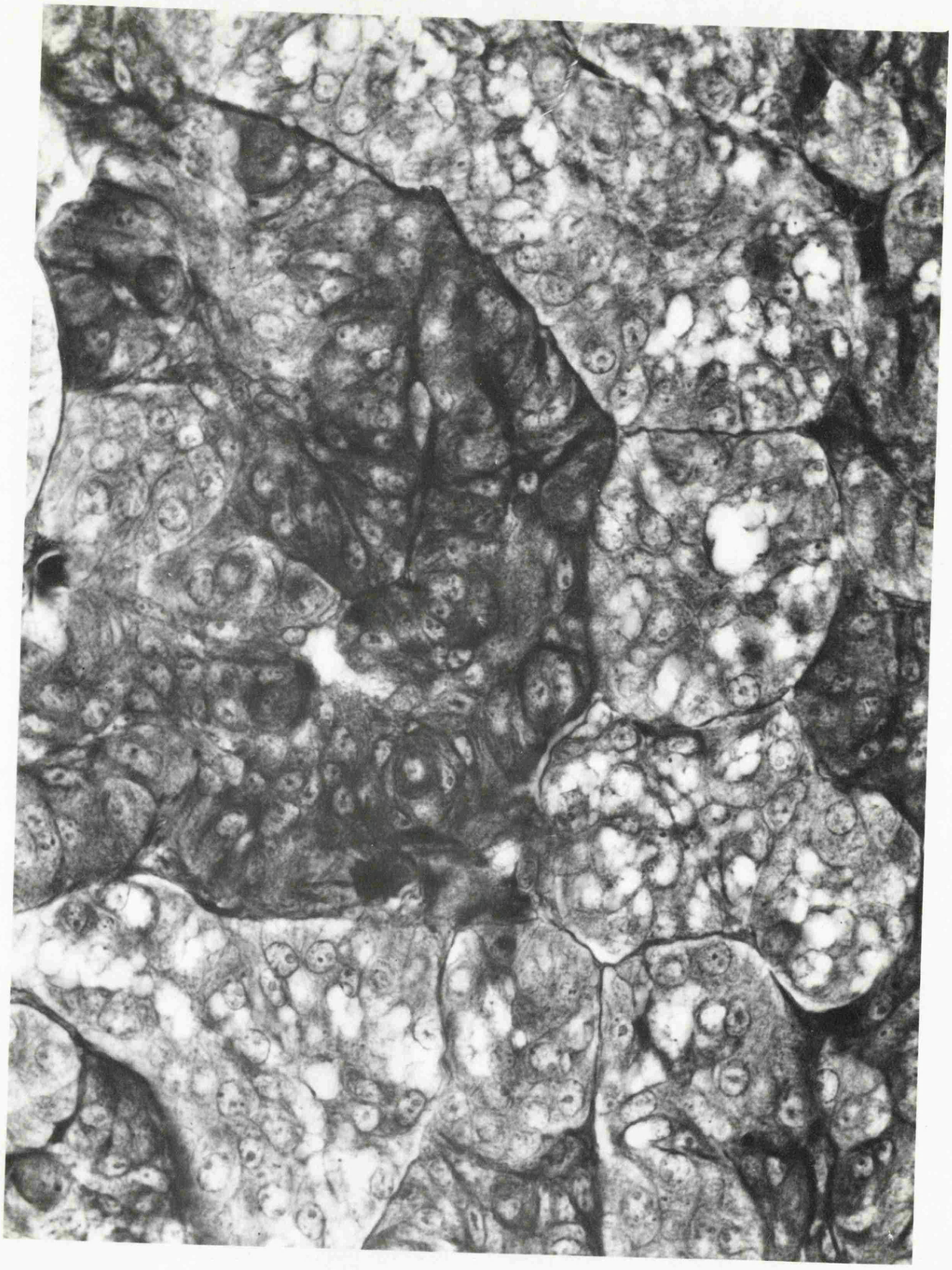


FIGURE 3.11

Surface of aorta with group of interrenal cells (left) and granular chromaffin cells (right).

Trichrome X 200.

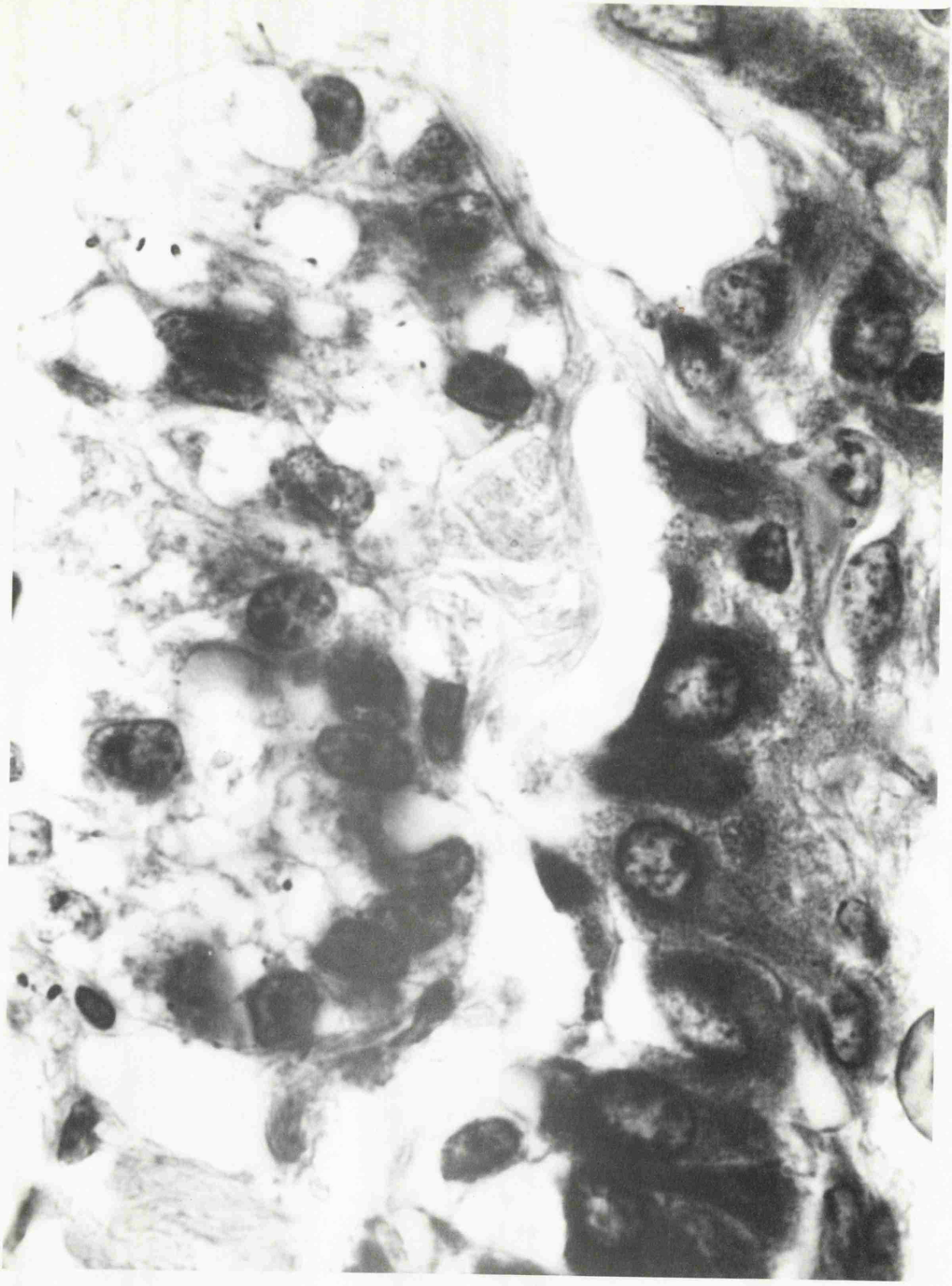


TABLE 3.7(A) MEASUREMENT OF NUCLEAR DIAMETERS IN ISLET, INTERRENAL AND CHROMAFFIN TISSUE IN HYPOXIA AND VARIOUS EXPERIMENTAL TREATMENTS

TREATMENT	NO OF ANIMALS	ISLET CELLS	NO OF ANIMALS	INTERRENAL	NO OF ANIMALS	CHROMAFFIN TISSUE
HYPOXIA						
18 h.	3	6.83 ± 0.05	5	5.79 ± 0.05	5	6.58 ± 0.05
3 Days	3	6.71 ± 0.06	4	5.73 ± 0.06	4	6.27 ± 0.07
Controls	3	6.79 ± 0.06	4	5.70 ± 0.06	4	5.82 ± 0.07
7 Days	3	6.20 ± 0.05	-	-	-	-
14 Days	3	6.33 ± 0.03	4	5.80 ± 0.07	4	6.60 ± 0.02
Controls	3	6.28 ± 0.03	4	5.79 ± 0.06	4	6.65 ± 0.07
HYPOPHYSECTOMY						
7 day Hypoxia	3	6.43 ± 0.04	4	5.32 ± 0.05	4	6.22 ± 0.06
Controls	3	6.34 ± 0.04	4	5.82 ± 0.05	4	6.33 ± 0.06
Unoperated Controls	3	6.28 ± 0.03	4	5.79 ± 0.08	4	6.65 ± 0.07
ADRENALINE INJECTED		6.71±0.05		6.32±0.05		6.60±0.07
Saline Injected Controls	3	6.27 ± 0.05	3	5.66 ± 0.05	3	6.38 ± 0.08
GLUCOSE LOADING						
24 h.	5	6.81 ± 0.03	4	5.87 ± 0.05	4	6.91 ± 0.10
Controls	3	6.31 ± 0.03	4	5.60 ± 0.05	4	6.25 ± 0.07

Mean nuclear diameters (μ) ± S.E.

TABLE 3.7(B) CHANGES IN NUCLEAR DIAMETER OF ISLET CELLS AFTER PARTIAL ISLETECTOMY

TREATMENT	NO OF ANIMALS	MEAN NUCLEAR DIAMETER + S.E. μ
ISLETECTOMY		
Caudal	4	7.02 ± 0.05
Control	6	6.31 ± 0.04
Cranial	6	6.53 ± 0.04
Control	4	6.36 ± 0.06

DIFFERENCES IN MEANS:

Caudal Isletectomy / Control $P < 0.001$

Cranial Isletectomy/ Control $P < 0.002$

Cranial Isletectomy/ Caudal Islectomy $P < .001$

3.5 ADRENALINE ADMINISTRATION

Adrenaline was administered in doses of 200 µg as 0.1 ml of a 2 mg/ml solution in lamprey Ringer. Injections of 0.1 ml of lamprey Ringer were given to the control group. All experimental animals showed marked de-pigmentation (Fig. 3.12) and hyper-ventilation. Ventilatory frequency 2 hr after injection was 162 ± 2.3 per min, compared with 88 ± 8.2 per min for the controls.

a) Glucose Levels

Hyperglycaemia was observed with mean glucose levels of experimental group 148 ± 14.8 mg/100 ml ($n = 4$) as against 62.8 ± 11.9 mg/100 ml ($n = 5$) for the control. This was highly significant ($t = 4.54$ $P < 0.001$).

b) Insulin Levels

In spite of the hyperglycaemia, the levels of insulin-like immunoreactivity appear to be lower in adrenaline treated animals than in Ringer injected controls (Table 3.8), and the analysis of variance (Table 3.9) indicates that this is significant. These determinations and those for insulin levels in serum samples for glucose tolerance and long term glucose loading were made on the same assay run.

FIGURE 3.12

Comparison of depigmented adrenaline treated animal (upper) and saline injected control (lower) X 3/4.

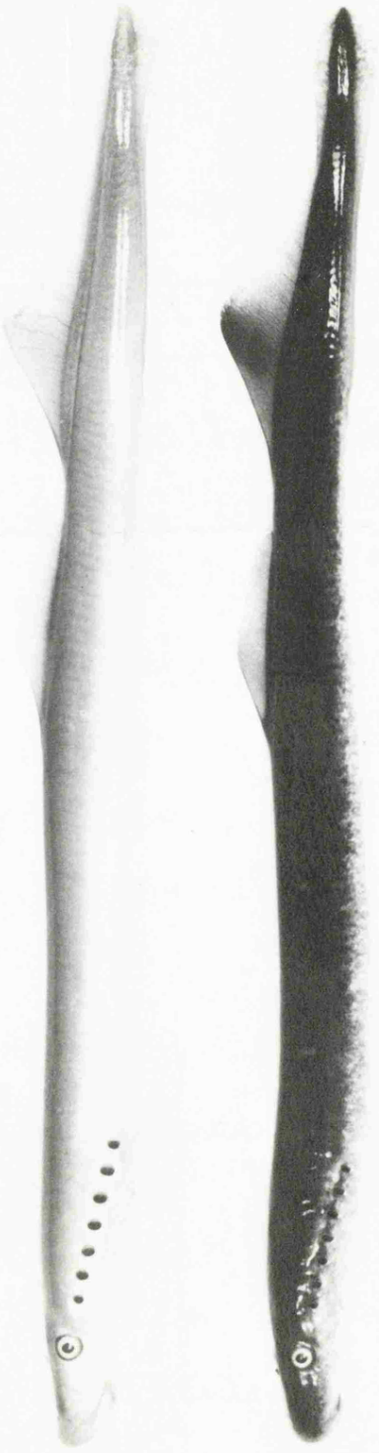


TABLE 3.8

ADRENALINE INJECTIONS (200 µg) 2 X DAY FOR 3 DAYS

V RINGER INJECTED CONTROLS

GROUP	NO.	SEX	cpm	INSULIN µU/ml
I Adrenaline Injected	110	Female	6098 5814	26.19
	111	Male	6369 6803 6579	15.88
	112	Female	6666 6494 6494	16.38
	113	Female	6173 5848 5875	26.67
	114	Female	5833 7408 7092	12.92
II Controls	120	Male	2749	109.68
	121	Male	4975 4762	48.90
	123	Male	2954 3497 3311	93.20
	124	Male	3937 4048 4525	66.04

Mean and Confidence Limits

Mean = 19.14
Upper 95% = 23.33
Lower 95% = 14.94

Mean = 76.13
Upper 95% = 83.04
Lower 95% = 69.2

$P < 0.001$

TABLE 3.9

ANALYSIS OF VARIANCE - INSULIN ASSAY - ADRENALINE TREATMENT AND CONTROLS

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	1.484375	1.4843750	356.6	< 0.00001
Between Animals (Within Treatments)	7	0.352293	0.0503273	18.873	< 0.00001
Between Animals	8	1.836670	0.2295887	86.041	< 0.00001
Residual	14	0.063477	0.0045348		
Residual (Reg. + Unknown)	26		0.002666		
Total	22	1.900146			

c) Islet Histology

No marked abnormalities were observed beyond increased vascularity but mitotic frequency was greater than in any other experimental group. The nuclear volume was enlarged in this group almost as much as in the glucose loaded animals.

d) Interrenal Histology

Although quantitative measurements are difficult because of the diffusiveness of this tissue, observations on serial sections from a number of experimental animals gave distinct indications of hyperplasia. The increase in nuclear volume of interrenal tissue was higher in the adrenaline treated animals than any other experimental group (Table 3.7) and the differences are highly significant.

e) Chromaffin Histology

Nuclear enlargement was observed which was statistically significant at a 5% level ($0.02 < P < 0.05$).

3.6 GLUCOSE TOLERANCE AND LONG TERM LOADING TESTS

Two types of glucose loading tests were employed; a glucose tolerance test, where glycaemia and insulin levels were monitored at intervals up to 24 h following a single glucose (100 mg) injection; and a long term glucose loading test, where animals received one or two daily injections of glucose for period of 8-11 days. In both cases control animals received similar number of injections of the same volume and osmolarity. as the controls.

a) Glucose Tolerance Tests

(i) Glucose Levels

In glucose loading tests on 77 intact lampreys (Fig.3.2). maximum blood glucose values were usually recorded within two hours of an injection of 100 mg glucose and at this point, individual animals showed considerable variability in blood sugar levels. At 24 h values were however, much more uniform and approached normal control levels. Analyses of the water in which the test animals had been kept, showed that maximum glucose losses occurred within the first two hours, but over the whole 24 h period these losses accounted for only about 0.5% of the injected glucose.

(ii) Insulin Levels

Insulin values were determined at periods of 2, 8 and 24 h following a single glucose injection (100 mg as 0.2 ml of 50% glucose). Insulin levels were also estimated at the same periods following a single control injection of 0.2 ml of 8.125% saline. Two hours after a glucose injection, insulin values did not differ significantly from the control group (Table 3.10, Fig. 3.13). However, after 8 and 24 h insulin values were elevated to mean levels of 46.5 and 42.9 μU/ml respectively (Tables 3.11, 3.12). All these determinations were performed on the same assay run and therefore comparisons between them are valid (see page 99). Comparisons between the raw c.p.m. data for each of the experimental groups is given in the analysis of variance tables (Tables 3.13, 3.14, 3.15). These show a highly significant difference between the experimental and control groups at 8 and 24 h after injection, but 2 h after injection the variation between individual animals within each group is greater than the differences between the two treatment groups.

(iii) Islet Tissue Histology

Animals killed 24 h after a single glucose injection showed marked degranulation, as judged by the diminished intensity of aldehyde-fuchsin staining of the dark cell cords (Fig. 3.14). Stimulation of cell division was also evident in the light cells.

Nuclear diameters increased from 6.31 ± 0.04 μM (n = 150) to 6.81 ± 0.03 μM (n = 250) which is statistically very significant ($P < 0.001$) (Table 3.7).

FIGURE 3.13

Time course of circulating levels of insulin following single injection (100 mg) of glucose.

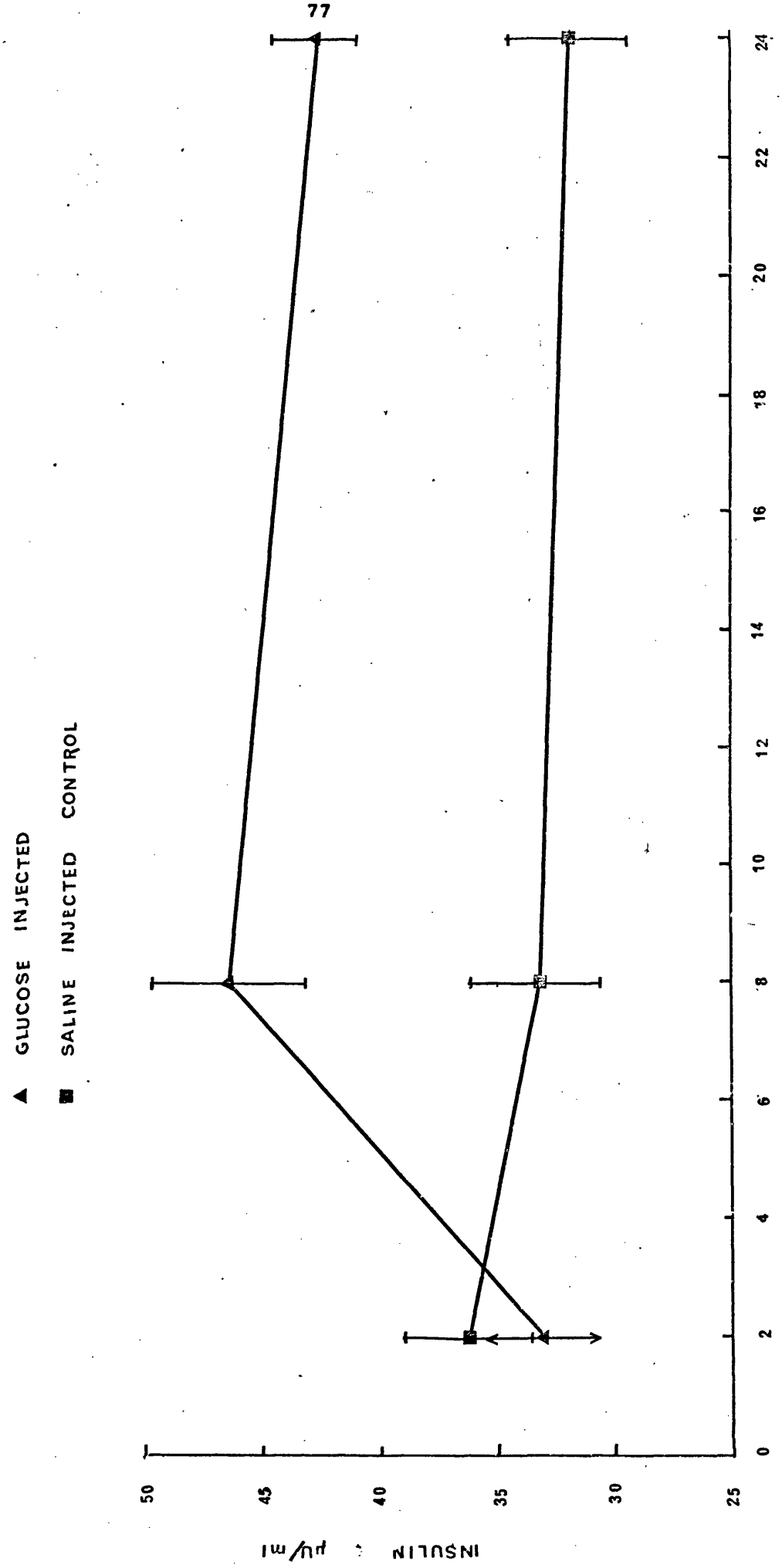

GLUCOSE INJECTED
SALINE INJECTED CONTROL
INSULIN : μU/ml
50
45
40
35
30
25
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (hrs)

TABLE 3.10

2 HRS GLUCOSE TOLERANCE

GROUP	NO.	SEX	cpm	INSULIN µU/ml
I Glucose Injection (2 hr)	130	Female	5556 5681 5714	33.44
	131	Female	5556 5376 5435	36.42
	132	Male	5650 5313 5435	36.20
	133	Male	6173 6173 5474	25.50
	134	Female	5474 5435 5682	33.17
II Saline Cont. (2 hr)	150	Female	5435 5291 5376	38.21
	151	Female	5236 4975 5405	41.62
	153	Female	5405 5814 5417	32.84
	154	Female	5618 5650	36.30

Mean and Confidence Limits

Mean = 33.05
Upper 95% = 35.46
Lower 95% = 30.65

Mean = 36.33
Upper 95% = 39.00
Lower 95% = 33.67

$P < 0.05$

TABLE 3.11

8 HRS GLUCOSE INJECTION

GROUP	NO.	SEX	cpm	INSULIN µU/ml
I Glucose Injection 8hr	135	Female	4902 5025	51.67
	136	Female	5556 5556 5618	34.01
	137	Male	4785 4831 4975	49.00
	138	Male	4739 5291 5000	45.85
	139	Male	4595 4444	57.04
II Saline Controls 8hr	155	Male	5263 5239 5464	39.15
	156	Female	5494 5291 5741	35.72
	157	Male	5314 5988 6024	30.32
	158		5988 5747 6173	24.55

Mean and Confidence Limits

Mean = 46.46
Upper 95% = 49.63
Lower 95% = 43.3

Mean = 33.15
Upper 95% = 36.19
Lower 95% = 30.12

$P > 0.001$

TABLE 3.12

24 HRS GLUCOSE TOLERANCE

GROUP	NO.	SEX	cpm	INSULIN µU/ml
I Glucose Loaded 24hr	140	Male	5780 6060 5644	29.2
	141	Male	4630 4785 4605	53.33
	142	Male	5128 5241	41.48
	143	Male	4879 4950 5025	47.05
II Saline Control 24hr	160	Female	6024 5291	30.00
	161	Male	5129 4830 5025	46.12
	162	Female	6494 6250	19.43
	163	Male	5882 6000 5780	27.72
	164	Female	5555 5917 5714	31.10

Mean and Confidence Limits

Mean = 42.88
Upper 95% = 45.46
Lower 95% = 41.30

Mean = 31.82
Upper 95% = 34.14
Lower 95% = 29.51

$P > 0.0001$

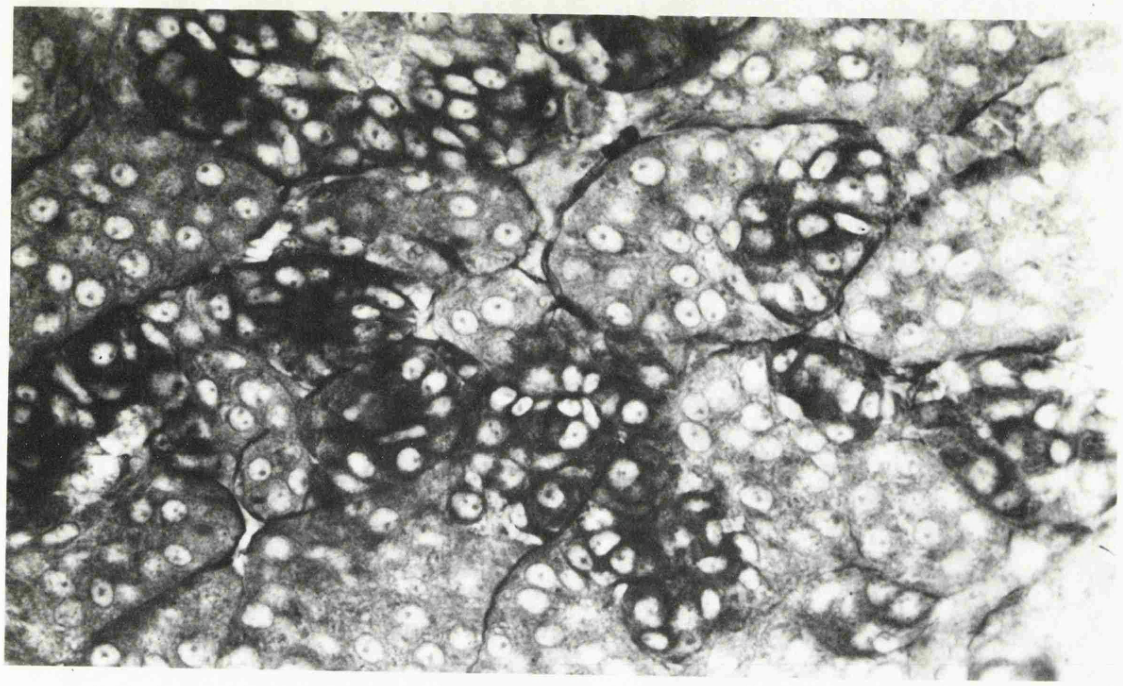
TABLE 3.13

ANALYSIS OF VARIANCE - INSULIN ASSAY - GLUCOSE TOLERANCE (2 HRS)

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	0.005615	0.0056152	6.119	0.02421
Between Animals (Within Treatments	7	0.034424	0.0049177	5.354	0.00223
Between Animals	8	0.020034	0.0050029	5.454	0.00183
Residual	17	0.020752	0.0012207		
Residual (Reg. + Unknown)	29		0.0009176		
Total	25	0.060741			

FIGURE 3.14

Comparison of islet tissue from saline injected control (upper) with degranulation of dark cells after 8 hr single glucose injection (lower).



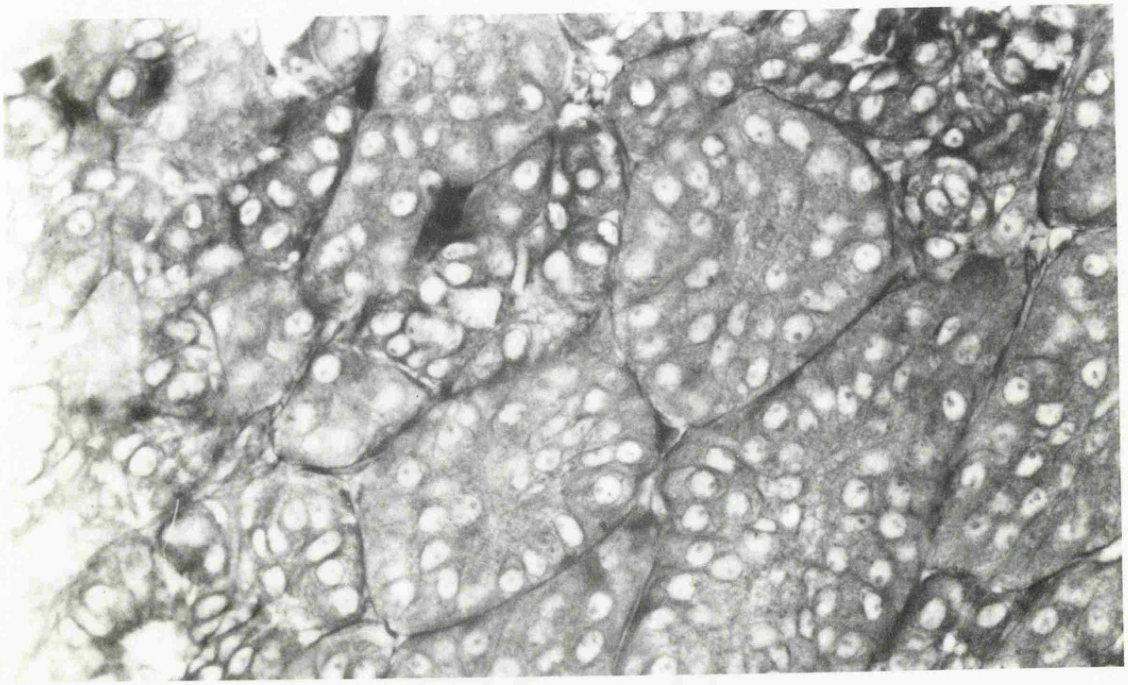


TABLE 3.14

ANALYSIS OF VARIANCE - INSULIN ASSAY - GLUCOSE TOLERANCE (8 HRS)

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	0.087646	0.0876465	72.336	< 0.00001
Between Animals (Within Treatments)	7	0.088135	0.0125907	10.391	0.00009
Between Animals	8	0.175781	0.0219727	18.134	< 0.00001
Residual	15	0.027100	0.0018066		
Residual (Reg. + Unknown)	27		0.0012117		
Total	23	0.202881			

TABLE 3.15

ANALYSIS OF VARIANCE - INSULIN ASSAY - GLUCOSE TOLERANCE AND CONTROLS (24 HRS)

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	0.060303	0.0603027	76.655	$\overline{<}$ 0.00001
Between Animals (Within Treatments)	7	0.159912	0.0228446	23.039	$\overline{<}$ 0.00001
Between Animals	8	0.220215	0.0275269	34.991	$\overline{<}$ 0.00001
Residual	15	0.015625	0.0010417		
Residual (Reg. + Unknown)	27		0.0007867		
Total	23	0.235840			

In general, the measurements of nuclear diameters of islet cells does not differentiate between cell types, and represents therefore a random mean of both light and dark cells. Separate measurements have, however, been made of the nuclei of both light and dark cells in sections stained by the aldehyde-fuchsin technique, both in saline injected control animals and in others killed within 24 h of receiving a single glucose injection of 100 mg. These measurements demonstrate the larger size of the nucleus in the light cells, but show that both cell types respond to glucose loading (Table 3.16).

Although in most cases the dark and light cells appear to be present in roughly equal proportions, this is not reflected in the data in Table 3.16, where the overall mean value for the nuclear diameters of both types measured indiscriminately does not fall, as expected, approximately mid-way between the values for the light and dark cells recorded separately. This apparent discrepancy is almost certainly due to the fact that the two sets of measurements include animals not common to both series and it has been the experience that there are often quite large individual differences between animals, which do not appear to be related to body size or sex.

TABLE 3.16

MEAN NUCLEAR DIAMETERS (μ) OF DARK AND LIGHT ISLET CELLS IN NORMAL AND GLUCOSE LOADED ANIMALS (SINGLE INJECTION)

	N	DARK	N	LIGHT	N	ALL CELL TYPES
Glucose Loaded	4	6.33 ± 0.04	4	6.89 ± 0.04	4	6.81 ± 0.03
Controls	3	5.98 ± 0.04	3	6.44 ± 0.04	3	6.31 ± 0.03

(iv) Nuclear Diameters of Interrenal and Chromaffin Cells

The nuclear volume of the interrenal cells was slightly but significantly ($P < 0.001$) increased 24 h after a single glucose injection (100 mg) as compared with saline injected controls; for the glucose injected animals the mean nuclear diameter was 5.87 ± 0.05 µM, compared with 5.60 ± 0.005 µM. These means were calculated from observations on 4 animals (20 measurements per animal). A considerable nuclear englargement was also noted in chromaffin cells after the same treatment. For the experimental group the mean nuclear diameter was 6.91 ± 0.10 µM, as compared with control nuclear diameter of 6.25 ± 0.07 µM. These were from observations on 4 animals (20 measurements per animal). The differences between the means is statistically highly significant ($P < 0.001$).

b) Long Term Glucose Loading

(i) Insulin

The glucose loaded animals (injected twice daily with 100 mg glucose for 12 days) exhibited considerable elevation in circulating insulin levels, (84.3 µM/ml) as compared with the saline injected controls (mean 31.3 µM/ml) (Table 3.17). These determinations were performed on the same assay run as that used for estimation of insulin levels at various time intervals after a single injection and therefore this data is comparable. The analysis of variance on the raw c.p.m. data also indicates a highly significant difference between the two groups (Table 3.18).

TABLE 3.17

GLUCOSE LOADED 2 X DAILY FOR 2 DAYS (100 mg) AGAINST

0.8125% NACL LOADED 2 X DAILY FOR 12 DAYS (0.2 ml)

GROUP	NO.	SEX	cpm	INSULIN µU/ml
I Glucose Loaded	80	Male	1485 1416	101.55
	81	Female	3734 2924	70.19
	82	Female	4785 3356	102.17
	83	Male	3355 3984	70.14
	84	Female	6098 5536	20.43
	85	Male	2398 2200	131.13
II NaCl Controls	90	Male	6098 6173	37.39
	91	Male	5744 6029 6048	28.43
	92	Male	6536 6538	16.61
	93	Male	5348 5050 5587	39.09
	94	Female	5263 4926	43.97
	95	Male	5618 6173 6495	24.47

Mean and Confidence Limits

Mean = 84.28
Upper 95% = 113.7
Lower 95% = 54.86

Mean = 31.39
Upper 95% = 48.24
Lower 95% = 15.57

P > 0.001

TABLE 3.18

ANALYSIS OF VARIANCE - INSULIN ASSAY - LONG TERM GLUCOSE LOADING

SOURCE	D.F.	SUM OF S.D.	MEAN SQUARE	VARIANCE RATIO	PROBABILITY
Between Treatments	1	1.568115	1.5681152	35.584	0.00002
Between Animals (Within Treatments)	10	1.284180	0.1284180	2.914	0.02759
Between Animals	11	2.852295	0.2592995	5.884	0.00081
Residual	16	1.228027	0.0767517		
Residual (Reg. + Unknown)	28		0.0440674		
Total	27	4.080322			

(ii) Histology of Islet Tissue

In a group of animals subjected during December to a twice daily injection of 100 mg glucose for periods of 8-11 days, the increase in nuclear diameters of the islet cells was slightly less than in the animals receiving only a single glucose injection (Table 3.7). Out of a total of 7 animals treated in this way, three showed extensive vacuolisation of the islet tissue.

3.7 RADIOIMMUNOASSAY FOR GLUCAGON IN ISLET TISSUE AND INTESTINAL EXTRACTS

Extracts of cranial islet tissue (Islet 1 and Islet 2) and the intestine from the pericardio-hepatic region (FG1, FG2) from two animals (Animal 1, Animal 2) were prepared in 1 ml of 0.65 saline.

The standard curve and the unknown c.p.m. are presented in Table 3.14 and the standard curve is illustrated in Fig. 3.15. The results clearly indicate the presence of glucagon-like immunoreactivity in both the "islet tissue" and the intestine, although no attempt can be made at quantification, as the unknowns are more concentrated than any of the standards. The presence of glucagon-like immunoreactivity in the cranial islet tissue seems anomolous but it is not possible to separate completely by dissection the cranial islet tissue from the epithelium of the intestinal diverticula and the glucagon-like immunoreactivity apparently present in this tissue could well be due to contamination from the tissues of the anterior intestine.

FIGURE 3.15

Glucagon radioimmunoassay standard calibration curve.

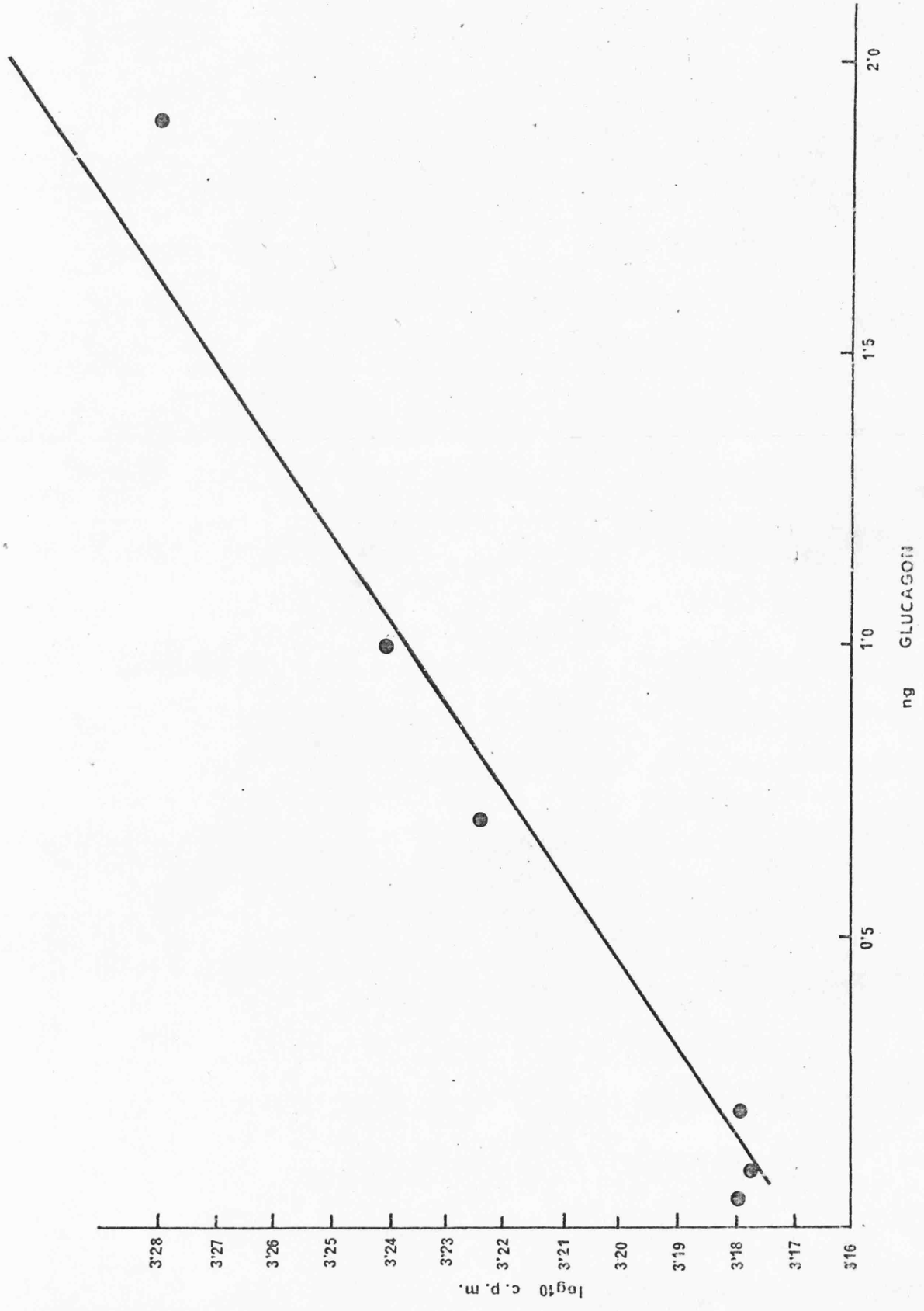

log10 c.p.m.
3'28
3'27
3'26
3'25
3'24
3'23
3'22
3'21
3'20
3'19
3'18
3'17
3'16
0'5
1'0
1'5
2'0
ng GLUCAGON

TABLE 3.19 GLUCAGON IMMUNOASSAY

DOSE	cpm	MEAN cpm	LOG_{10} MEAN cpm
STANDARD			
0.05	1555.5 1552.4 1434.6	1514	3.180
.1	1451.8 1475.4 1587.7	1505	3.176
0.2	1517.5 1467.6 1547.6	1510	3.179
0.5	1610.3 1623.9 1604.1	1613	3.207
0.7	1701.0 1629.6 1709.2	1680	3.225
1	1704.4 1754.3 1760.8	1740	3.241
2	1847.5 1827.4	1862	3.270
UNKNOWN			
FG1	2057.4 2065.2 2156.3	2086	3.319
FG2	4262.6 4266.8 4098.4	4209	3.624
Islet 1	2531.6 2149.2 2542.6	2408	3.382
Islet 2	2236.4 2119.5 2074.3	2143	3.331

SECTION 4

DISCUSSION

4. DISCUSSION

4.1 ISLET TISSUE VOLUMES

Both in absolute volume and in relation to body weight, the islet organ of the two cyclostome groups is remarkably similar (Table 4.1). In *Myxine*, the total weight of islet tissue dissected from 347 animals with body weights of 40-50 g has been given as 0.76 g (Falkmer and Matty, 1966), representing an average of 2.2 mg per animal. Thus the ratio, islet tissue mg/100 g body weight, must be about 4.8, which is very similar to the figures obtained for *L. fluviatilis* and *L. planeri*. A similar ratio has also been reported for the principal islets of the teleost, *Cottus scorpius* (Falkmer, 1961). These values are much higher than the approximate ratio of 0.8 derived from data on the total volume of the islets in the pancreas of the 480 day rat (Hellman *et al.*, 1964) but are similar to the ratio of 4.8 for human islets at the age of 21 (Ogilvie, 1937). Such comparisons are all the more remarkable, bearing in mind difference in metabolic rates and low carbohydrate content of the cyclostome diet. Since it is likely that the proportion of B cells in the cyclostome islet tissue is at least equal to, if not greater than, in mammals, it must be presumed either that the insulin secretory potency of the lamprey and hagfish B cells or the sensitivity of the target organs is lower than in the rat, or alternatively that this hormone plays a larger part in some aspects of metabolism other than that of carbohydrates.

TABLE 4.1

RELATIVE WEIGHT OR VOLUME OF ISLET TISSUE IN VARIOUS VERTEBRATE SPECIES

SPECIES	TOTAL ISLET VOLUME mm^3 OR WEIGHT mg/ 100 g BODY WEIGHT	SOURCE
Lampetra fluviatilis		
macrophthalmia	3.4	
adults	4.1 - 4.6	
Lampetra planeri		
adults	3.6 - 4.9	
Myxine glutinosa	4.8	Falkmer and Matty (1966)
Cottus scorpius	5.0 - 6.0	Falkmer (1961)
Rat (480 day)	0.8	Hellman *et al* (1964)
Human (21 yr)	4.8	Ogilvie (1937)

For example, the suggestion has been made that in the lower vertebrates insulin may play a primary role in protein metabolism (Tashima and Cahill, 1968) and evidence has been obtained that this hormone is involved in the metabolism of lipids in teleosts (Minick and Chavin, 1972; Lewis and Epple, 1972).

4.2 LIVER GLYCOGEN

In both the author's own experience and that of other workers, liver glycogen values in river lampreys tend to show extreme variability. While, to some extent this may be due to the techniques involved in sampling and more especially, handling, anaesthetisation or temperature, a major factor is likely to be the decline in glycogen reserves that normally accompanies the prolonged starvation during the migratory period. Thus, by the time of spawning, liver glycogen reserves have been found to be almost completely depleted (Bentley and Follett, 1965a) and for this reason considerable individual variability would be expected, depending on the period that has elapsed since feeding ceased and on the degree of sexual maturity.

Except for a single report (Plisetskaya and Zheludkova, 1971), determination of liver glycogen levels in river lampreys (Bentley and Follett, 1965a; Leibson and Plisetskaya, 1968; Plisetskaya and Leibush, 1972) have not distinguished between the sex of the experimental animals. However, the values reported independently by Plisetskaya and Zheludkova (1971) are very similar to those reported here. Thus the mean glycogen content of male lamprey liver was 236 $\pm$ 21 (compared with 261 $\pm$ 21 reported here) and for the females 150 $\pm$ 20 (compared with the value of 141 $\pm$ 16 obtained in this work) (Plisetskaya, personal communication). Plisetskaya and Leibson (1973) have also found that the glycogen synthetase activity is significantly higher in females than in males. Thus, the rate of utilisation of hepatic glycogen reserves

appears to be greater in the female, because even though glycogen synthetase activity and therefore, the rate of glycogen biosynthesis is greater in females, the hepatic glycogen reserves appear to be lower.

In view of these findings on sex differences in liver glycogen values, pooling of results from both sexes could give rise to spurious results should there be wide variations in the proportions of the two sexes in various experimental or control groups. Moreover, the situation may be further complicated by sex differences in liver weight relative to body weight and by differences in the liver lipid levels of males and females (Larsen, 1973; Bentley and Follett, 1965b; Moore, 1975).

4.3 COMPARISON OF THE RADIOIMMUNOASSAY STANDARD CALIBRATION FOR HUMAN INSULIN WITH THE DILUTION CURVE FOR LAMPREY ISLET TISSUE EXTRACTS

Comparison of the slopes of these two curves reveals that although there is cross reactivity between the two types of insulin, the immunological properties of human and lamprey insulin are different and this renders quantification of the circulating lamprey insulin very difficult. The calibration curve has little meaning, but the raw c.p.m. data for each of the samples can be compared by analysis of variance.

Insulin values have also been given, although they do not mean much in absolute terms, significant differences between serum samples can be detected in any single assay run. Thus, one can say that a value of 25 μU/ml is higher than 15 μU/ml but one cannot say the the difference (apparently 10 μU) is equal to the difference between 45 μU/ml and 35 μU/ml.

4.4 EFFECTS OF ISLETECTOMY

Differences in cytological and physiological response to sub-total isletectomy, affecting either the caudal or cranial region, are readily comprehensible in the context of the relative volumes of the two regions, and there is at present no indication of cytological differences, other than the frequent occurrence of cysts and other lesions in the cranial islets (Hardisty, 1972b; 1976).

a) Blood Sugar and Histology of Remaining Islet Tissue

The fact that normal or near normal blood glucose levels are apparently maintained in the fasting river lamprey when either the cranial or caudal islet tissue alone is removed, suggests that at this period in the life cycle, the total insulin secreting capacity of the islet tissue may be more than equal to the physiological demands of the tissue. This suggestion is reinforced by the fact that, after 12 days glucose loading both histological observation (vacuolisation in only 3 out of 7 animals) and biochemical evidence (insulin levels very high except in one animal) agree in the conclusion that the islet tissue is not in an advanced state of exhaustion.

Removal of the caudal islet tissue gave a rather higher mean value for blood glucose concentration compared with the cranially isletectomised group, although the difference in the means is not statistically significant. Such a result would,

however, be consistent with the view that the larger caudal islets have greater insulin secreting capacity than the cranial component. This conclusion is supported by the degranulation observed in the cranial region after caudal isletectomy and by the more marked increases in nuclear volume in response to this form of sub-total isletectomy.

b) Insulin

Six days after total isletectomy, a significant dimunition in levels of insulin-like immunoreactivity is observed. If the secretion of insulin-like immunoreactivity is confined to the islet tissue the question arises as to the source of a detectable amount of this activity in the blood after removal of the islet tissue. Two possibilities may be suggested. In the first place there may exist extra insular tissue capable of secreting insulin, perhaps in the scattered intermediate cords or even in the anterior intestine or its cranial diverticula. Alternatively, the rate of inactivation of circulating insulin may be slow. In fact the data would be explicable if a "half-life" for insulin of about six days is assumed. Such an assumption tends to be supported by the course of the insulin response to glucose loading, where the insulin level after 24 h is still almost as high as the insulin concentration level observed after 8 h. A long half life for insulin is also consistent with the data of Leibson and Plisetskaya (1968) which shows that the duration of the hypoglycaemic response to insulin was 7-11 days. This prolonged response is in fact typical of lower vertabrates generally.

c) Glucose Tolerance

The large individual variations observed in blood sugar levels, especially in the early periods after a single injection of glucose might be attributed to a number of factors, among them the rate of uptake of the injected glucose into the extracellular compartment, the intensity of insulin secretion, the rate of metabolism of glucose, the storage of glycogen in the liver or the rate of renal excretion of glucose. Like the teleosts, the lamprey shows a much lower tolerance to glucose than the mammal and this has been attributed to either low rates of insulin secretion or to the low carbohydrate diet (Ince and Thorpe, 1974). In the intact lamprey, urine losses are not an important factor and both our estimates and those of Bentley and Follett (1965b) put these at between 0.5 and 2.0% of injected glucose; much lower than the estimates of 10-15% in the hagfish (Falkmer and Matty, 1966).

Assuming that the whole of the injected glucose is distributed initially in the extracellular phase and neglecting any losses in the urine, it may be estimated that in a 50 g lamprey with an extracellular fluid volume of 22% (Morris, 1972) the glucose concentration might be raised by about 900 mg/100 ml above the baseline values; a level far in excess of those observed in glucose loaded intact animals. Allowing for a baseline level of about 280 mg/100 ml, this corresponds quite closely to the kind of values obtained in isletectomised animals within 2 h of injection. In view of the extent of glucose losses to the

ambient water and the absence of any indication of increased liver glycogen concentrations; it is reasonable to suppose that the totally isletectomised lampreys have substantially lost their normal capacity to regulate blood sugar concentrations. In the larval lamprey, the kidney threshold for glucose has been given as about 80 mg/100 ml (Morris and Islam, 1969b) but the lower rates of glucose losses in the intact glucose loaded river lamprey indicate much higher threshold values at this stage of the life cycle. Such differences might well be related to the extensive modification in renal structure that occurs during metamorphosis (Morris, 1972).

Indeed, the fact that totally isletectomised lampreys were able to maintain blood sugar levels of around 300 mg/100 ml for periods of up to three weeks after operation suggests that threshold values for glucose proably approximate to this figure. However, in both intact or isletectomised lampreys, differences in renal excretion rates may well be an important factor in the variability of blood sugar values.

d). Comparison with Myxinoids

A recent review of the histophysiology of the vertebrate islet tissue (Epple and Lewis, 1973) has emphasised the dangers of extrapolating from conditions in mammals to those of the lower vertebrates and especially in assuming pancreatectomy must invariably result in diabetes mellitus; a situation that apparently

applies generally only to anurans, reptiles and mammals. In the chondrichthyes, teleosts and birds, the outcome of pancreatectomy varies considerably and there appears to be no close correlation between the percentage of B cells in the islet tissue and the development of permanent hyperglycaemia. While it is true that functional cell types other than B cells have not as yet been identified in cyclostome islet tissue, in *Myxine* a second type of granular cell has recently been observed in electron micrographs (Thomas, *et al*, 1973). Until the functional significance of this cell type has been clarified, it would be unwise to speculate on the reasons for the marked differences between the two groups of cyclostomes in their response to isletectomy.

4.5 GLUCOSE LOADING

a) Histology

Vacuolisation of B cells after glucose loading has been reported in a number of teleost species (Khanna and Mehrotra, 1969; Khanna and Rekhari, 1972; Bhatt, 1974). It has also been observed in the islet tissue of larval lampreys follwing glucose injections (Barrington, 1942; Ermisch, 1966), but significantly was not reported by the latter author in the adult stage of either *L. planeri* or *L. fluviatilis*, even when they were subjected to daily glucose injections over periods of up to 11 days. In both ammocoete and adult of *L. planeri*, the terminal phase of serial glucose injection was necrosis, but this was not seen in the adult of *L. fluviatilis*. In these experiments on the river lamprey, necrosis did not occur after daily glucose injections over a period of 8-11 days and vacuolisation was observed in only three out of seven animals. These differences in response to glucose loading between adult and ammocoete, or between species, may reflect differences in the insulin secreting capacity of the B cells or even in the relative volumes of active tissue.

b) Plasma Insulin Levels

The measurements of plasma insulin-like immunoreactivity after a single glucose injection and after long term glucose loading indicate that this activity is released after glucose load, and confirms the results obtained from histological studies

that when in the fasting stage of the animal's life cycle, the islet tissue retains its ability to respond to a glucose load. The B cells seem to retain the ability to secrete even after 12 days loading, as with one exception out of six, there appears to be no biochemical evidence of 'exhaustion'. The time course of the insulin response to glucose injection reveals no significant elevation at 2 h but significant elevation at 8 h and 24 h, which is consistent with glucose tolerance curves which showed a maximum at 2 h and gradually, most probably under the influence of insulin, fell towards baseline levels at 24 h.

Plisetskaya and her co-workers have not been able to show any significant change in insulin-like immunoreactivity in response to glucose, even though the glucose dose (4 gm/kg) and the time after injection (3½ h) were very similar to those which were employed in this study (Plisetskaya and Leibush, 1972; Plisetskaya, personal communication). The reason for this different result in the two different laboratories is not clear but possibly these workers did not estimate the insulin in the serum samples on the same assay run, or possibly did not have an antiserum as sensitive to lamprey insulin as the one used in this study.

4.6 HYPOXIA

The physiological and behavioural responses of the lamprey to hypoxia are strongly suggestive of heightened chromaffin tissue activity. Increases in heart rate, intense motor activity and hyperventilation have also been recorded in teleosts under catecholamine stimulation (Serfaty *et al.*, 1964; Waitznegger and Serfaty, 1967; Pickford *et al.*, 1971). Analyses have been made of catecholamine levels in the heart tissue and blood of lampreys, both under normal and stress conditions (Bloom *et al.*, 1963; Stabrovsky, 1967; Dahl *et al.*, 1970; Mazeaud, 1969, 1972). In *L. fluviatilis*, asphyxia was found to increase the adrenalin content of the heart and skeletal muscle, while forced motor activity decreased heart concentrations, but increased hormone levels in the liver (Stabrovsky, 1967). In the same species, deficient aeration or transport of animals out of water, elevated blood adrenalin levels, particularly in the male (Plisetskaya and Prozorovskay, 1971), although in the sea lamprey, *Petromyzon marinus*, concentrations of adrenalin were found to be twice as high in the females as in males (Mazeaud, 1969). This last observation is of particular interest in view of the trend towards higher blood sugar levels in female river lampreys exposed to hypoxia. In *P. marinus*, forced swimming increased adrenalin levels by a factor of about 8, but in animals with severe skin damage, blood concentrations were about 19-20 times higher than in controls (Mazeaud, 1969, 1972).

In view of the increases observed in the nuclear volumes of

chromaffin cells under hypoxic conditions, the involvement of this tissue may be safely assumed, but it is difficult to decide how far the hyperglycaemia is a direct result of reduced oxygen tensions or is an indirect consequence of the hyperactivity associated with hypoxia. Characteristically, both in teleosts and lampreys, exogenous adrenalin evokes a rapid, but often transient hyperglycaemia, developing within a few hours (Young and Chavin, 1963; Mazeaud, 1964; Bentley and Follett, 1965a; Banerji and Ghosh, 1973; Thorpe and Ince, 1974). On the other hand, in hypoxia treatment, hyperglycaemia in the lamprey developed only gradually, taking 7 days to reach maximum values. This appears to differentiate this condition from the usual 'stress' or 'asphyxial' hyperglycaemia in teleosts and lampreys, where the elevation in blood sugar concentrations is usually very rapid, attaining maximum levels within only a few hours (McCormick and Macleod, 1925; Simpson, 1926; Menten, 1927; Al-Gauhari, 1958; Black and Tredwell, 1967; Leibson *et al.*, 1969; Chavin and Young, 1970; Plisetskaya *et al.*, 1971; Tandon and Joshi, 1973).

The absence of a close correlation between the degree of hyperglycaemia and liver glycogen levels that has been noted in some studies of 'asphyxial' hyperglycaemia in teleosts, has been attributed to variations in nutritional status and liver glycogen reserves (Menten, 1927; Nakano *et al.*, 1967). Since, during the migratory period, liver glycogen levels in the river lamprey are notoriously variable and subject to continuous depletion, similar factors may explain the failure to find evidence of liver glycogenolysis in the present experiments. However, since exogenous

adrenalin is reported to decrease muscle, but not liver glycogen, in the lamprey (Bentley and Follett, 1965a), mobilisation of carbohydrate reserves in the musculature of the body wall may be a major factor in the maintenance of hyperglycaemia in the fasting river lamprey.

Interpretation of the possible role of insulin under hypoxic conditions is difficult to evaluate, particularly in view of the absence of changes in the volume and islet cell nuclei. In mammals, hyperglycaemia is the main factor in the release of insulin from the B cells (Ashcroft *et al*, 1971), but this glucose stimulated secretion is inhibited by adrenalin in *vivo* and in *vitro* (Howell and Taylor, 1966; Milner and Hales, 1969; Bassett, 1971).

An in *vivo* inhibition of insulin secretion by adrenaline in the lamprey has also been shown in this study. The levels of circulating catecholamines in the adrenalin injected animals must be considerably higher than in the hypoxically stressed animals, since the adrenalin injected animals always appear to be de-pigmented,whereas the hypoxic animals are normal in shade. It is possible that at this lower circulating level of adrenalin there is no inhibition of insulin release.

Furthermore, in isolated islets and pancreatic slices, anoxia is said to reduce the effects of insulin-secretory agents, including glucose (Hales, 1971). Thus, in addition to any direct effects of adrenalin on glucogenesis or glycogenolysis, its inhibitory actions may play an important part in the generation or maintenance of hypoxial hyperglycaemia. A role for glucocorticoids in stress hyperglycaemia is well established in teleosts, but the

measurements on interrenal nuclei lend no support to the involvement of this tissue in the present experiments.

The results obtained in experiments involving the subjection of hypophysectomised lampreys to reduced oxygen tensions, although based on small numbers, are consistent in showing the retention of normal blood sugar levels, combined with extensive 'hydropic degeneration'. Neither these observations, nor those of other workers (Matty and Falkmer, 1965; Falkmer and Matty, 1966; Larsen, 1969b; 1973), have demonstrated significant changes in glycaemia in cyclostomes after hypophysectomy, although this operation is said to prevent the rise in blood sugar at sexual maturity (Larsen, 1973).

In birds and mammals, direct or indirect pituitary control of adrenalin synthesis via ACTH or glucocorticoid regulation of phenylethanolamine-N-methyl transferase (PNMT) activity is well established (Wurtman and Axelrod, 1965; Pohorecky *et al.*, 1971; Wasserman *et al.*, 1971; Newcomer *et al.*, 1972; Manelli *et al.*, 1973) and in Amphibia, adrenalin levels are reduced after hypophysectomy (Rapela *et al.*, 1956). In fishes, there is evidence of pituitary stimulation of glycogenolysis which is enhanced by ACTH or cortisol (Chester-Jones *et al.*, 1974) and hypertrophy of chromaffin tissue has been reported after administration of these hormones (Mahon *et al.*, 1962; Olivereau, 1966). On the other hand, in *Salmo gairdneri*, no alteration of PNMT activity or catecholamine levels were detected after hydrocortisone administration (Mazeaud, 1972) and similarly, in the dogfish,

Scylliorhinus canicula, no significant alterations in the methylating capacity of the chromaffin tissues were noted after hypophysectomy (Perrin and Pérès, 1970). The former author, after an examination of the distribution and intensity of PNMT activity in relation to catecholamine concentrations in the heart of the sea lamprey, *P. marinus* concluded that the chromaffin tissue itself is capable of the methylation of noradrenalin, and is not dependent on corticosteroids. In his view, this situation would be consistent with a lack of contact between interrenal and chromaffin tissues, which he believed was characteristic of lampreys. This belief is, however, unjustified and especially in the walls of the cardinal veins and sinus venosus, small groups of the two tissues are often directly contiguous (Fig.3.11).Both in the hypophysectomised control group, as well as those subjected to hypoxia, there has been some evidence from nuclear measurements that chromaffin cell activity is reduced by this operation, but it must be recognised that there are considerable difficulties in postulating the involvement of either ACTH or glucocorticoids in catecholamine biosynthesis. There are some indications that the adenohypophysis of lampreys may produce an adrenocorticotrophic principle (Larsen and Rothwell, 1972; Youson, 1973), but neither mammalian ACTH nor cortisol have produced significant elevation in blood sugar levels (Bentley *et al*, 1965). Furthermore, biochemical evidence for the existence of steroidogenic activity or the production of the normal vertebrate corticoids by the interrenal is at very least uncertain (Seiler *et al.*, 1970; Hardisty, 1972; Weisbart *et al.*, 1970; Weisbart, 1975) and the assumption that this tissue is homologous with the adrenocortical

tissue of gnathostomes rests mainly on morphological criteria (Hardisty and Baines, 1971; Youson, 1972). However, until a more complete analysis has been made of hormonal interactions, the possibility should be borne in mind that the absence of hyperglycaemia in hypophysectomised lampreys might result from more general metabolic changes, reflected in locomotory inertness, loss of reactivity and reduced rate of tissue mobilisation particularly in the body wall (Larsen, 1973).

Striking and significant nuclear hypertrophy of the interrenal, probably accompanied by hyperplasia was noted after adrenalin injections and similar responses have been reported in the teleost, *Heteropneustes fossilis* (Yadov *et al.*, 1970). On the other hand, in *Scorpaena porcus* (Chuiko, 1970) no changes were observed in blood corticosteroids following exogenous adrenalin treatment. That the changes observed in the lamprey interrenal are not a direct response to hyperglycaemia is suggested by the absence of similar changes in hypoxia or glucose loading, and although the mechanism remains obscure, the topographical relationship between chromaffin and interrenal tissues suggests the possibility of some interactions, other than the usual corticosteroid stimulation of PNMT activity.

Certain aspects of hydropic degeneration under hypoxic conditions are difficult to interpret. Vacuolisation resulting from an overloading of the functional capacity of the β cells has been widely reported in teleosts after glucose loading (Khanna and Rekhari, 1972) and has been shown to be a temporary and reversible

condition (Bhatt, 1974), apparently differing qualitatively from the more severe and extensive hydropic degeneration observed in the present experiments. In mammalian islets also, hydropic degeneration of B cells has been reported as only a transitory lesion, reflecting extensive functional strain in cells that escape necrosis, but are nevertheless affected by hyperglycaemia (Warren *et al.*, 1966). Apart from the effects of reduced oxygen tensions, where these lesions have been observed in other groups of lampreys, for example after repeated glucose loading or sub-total isletectomy, they have been associated with marked increases in nuclear volumes, unlike the situation in hypoxia, where in spite of the hyperglycaemia, the nuclear volumes remained unchanged. In mammalian B cells, increased nuclear volume has been noted within 30 min. of a single dextrose injection and although some decrease occurred thereafter with the disappearance of Gomori-positive granulation, the enlargement of the nucleus persisted for some hours during the degranulatory and secretory phase (Mohnicke and Moritz, 1964).

Equally paradoxical is the observation of hydropic degeneration in the hypophysectomised lampreys subjected to hypoxia, where it was accompanied by normal blood sugar levels. The occurrence of these lesions, in the absence of hyperglycaemia and without the usual indications of islet cell stimulation, must cast some doubt on the assumption that vacuolisation represents a simple physiological overloading of the insulin secretory capacity of the cell and is a direct consequence of elevated blood sugar levels. In this connection, it is significant that both glycogen stores and

glycolytic intermediates are reported to accumulate in the B cell after adrenalin inhibition of insulin release (Hellman, 1970). The apparent lack of a cellular response under conditions of hypoxia might be related directly to the reduced oxygen tensions and their effects on cellular metabolism, and in this connection the reports of anoxic inhibition of B cell activity may be recalled (Hales, 1971).

In a marine teleost, exogenous adrenalin has been shown to affect the water permeability of the gills, with wider repercussions on plasma osmolarity and sodium levels as well as having implications for some aspects of stress responses (Pic *et al.*, 1974). In view of the possibility that similar changes might affect cell and nuclear volumes generally, measurements were made of the diameters of erythrocytes in the adrenalin injected and control groups of lampreys, but without disclosing any evidence of changes in mean values.

In certain respects, the cytological observations have some relevance to the functional status of the dark and light cells. In the later stages of hypoxia both in intact and hypophysectomised animals, there was no marked degranulation of the dark cell cords, such as occurs in glucose loading. On the contrary, these cells often showed an abnormally high concentration of coarse aldehyde-fuchsinophil granulation, contrasting with the vacuolated areas which appear to correspond to light cell lobules. Similar differential lesions have also been seen in glucose loading and in cases of 'spontaneously' occurring vacuolisation. These observations would be consistent with the view that both light and dark cells should be

regarded as developmental stages of a single functional cell type (Barrington, 1972) and the reaction of the dark cell granules to both aldehyde-fuchsin and pseudoisocyanin techniques points to the latter as being the terminal or storage phase in the B cell cycle. Moreover, it may be significant that where increased vascularity has been noted in the islet parenchyma, accumulations of erythrocytes appear to be mainly confined to the lumina of the dark cell cords which would presumably be concerned with insulin release. A similar interpretation is also supported by the nuclear measurements, which show that both cell types are stimulated by glucose loading, although the increase in volume tended to be rather greater in the light cells. If this interpretation is valid, the development of vacuolisation in the light cell lobules suggests that earlier phases in insulin biosynthesis and granule development may be more sensitive than later stages to whatever factors may be immediately involved in the genesis of these lesions. The degranulation of the dark cells under glucose loading and their retention of aldehyde-fuchsinophil granulation under hypoxic conditions, would thus represent the influence of factors which respectively stimulate or inhibit insulin release. However, the importance of distinguishing insulin biosynthesis from insulin release is illustrated by the results of adrenalin injection, which have led to very marked increases in the nuclear volumes of islet cells. In the mammalian B cells, mitoses have been increased by glucose injection (Korcakova, 1971) and in the lamprey increased mitotic frequency has occured in hypoxia, glucose loading and above all, after exogenous adrenalin, all of which raise blood glucose

levels. Cell division has, however, been observed only in the light cells; again pointing to these elements as representing an earlier stage in the B cell cycle.

The possible ecological effects, under field conditions, of reduced oxygen tensions in the aetiology of islet cell lesions is difficult to assess. Whether in polluted estuaries or rivers, prolonged exposure to sub-optimal, but much less severe reductions in oxygen tensions, would be capable of producing extensive and permanent hydropic degeneration is open to question. Certainly, a number of examples of these lesions in fungus infected river lampreys have been observed, but in these cases it is possible that the immediate causal factor is an interference with normal gill function. Apart from this, the occurrence of 'spontaneous' islet cell damage of the same type in animals maintained under laboratory conditions, or stored in live boxes in the river, suggests that severe vacuolisation may develop under conditions which, although unfavourable, are nevertheless compatible with survival.

4.7 GLUCAGON-LIKE IMMUNOREACTIVITY

Barrington and Dockray (1970) have found that intestinal extracts of *L. fluviatilis* and *P. marinus* when administered to rats, evoke discharge of pancreatic juice (secretin-like activity) and cause the release of enzymes from the pancreas (pancreozymin-like activity).

Van Noorden and Pearse (1974), using immunofluorescent techniques, have shown caerulin-like, gastrin-like and glucagon-like immunoreactivity in the cells of the intestinal epithelium. All these immunoreactivities appeared to be synthesized in the same cell.

Comparisons of the amino-acid sequences of the gastro-intestinal hormones of mammals have revealed two 'families' of hormones (Weinstein, 1969; Grossman, 1976). Thus, one family comprises secretin, gastrin, gastric inhibitory peptide (G.I.P.), glucagon, vasoactive intestinal peptide (V.I.P.). The other group includes gastrin and cholecystokinin-pancreozymin. It has even been suggested (Weinstein, 1972) that these two families may have originated from one molecule. It is also well established that extracts of the gut of higher vertebrates possess a glucagon-like immunoreactivity which is not identical to pancreatic glucagon (Samols *et al*, 1966; Heding, 1971; Buchanan, 1976).

Active factors from cod and pike intestine, which have secretin-like effect when tested on turkeys and rats cross react in the radioimmunoassay for mammalian vasoactive intestinal peptide (V.I.P.) (Dockray, 1976). It could well be therefore, that V.I.P., which does not apparently have a physiological role in mammals, could be the factor from which the members of the secretin and glucagon family are derived. The lamprey glucagon-like immunoreactivity (Van-Noorden and Pearse, 1974) and the secretin-like activity of (Barrington and Dockray, 1970) could therefore be due to the same (probably V.I.P.-like) principle

Although secretin or pancreozymin function is not possible, in the sense of stimulation of secretion into a pancreatic duct in an animal lacking a discrete exocrine pancreas, the principle(s) responsible for the secretin and pancreozymin-like effects demonstrated in rats could well be involved in regulation of zymogen cell secretion.

Bentley and Follett (1965a) have failed to obtain a hyperglycaemic effect after administration of mammalian glucagon in the lamprey, but a delayed hypoglycaemia, possibly due to stimulation of insulin secretion was observed. The glucagon-like substance in the lamprey gut, however, may have a metabolic function in the lamprey. Indeed some of the apparently anomolous effects of partial isletectomy on blood glucose and liver glycogen values would be explicable if it were assumed that the glucagon-like immunoreactivity in the anterior intestine and its diverticula possessed glycogenolytic and hyperglycaemic properties. Many of the

glucagon containing cells might be destroyed during cranial isletectomy, since part of the anterior intestine and it cranial diverticula must inevitably be removed in this operation. On the other hand, caudal isletectomy would leave the glucagon cells intact. Thus both types of partial isletectomy might interfere with the glucagon/insulin ratio in the blood. Cranial isletectomy could result in a decrease in the glucagon/insulin ratio, and caudal isletectomy in an increase in this ratio. This might provide a hypothesis which would explain the results in Table 3.3. A possible increase in the glucagon/insulin ratio could result in the hepatic glycogenolysis suggested in Table 3.3 in the caudal isletectomised group. Moreover, the slight, but statistically not significant, decrease in blood sugar in the cranial isletectomised animals compared with intact controls, would be consistent with a decrease in the glucagon/insulin ratio. It would be interesting to repeat Bentley and Follett's (1965) test using a purified extract of lamprey glucagon-like immunoreactivity.

4.8 GENERAL CONCLUSIONS

The results obtained are generally consistent with the concept that the islet tissue secretes insulin in response to glucose loading and the presence of the islet tissue is required to enable the animal to regulate its glucose metabolism. Thus, if the islet tissue is removed, the circulating levels of insulin-like immunoreactivity decline, the blood sugar levels rise and the injected animal does not significantly 'metabolise' injected glucose but eliminates most of this injected load by urinary excretion.

The changes in cytological appearance of the islet tissue and circulating levels of insulin-like immunoreactivity consequent upon glucose loading, both indicate that insulin is secreted by the islet tissue in response to a glucose load. Thus, it appears that the B cell of a river lamprey in its upstream migrating phase, far from being "functionless and degenerate" (Boenig, 1929; Sterba, 1955) has all the functional capabilities of a typical vertebrate B cell.

The time course of the hyperglycaemic response to hypoxia has been established and it seems clear that the chromaffin tissue is involved in this hyperglycaemic response to hypoxia but a role for the interrenal tissue does not appear to have been established.

The hydropic degeneration of the islet tissue in hypophysectomised animals, where the sugar levels remained normal has not been completely explained.

These investigations have accomplished some of the aims stated in the Introduction but some further questions are posed:

> "What are the circulating levels of insulin-like immunoreactivity and of catecholamines during hypoxia in intact, sham-operated and hypophysectomised lampreys?'
>
> "What is the nature of the glucagon-like immunoreactivity? Does it have hyperglycaemic and glycogenolytic properties in the lamprey? Is the same chemical substance responsible for it and the gastrin, secretin and pancreozymin-like activity, or are all these activities associated with a single substance of multiple antigenic and biological properties?"

APPENDIX

THE STATISTICAL EVALUATION OF RADIOIMMUNOASSAY DATA

THE STATISTICAL EVALUATION OF RADIOIMMUNOASSAY DATA

A computer programme for radioimmunoassay data has been published (Ayery and Evans, 1972), but this did not include any method for determining confidence limits in the results obtained. For experimental work it is important to be able to determine whether one set of serum samples obtained from a group of animals, given a certain treatment, are significantly different from a control set of serum samples.

Various mathematical models, based on physico-chemical considerations have been published (Yallow and Berson, 1970; Ekins *et al.*, 1970) but although these give an understanding of the kinetics of antigen-antibody interaction, schemes for determining confidence limits from radioimmunoassay data do not appear to have been developed (see Ekins, 1973).

A scheme has therefore, been evolved for the determination of radioimmunoassay data and incorporated into an interactive FORTRAN computer programme. This scheme will be illustrated in this Appendix by explaining the various steps in the computer programme (RIA) and outlining some of the basic mathematical and statistical considerations.

Lines 1-29 of the programme declare the arrays and place a p = .95 t distribution table into variable array TT and the table for the upper 5% points of Hartley's table of ratios of maximum and minimum variance. The function X LOGIT (logit) and

ALOGIT (anti-logit) are defined:

$$\text{logit } a = \ln \left(a \,/\, (1-a) \right)$$

$$\text{anti logit} = \frac{e^a}{1 + e^a}$$

Lines 30-40 are concerned with the input of the new data relating to the standard curve.

Lines 41-51 cause a table for this data to be printed.

Lines 52-81 calculate the mean c.p.m. for each concentration. value on the standard curve and determine the variance for each concentration group.

Because the c.p.m. dose curve is not linear, a linearising transform has to be executed. Three different transforms can be selected; ln c.p.m. against dose, (c.p.m./c.p.m. at zero concentration) against dose (Ayery and Evans, 1972); and logit (c.p.m./c.p.m. at zero concentration) against ln dose (Yallow and Berson, 1970).

If the third transform is used then those logit values lying outside the limits $\pm$ 2.2 must be rejected (Yallow and Berson, 1972). Lines 82-127 enable the user to select these transforms and execute them.

Lines 128-136 print a table of the transformed data. The mean and variance of the transformed data for each concentration group are determined in Lines 137-164.

The data is then tested for homoscedasticity by Hartley's Maximum F Ratio Technique (Acton, 1959; Hartley, 1950). First the untransformed data (Lines 165-173) and then the transformed data (Lines 174-183) are analysed by this technique. The more crucial test is whether the transformed data is homoscedastic.

Lines 184-193 calculate the weighted means $\bar{x}$ and $\bar{y}$, allowance being made to put less weight on those concentrations in which there were fewer c.p.m. observations.

The next section, Lines 193-208 calculates the parameters Sxx, Sxy and Syy (Acton, 1959):

$$Sxx = \sum_i ki\ (x_i - \bar{x})^2$$

where i is the number of concentration, ki is the number of c.p.m. observations in each concentration group, and x_i is the concentration (transformed if required):

$$Syy = \sum_i ki\ (x_i - \bar{x})\ \ (y_i - \bar{y})$$

where y_i is the mean of the transformed c.p.m. values for each concentration group:

$$Syy = \sum ki\ (y_i - \bar{y})$$

Slope b and the intercept a are then calculated (Lines 209-210).

$$b = Sxx$$

$$a = \bar{y} - b\bar{x}$$

Now that the values of the constants a and b in the equation

$$y = a + b\,x$$

are known, the c.p.m. values which could be predicted from each concentration group can be worked out (Lines 211-220). Then the 95% confidence limits for the predicted value of y are calculated. The variance of y within each group and the total variance due to within group variability var(y) has already been calculated (Lines 138-162), where it was assigned the variable TAMSW. Now the variance of each point on the regression line var(Y) is given by (Colquohoun):

$$\text{var}(Y) = \text{var}(y)\left(\frac{1}{N} + \frac{(x - \bar{x})^2}{Sxx}\right)$$

where N is the total number of observations:

$$N = \sum_i ki$$

These calculations are performed in Lines 221-236.

The next part of the programme control the printout of the results; Lines 237-247 give instructions for the printing of Hartley's ratio for untransformed data. Lines 248-289 print out the Hartley's ratio for untransformed data, and Lines 258-268 the output of Sxx, Sxy, Syy, and the slope and intercept.

The next part of the programme, Lines 264-313 calculates and prints the analysis of variance table. The probability level

for each F ratio is calculated by a subroutine DOUBLE which converts the data into double precision, so that it can be calculated on a further subroutine F TEST.

The correlation coefficient is calculated in Lines 314-320.

$$r = \frac{Sxy}{\sqrt{Sxx \cdot Syy}}$$

Lines 321-328 calculate Fiellers Constant g (Finney, 1964):

$$g = \frac{t^2 \text{ var } y}{Sxx\ b^2} Sxx$$

The next section, Lines 329-335 asks the user if he desires that the data be transformed in another way, and if so, transfers the user back to Line 83, if no further transforms are to be tried, the c.p.m. values for the unknown samples are fed in (Lines 336-356).

Each repeat sample from each individual animal are grouped and the individual animals are organised into two groups. This is to enable easy comparison to be made between treatment groups, for example experimental and control.

The unknown c.p.m. values subjected to an hierarchical analysis of variance (Lines 376-462) which enables one to compare the variability between treatment groups, between animals and between repeat samples in each animal. This enables one to state whether or not there is a significant difference between animals from the different groups, without any assumptions as to the relationship

between c.p.m. and dose. This approach is necessary as it was found, not surprisingly, that the dose - c.p.m. curves for human insulin and lamprey insulin are not parallel. During the analysis the mean c.p.m. for each animal and the mean c.p.m. for each treatment group is calculated.

Lines 463-497 seek the subroutine UNCONC which calculates the concentration in the unknown samples from the regression equation, and also calls CONLIM which calculates the confidence limits in the results obtained, using a simplified version of Fieller's Theorem (Colquhoun, 1971).

Lines 498-505 asks the users if further pairs of treatment groups are to be analysed in the same assay run. If so, control is transferred back to Line 337, if not the programme run is terminated.

Lines 506-507 contain the FORMAT statements which control the input and output.

This programme (RIA) is set out in Figures A.1 - A.12. The subroutine F TEST which is a translation of the programme of Morris (1969) from ALGOL into FORTRAN is illustrated in Figures A.13 and A.14. Subroutines DOUBLE, UNCONC and CONLIM are illustrated in Figure A.15. A typical interactive run of the programme RIA is illustrated in Figures A.16 - A.28. The explanation to these figures (A.16 - A.28) explain how the programme is used in practice.

FIGURE A.1

PROGRAMME RIA (Lines 1–54)

```
 1 C      THIS PART INPUTS X AND Y VALUES - RAW DATA
 2        DIMENSION X(7),Y(7,4),K(7)
 3        DIMENSION YP(7),UCL(7),SCL(7)
 4        DIMENSION YMEAN(7),YVAR(7)
 5        DIMENSION YU(4)
 6        DIMENSION XT(7),YT(7,4),KT(7),KTM(7)
 7        DIMENSION TT(30),HART(10,10)
 8        DIMENSION TYME(7),TYVAR(7)
 9        DIMENSION YU1(4,20),YU2(4,20),KU1(20),KU2(20),U1(20),U2(20)
10        DIMENSION YUT1(4,20),YUT2(4,20),AVYU1(20),AVYU2(20),XU1(20),
11       /XU2(20)
12        DIMENSION UICLM1(20),CLIL1(20),CLIU1(20)
13        DIMENSION UICLM2(20),CLIL2(20),CLIU2(20)
14        DATA TT/12.706,4.303,3.182,2.776,2.571,2.447,2.365,2.306,2.262,
15       /2.228,2.201,2.179,2.160,2.145,2.131,2.120,2.110,2.101,2.093,2.086,
16       /2.080,2.074,2.069,2.064,2.060,2.056,2.052,2.048,2.045,2.042/
17        DATA HART/ 11*1,39.0,15.4,9.6,7.15,5.82,4.99,4.43,4.03,3.72,
18       /1,87.5,27.8,15.5,10.8,8.38,6.94,6.0,5.34,4.85,
19       /1,142.0,39.2,20.6,13.7,10.4,8.44,7.18,6.31,5.67,
20       /1,202.0,50.7,25.2,16.3,12.1,9.7,8.12,7.11,6.34,
21       /1,266,62,29.5,18.7,13.7,10.8,9.03,7.8,6.92,
22       /1,333,72.9,33.6,20.8,15.0,11.8,9.7,8.41,7.42,
23       /1,403,83.5,37.5,22.9,16.3,12.7,10.5,8.95,7.87,
24       /1,475,93.9,41.1,24.7,17.5,13.5,11.1,9.45,8.28,
25       /1,550,104,44.6,26.5,18.6,14.3,11.7,9.91,8.66/
26        DATA X/7*1000000/,XT/7*1000000/,Y/28*1000000/,YT/28*1000000/
27        REAL YES/'YES'/,NO/'NO'/
28        XLOGIT(A)=ALOG(A/(1-A))
29        ALOGIT(A)=EXP(A)/(1+EXP(A))
30        WRITE(2,10)
31        READ(1,20)N
32        WRITE(2,30)
33        READ(1,40) (X(I),I=1,N)
34        DO 100 I=1,N
35        WRITE(2,50)
36        READ(1,20) K(I)
37        WRITE(2,60)
38        L=K(I)
39        READ(1,40) (Y(I,J),J=1,L)
40   100  CONTINUE
41 C      THIS PART PRINTS TABLE OF RAW DATA
42        WRITE(2,120)
43        WRITE(2,110)(X(I),I=1,7)
44        WRITE(2,150)
45        WRITE(2,130)
46        DO 111 J=1,3
47        WRITE(2,110)(Y(I,J),I=1,7)
48   111  CONTINUE
49        WRITE(2,150)
50        WRITE(2,140)
51        WRITE(2,160)(K(I),I=1,7)
52 C      THIS PART CALCULATES YMEAN AND Y
53 C      VARIANCE FOR EACH X GROUP
54        SSW=0.0
```

FIGURE A.2

PROGRAMME RIA (Lines 55–108)

```
55          G=0.0
56          DO 170 I=1,N
57          L=K(I)
58          SUMY=0.0
59          SUMYSQ=0.0
60          DO 180 J=1,L
61          SUMY=SUMY+Y(I,J)
62          YSQ=Y(I,J)*Y(I,J)
63      180 SUMYSQ=SUMYSQ+YSQ
64          G=G+K(I)
65          YMEAN(I)=SUMY/L
66          SYMYB=SUMYSQ-((SUMY*SUMY)/K(I))
67          IF(L.LE.1)GO TO 171
68          YVAR(I)=SYMYB/(L-1)
69          GO TO 172
70      171 YVAR(I)=1
71      172 SSW=SSW+SYMYB
72      170 CONTINUE
73          NDFW=G-N
74          AMSW=SSW/NDFW
75          WRITE(2,150)
76          WRITE(2,200)
77          WRITE(2,110)(YMEAN(I),I=1,7)
78          WRITE(2,150)
79          WRITE(2,210)
80          WRITE(2,110)(YVAR(I),I=1,7)
81          YO=YMEAN(1)
82  C       THIS PART SELECTS TRANSFORMS
83      179 WRITE(2,211)
84          READ(1,20)M
85          GO TO (186,186,187),M
86      186 DO 181 I=1,N
87          L=K(I)
88          DO 182 J=1,L
89          IF(M.EQ.1)YT(I,J)=ALOG(Y(I,J))
90          YYO=Y(I,J)/YO
91          IF(M.EQ.2)YT(I,J)=YYO
92      182 CONTINUE
93          XT(I)=X(I)
94          KT(I)=K(I)
95      181 CONTINUE
96          NT=N
97          GT=G
98          GO TO 188
99  C       THIS PART EXECUTES LOGIT TRANSFORM AND INCORPORATES TEST TO REJEC
100 C       THOSE READINGS WHERE X=0 OR MEAN LOGIT IS OUTSIDE LIMITS +/-2.2
101     187 IT=1
102         I=1
103         NT=N
104         NN=0.0
105         IF(X(I).GT.0.0)GO TO 191
106     192 NN=NN+1
107         NT=N-NN
108     191 KOUNT=0.0
```

FIGURE A.3

PROGRAMME RIA (Lines 109–162)

```
109    196 I=1+KOUNT+NN
110        IT=1+KOUNT
111        XT(IT)=ALOG(X(I))
112        KT(IT)=K(I)
113        L=K(I)
114        SYT=0.0
115        DO 194 J=1,L
116        YYO=Y(I,J)/YO
117        YT(IT,J)=XLOGIT(YYO)
118    194 SYT=SYT+YT(IT,J)
119        YTM=SYT/L
120        IF(YTM.GE.2.2)GO TO 192
121        IF(YTM.LE.-2.2)NT=NT-1
122        IF(YTM.LE.-2.2)GO TO 193
123        KOUNT=KOUNT + 1
124        IF(KOUNT.LT.NT) GO TO 196
125    193 GT=0.0
126        DO 199 I=1,NT
127    199 GT=KT(I)+GT
128 C      THIS PART PRINTS TABLE OF TRANSFORMED DATA
129    188 WRITE(2,125)
130        WRITE(2,110)(XT(I),I=1,7)
131        WRITE(2,150)
132        WRITE(2,135)
133        DO 112 J=1,3
134        WRITE(2,115)(YT(I,J),I=1,7)
135    112 CONTINUE
136        WRITE(2,150)
137 C      THIS PART CALCULATES Y MEAN AND VARIANCE FOR TRANSFORMED DATA
138        TSSW=0.0
139        DO 201 I=1,NT
140        TSUMY=0.0
141        L=KT(I)
142        TSUMY=0.0
143        TSMSQ=0.0
144        DO 202 J=1,L
145        TSUMY=TSUMY+YT(I,J)
146        TYSQ=YT(I,J)*YT(I,J)
147    202 TSMSQ=TSMSQ+TYSQ
148        TYME(I)=TSUMY/L
149        TSYMYB=TSMSQ-((TSUMY*TSUMY)/KT(I))
150        IF(L.LE.1) GO TO 207
151        TYVAR(I)=TSYMYB/(L-1)
152        GO TO 206
153    207 TYVAR(I)=1
154    206 TSSW=TSSW+TSYMYB
155    201 CONTINUE
156        NTDFW=GT-NT
157        IF(NTDFW.LE.30)T=TT(NTDFW)
158        IF(NTDFW.GT.30)T=2.0
159        TAMSW=TSSW/NTDFW
160        WRITE(2,205)
161        WRITE(2,115)(TYME(I),I=1,7)
162        WRITE(2,150)
```

FIGURE A.4

PROGRAMME RIA (Lines 163–216).

```
163         WRITE(2,215)
164         WRITE(2,115)(TYVAR(I),I=1,7)
165 C       THIS PART CALCULATES HARTLEYS RATIO
166         VARMAX=YVAR(1)
167         VARMIN=YVAR(1)
168         DO 230 I=2,N
169         IF (YVAR(I).GT.VARMAX)VARMAX=YVAR(I)
170         IF (YVAR(I).LT.VARMIN)VARMIN=YVAR(I)
171     230 CONTINUE
172         DEGFRE=G/N
173         HARTLY=VARMAX/VARMIN
174 C       THIS PART CALCULATES HARTLEYS RATIO FOR TRANSFORMED DATA
175         TVMAX=TYVAR(1)
176         TVMIN=TYVAR(1)
177         DO 208 I=2,NT
178         IF(TYVAR(I).GT.TVMAX) TVMAX=TYVAR(I)
179         IF(TYVAR(I).LT.TVMIN) TVMIN=TYVAR(I)
180     208 CONTINUE
181         TDFR=FT
182         TDFR=GT/NT
183         THART=TVMAX/TVMIN
184 C       CALCULATION OF WEIGHTED MEANS XBAR AND YBAR
185         SUMKX=0.0
186         SUMKY=0.0
187         DO 270 I=1,NT
188         SUMKX=SUMKX +(KT(I) *XT(I))
189         SUMKY=SUMKY +(KT(I) *TYME (I))
190     270 CONTINUE
191         YBAR=SUMKY/GT
192         XBAR=SUMKX/GT
193 C       CALCULATION OF SXX,SYY,SXY
194         SXX=0.0
195         SYY=0.0
196         SXY=0.0
197         DO 290 I=1,NT
198         AKXX=KT(I)*XT(I)*XT(I)
199         AKYY=KT(I)*TYME(I)*TYME(I)
200         AKXY=KT(I)*XT(I)*TYME(I)
201         SXX=SXX+AKXX
202         SXY=SXY+AKXY
203         SYY=SYY+AKYY
204     290 CONTINUE
205         SXX=SXX-(GT*XBAR*XBAR)
206         SYY=SYY-(GT*YBAR*YBAR)
207         SXY=SXY-(GT*XBAR*YBAR)
208         WRITE(2,150)
209         B=SXY/SXX
210         A=YBAR-(B*XBAR)
211 C       THIS PART CALCULATES Y PREDICTED
212         DO 305 I=1,NT
213         YP(I)=A+B*X(I)
214         IF(M.EQ.1)YP(I)=EXP(YP(I))
215         IF(M.EQ.2)YP(I)=YO*YP(I)
216         IF(M.EQ.3)YP(I)=YO*(ALOGIT(YP(I)))
```

FIGURE A.5

PROGRAMME RIA (Lines 217–270)

```
217    305 CONTINUE
218        WRITE(2,306)
219        WRITE(2,110)(YP(I),I=1,7)
220        WRITE(2,150)
221  C     THIS PART CALCULATES 95% CONFIDENCE LIMITS FOR Y PREDICTED
222        DO 292 I=1,NT
223        VARY=TAMSW*((1.0/GT)+((X(I)-XBAR)**2/SXX))
224        CLW=T*SQRT(VARY)
225        IF(N.EQ.1)CLW=EXP(CLW)
226        IF(N.EQ.2)CLW=YO*CLW
227        IF(N.EQ.3)CLW=YO*(ALOGIT(CLW))
228        UCL(I)=YP(I)+CLW
229        SCL(I)=YP(I)-CLW
230    292 CONTINUE
231        WRITE(2,293)
232        WRITE(2,110)(UCL(I),I=1,7)
233        WRITE(2,295)
234        WRITE(2,110)(SCL(I),I=1,7)
235        WRITE(2,150)
236        WRITE(2,709)
237  C     THIS PART PRINTS HARTLEYS RATIO FOR UNTRANSFORMED DATA
238        IDEGFR=DEGFRE+0.5
239        HARTT=HART(N,IDEGFR)
240        WRITE(2,250)HARTLY
241        WRITE(2,260)N,DEGFRE
242        WRITE(2,281)HARTT
243        IF (HARTLY .GT. HARTT) GOTO 286
244        WRITE(2,283)
245        GOTO 287
246    286 WRITE(2,284)
247    287 WRITE(2,150)
248  C     THIS PART PRINTS HARTLEYS RATIO FOR TRANSFORMED DATA
249        ITDFR=TDFR+0.5
250        THARTT=HART(NT,ITDFR)
251        WRITE(2,255)THART
252        WRITE(2,260)NT,TDFR
253        WRITE(2,281)THARTT
254        IF(THART.GT.THARTT) GO TO 288
255        WRITE(2,283)
256        GO TO 289
257    288 WRITE(2,284)
258    289 WRITE(2,150)
259  C     THIS PART PRINTS SXX ETC, SLOPE AND INTERCEPT
260        WRITE(2,280)XBAR,YBAR
261        WRITE(2,300)SXX,SYY,SXY
262        IF(N.EQ.1)AA=EXP(A)
263        IF(N.EQ.2)AA=YO*A
264        IF(N.EQ.3)AA=YO*(ALOGIT(A))
265        WRITE(2,150)
266        WRITE(2,307)AA,B
267        WRITE(2,150)
268        WRITE(2,709)
269  C     CALCULATION OF CORRECTED TOTAL SUM OF SQUARED DEVIATIONS
270        SY=0.0
```

FIGURE A.6

PROGRAMME RIA (Lines 271–324)

```
271         SYSQ=0.0
272         DO 310 I=1,NT
273         L=KT(I)
274         DO 320 J=1,L
275         YSQ=YT(I,J)**2
276         SY=SY+YT(I,J)
277         SYSQ=SYSQ+YSQ
278     320 CONTINUE
279     310 CONTINUE
280         TOTSQ=SYSQ-((SY*SY)/GT)
281   C     CALCULATION OF DEGREES OF FREEDOM, MEAN SQUARES ETC
282   C     FOR ANOVAR TABLE
283         SSR=B*SXY
284         SSD=SYY-SSR
285         SSWA=TOTSQ-SYY
286         NDFR=1
287         NDFD=NT-2
288         NDFB=NT-1
289         NDFT=GT-1
290         NDFW=NTDFW
291         AMSR=SSR/NDFR
292         AMSD=SSD/NDFD
293         AMSB=SYY/NDFB
294         AMSW=SSWA/NDFW
295         AMST=TOTSQ/NDFT
296         FR=AMSR/AMSW
297         FD=AMSD/AMSW
298         FB=AMSB/AMSW
299         CALL DOUBLE(FR,NDFR,NDFW,PR)
300         CALL DOUBLE(FD,NDFD,NDFW,PD)
301         CALL DOUBLE(FB,NDFB,NDFW,PB)
302   C     THIS PART PRINTS ANOVA TABLE
303         WRITE(2,321)
304         WRITE(2,325)
305         WRITE(2,350)
306         WRITE(2,330)
307         WRITE(2,360)NDFR,SSR,AMSR,FR,PR
308         WRITE(2,370)NDFD,SSD,AMSD,FD,PD
309         WRITE(2,330)
310         WRITE(2,380)NDFB,SYY,AMSB,FB,PB
311         WRITE(2,390)NDFW,SSWA,AMSW
312         WRITE(2,330)
313         WRITE(2,400)NDFT,TOTSQ
314   C     THIS PART CALCULATES THE CORRELATION COEFFICIENT
315         D=SQRT(SXX*SYY)
316         R=SXY/D
317         WRITE(2,150)
318         WRITE(2,709)
319         WRITE(2,410)R
320         WRITE(2,150)
321   C     THIS PART CALCULATES FIELLERS CONSTANT G AND PRINTS T
322         WRITE(2,415)NDFW,T
323     415 FORMAT(' THE VALUES OF T FOR  ',I2,
324        /'  DEGREES OF FREEDOM IS  ',F6.3)
```

FIGURE A.7

PROGRAMME RIA (Lines 325–378)

```
325         FG=(T*T*ANSW)/(SXX*B*B)
326         WRITE(2,420)FG
327         WRITE(2,150)
328         WRITE(2,709)
329 C       THIS PART ASKS IF FURTHER TRANSFORMS REQUIRED
330         WRITE(2,610)
331    610   READ(1,440)ANSW
332         IF(ANSW.EQ.YES)GO TO 179
333         IF(ANSW.EQ.NO)GO TO 600
334         WRITE(2,550)
335         GO TO 610
336 C       THIS PART INPUTS UNKNOWN
337    600 WRITE(2,430)
338         READ(1,20),NU1
339         DO 438 I=1,NU1
340         WRITE(2,432)
341         READ(1,440)U1(I)
342         READ(1,20)KU1(I)
343         WRITE(2,433) U1(I)
344         L=KU1(I)
345         READ(1,40)(YU1(I,J),J=1,L)
346    438 CONTINUE
347         WRITE(2,431)
348         READ(1,20)NU2
349         DO 439 I=1,NU2
350         WRITE(2,432)
351         READ(1,440) U2(I)
352         READ(1,20) KU2(I)
353         WRITE(2,433) U2(I)
354         L=KU2(I)
355         READ(1,40)(YU2(I,J),J=1,L)
356    439 CONTINUE
357 C       THIS PART TRANSFORMS UNKNOWN
358         DO 630 I=1,NU1
359         L=KU1(I)
360         DO 629 J=1,L
361         IF(N.EQ.1) YUT1(I,J)=ALOG(YU1(I,J))
362         YYO=YU1(I,J)/YO
363         IF(N.EQ.2)YUT1(I,J)=YYO
364         IF(N.EQ.3)YUT1(I,J)=ALOGIT(YYO)
365    629 CONTINUE
366    630 CONTINUE
367         DO 632 I=1,NU2
368         L=KU2(I)
369         DO 631 J=1,L
370         IF(N.EQ.1) YUT2(I,J)=ALOG(YU2(I,J))
371         YYO=YU2(I,J)/YO
372         IF(N.EQ.2)YUT2(I,J)=YYO
373         IF(N.EQ.3)YUT2(I,J)=ALOGIT(YYO)
374    631 CONTINUE
375    632 CONTINUE
376 C       THIS  PART  CALCULATES  ANOVA  FOR  UNKNOWN AND CALCULATES BOTH
377 C       INDIVIDUAL AND GRAND MEANS FOR EACH TREATMENT  GROUP
378         SYUT1=0.0
```

FIGURE A.8

PROGRAMME RIA (Lines 379-432)

```
379         SYUT1S=0.0
380         YUA1SN=0.0
381         SYUT2=0.0
382         SYUT2S=0.0
383         YUA2SN=0.0
384         NGU1=0
385         NGU2=0
386         DO 640 I=1,NU1
387         L=KU1(I)
388         AYUT1=0.0
389         AYUT1S=0.0
390         DO 639 J=1,L
391         AYUT1=YUT1(I,J)+AYUT1
392         YUT1S=YUT1(I,J)*YUT1(I,J)
393         AYUT1S=YUT1S+AYUT1S
394      639 CONTINUE
395         AVYU1(I)=AYUT1/L
396         NGU1=NGU1+L
397         SYUT1=SYUT1+AYUT1
398         SYUT1S=SYUT1S+AYUT1S
399         YA1SN=(AYUT1*AYUT1)/L
400         SYA1SN=SYA1SN+YA1SN
401      640 CONTINUE
402         GAVYU1=SYUT1/NGU1
403         DO 642 I=1,NU2
404         L=KU2(I)
405         AYUT2=0.0
406         AYUT2S=0.0
407         DO 641 J=1,L
408         AYUT2=YUT2(I,J)+AYUT2
409         YUT2S=YUT2(I,J)*YUT2(I,J)
410         AYUT2S=YUT2S+AYUT2S
411      641 CONTINUE
412         AVYU2(I)=AYUT2/L
413         NGU2=NGU2+L
414         SYUT2=SYUT2+AYUT2
415         SYUT2S=SYUT2S+AYUT2S
416         YA2SN=(AYUT2*AYUT2)/L
417         SYA2SN=SYA2SN+YA2SN
418      642 CONTINUE
419         GAVYU2=SYUT2/NGU2
420         CSYUT=SYUT1+SYUT2
421         CSYUTS=SYUT1S+SYUT2S
422         CSYASN=SYA1SN+SYA2SN
423         NGUC=NGU1+NGU2
424         BT=((SYUT1*SYUT1)/NGU1)+((SYUT2*SYUT2)/NGU2)
425         CF=(CSYUT*CSYUT)/NGUC
426         SSBT=BT-CF
427         SSBA=CSYASN-BT
428         SSTOT=CSYUTS-CF
429         NUC=NU1+NU2
430         NDFBA=NUC-2
431         NDFBB=NUC-1
432         NDFRU=NGUC-NUC
```

FIGURE A.9

PROGRAMME RIA (Lines 433–486)

```
      NDFTU=NGUC-1
      SSBB=SSBA+SSBT
      SSRU=SSTOT-SSBB
      AMSBT=SSBT
      AMSBA=SSBA/NDFBA
      AMSBB=SSBB/NDFBB
      AMSRU=SSRU/NDFRU
      AMSTU=SSTOT/NDFTU
      NDFRUR=NDFRU+NDFW
      AMSRUR=(SSWA+SSRU)/NDFRUR
      VRBT=AMSBT/AMSRUR
      VRBA=AMSBA/AMSRUR
      VRBB=AMSBB/AMSRUR
      CALL DOUBLE(VRBT,1,NDFRU,PBT)
      CALL DOUBLE(VRBA,NDFBA,NDFRU,PBA)
      CALL DOUBLE(VRBB,NDFBB,NDFRU,PBB)
      NT=1
      WRITE(2,620)
      WRITE(2,150)
      WRITE(2,621)NT,SSBT,AMSBT,VRBT,PBT
      WRITE(2,622)
      WRITE(2,623)NDFBA,SSBA,AMSBA,VRBA,PBA
      WRITE(2,150)
      WRITE(2,624)NDFBB,SSBB,AMSBB,VRBB,PBB
      WRITE(2,625)NDFRU,SSRU,AMSRU
      WRITE(2,150)
      WRITE(2,627)
      WRITE(2,628)NDFRUR,AMSRUR
      WRITE(2,150)
      WRITE(2,626)NDFTU,SSTOT
C THIS PART CALLS SUBROUTINE ,WHICH CALCULATES UNKNOWN CONCENTRATION
C   VALUES BOTH FOR THE MEAN AND INDIVIDUAL ANIMALS IN EACH TREATMET
C   GROUP
      IF(NDFRUR.LE.30)T=TT(NDFRUR)
      IF(NDFRUR.GT.30)T=2.0
      DO  650 I=1,NU1
      CALL UNCONC (XU1(I),AVYU1(I),A,B,M)
      CALL CONLIM(XU1(I),G,B,XU1(I),SXX,AMSRUR,T,YBAR,M,UICLW1(I))
      CLIU1(I)=XU1(I)+UICLW1(I)
      CLIL1(I)=XU1(I)-UICLW1(I)
  650 CONTINUE
      CALL UNCONC(GAVXU1,GAVYU1,A,B,M)
      DO 655 I=1,NU2
      CALL UNCONC (XU2(I),AVYU2(I),A,B,M)
      CALL CONLIM(XU2(I),G,B,XU2(I),SXX,AMSRUR,T,YBAR,M,UICLW2(I))
      CLIU2(I)=XU2(I)+UICLW2(I)
      CLIL2(I)=XU2(I)-UICLW2(I)
  655 CONTINUE
      CALL UNCONC(GAVXU2,GAVYU2,A,B,M)
      CALL CONLIM(NGU1,G,B,GAVYU1,SXX,AMSRUR,T,YBAR,M,UCLW1)
      CLU1=GAVXU1+UCLW1
      CLL1=GAVXU1-UCLW1
      CALL CONLIM(NGU2,G,B,GAVYU2,SXX,AMSRUR,T,YBAR,M,UCLW2)
      CLU2=GAVXU2+UCLW2
```

FIGURE A.10

PROGRAMME RIA (Lines 487–540)

```
      CLL2=GAVXU2-UCLW2
      WRITE(2,700)
      DO 670 I=1,NU1
      WRITE(2,701) U1(I),XU1(I)
  670 CONTINUE
      WRITE(2,702)GAVXU1,CLU1,CLL1
      WRITE(2,703)
      DO 680 I=1,NU2
      WRITE(2,701)U2(I),XU2(I)
  680 CONTINUE
      WRITE(2,702)GAVXU2,CLU2,CLL2
  636 WRITE(2,635)
      READ(1,440)ANSR
      IF(ANSR.EQ.YES) GO TO 600
      IF(ANSR.EQ.NO) GO TO 899
      WRITE(2,550)
      GO TO 636
  899 WRITE(2,710)
  900 STOP
   10 FORMAT(' TYPE IN THE NUMBER OF CONCENTRATIONS IN',
     /' STANDARD CURVE')
   20 FORMAT(I3)
   30 FORMAT(' NOW TYPE IN THE VALUES OF THESE CONCENTRATIONS',
     /'- X VALUES'/' IN ASCENDING ORDER PLEASE')
   40 FORMAT(F6.0)
   50 FORMAT(' TYPE IN THE NO OF CPM READINGS IN NEXT ',
     /'CONCENTRATION GROUP')
   60 FORMAT(' NOW TYPE IN THE CPM READINGS - Y VALUES ',
     /'- IN THIS GROUP')
  110 FORMAT(1X,7(F7.1,1X),T63,1H*)
  115 FORMAT(1X,7(F7.4,1X),T63,1H*)
  120 FORMAT(' X VALUES',T63,1H*)
  125 FORMAT(' TRANSFORMED X VALUES',T63,1H*)
  130 FORMAT(' Y VALUES',T63,1H*)
  135 FORMAT(' TRANSFORMED Y VALUES',T63,1H*)
  140 FORMAT(' K VALUES',T63,1H*)
  150 FORMAT(1X,62(1H*))
  160 FORMAT(1X,7(I7,1X)T63,1H*)
  200 FORMAT(' Y MEAN',T63,1H*)
  205 FORMAT(' MEAN OF Y TRANSFORMED',T63,1H*)
  210 FORMAT(' VARIANCE OF Y WITHIN EACH GROUP',T63,1H*)
  211 FORMAT(' TRANSFORM REQUIRED ?'/
     /' TYPE IN 1 FOR LOG Y V. X'/
     /' TYPE IN 2 FOR Y/YO V. X'/
     /' TYPE IN 3 FOR LOGIT(Y/YO) V. LOG X')
  215 FORMAT(' VARIANCE OF Y TRANSFORMED',T63,1H*)
  250 FORMAT(' HARTLEYS RATIO IS ',F6.2)
  255 FORMAT(' HARTLEYS RATIO FOR TRANSFORMED DATA',
     /' IS ',F6.2)
  260 FORMAT(' NO OF OPS(K)=',I3,'  DEGR. OF FREEDOM = ',F6.2)
  280 FORMAT(5X,'XBAR = ',F9.3,5X,'YBAR =',F9.3)
  281 FORMAT(' THE 5% HARTLEY RATIO FOR THIS DEGREE '
     /,'OF FREEDOM IS  ',F7.3)
  283 FORMAT(' HARTLEYS TEST SHOWS THAT THE DATA IS ',
```

FIGURE A. 11

PROGRAMME RIA (Lines 541–594)

```
541       /'HOMOSCEDASTIC')
542     284 FORMAT(' HARTLEYS TEST SHOWS THAT THE DATA IS ',
543       /'HETEROSCEDASTIC ')
544     293 FORMAT(' UPPER 95% CONFIDENCE LIMITS OF Y PREDICTED',T63,1H*)
545     295 FORMAT(' LOWER 95% CONFIDENCE LIMITS OF Y PREDICTED',T63,1H*)
546     300 FORMAT(2X,'SXX =',F13.4,2X,'SYY =',
547       / F13.4,'SXY= ',F13.4)
548     306 FORMAT(' Y PREDICTED',T63,1H*)
549     307 FORMAT(' INTERCEPT =',F13.6,3X,'SLOPE = ',F13.6)
550     321 FORMAT(24X,'ANOVA  TABLE')
551     325 FORMAT(24X,12(1H*))
552     330 FORMAT(55(1H*))
553     350 FORMAT('  SOURCE  *D.F*   SSD   *',
554       /'   M.S   *    F    *     P   *')
555     360 FORMAT('LIN. REGR.*',I3,1H*,4(F9.3,1H*))
556     370 FORMAT('DEV.F.LIN.*',I3,1H*,4(F9.3,1H*))
557     380 FORMAT('  BETWEEN *',I3,1H*,4(F9.3,1H*))
558     390 FORMAT('  WITHIN  *',I3,1H*,4(F9.3,1H*))
559     400 FORMAT('   TOTAL  *',I3,1H*,4(F9.3,1H*))
560     410 FORMAT('CORRELATION COEFFICIENT =',F8.5)
561     420 FORMAT(' FIELLERS CONSTANT ,G=  ',F11.7)
562     430 FORMAT(' TYPE IN NUMBER OF ANIMALS IN FIRST TREATMENT GROUP')
563     431 FORMAT(' TYPE IN NUMBER OF ANIMALS IN SECOND TREATMENT GROUP')
564     432 FORMAT(' TYPE IN CODE NUMBER OF THIS ANIMAL'/' THEN NUMBER OF',
565       /' OBSERVATIONS ON IT ')
566     433 FORMAT(' TYPE IN THE CPM OBSERVATIONS FOR ',A4,' ANIMAL')
567     440 FORMAT(A4)
568     480 FORMAT('THE MEAN CPM FOR ',
569       /A4,' GROUP IS ',F12.6)
570     490 FORMAT(' THE ESTIMATED CONCENTRATION IN ',
571       /A4,' GROUP IS ',F12.6)
572     500 FORMAT(' RATIO G = ',F12.6)
573     510 FORMAT('ASSYMETRICAL CONFIDENCE LIMITS FOR ',
574       /A4)
575     520 FORMAT(' UPPER = ',F12.6,' LOWER = ',F12.6)
576     530 FORMAT(' FOR ',A4,'GROUP :"VARIANCE" = ',F12.6)
577     540 FORMAT( 'SYMMETRICAL CONFIDENCE LIMITS FOR ',
578       /A4,' GROUP ')
579     550 FORMAT(' ARE THERE ANY MORE UNKNOWNS TO BE EVALUATED ?'/
580       /' TYPE IN YES OR NO')
581     570 FORMAT(' TYPE IN "YES" OR "NO"')
582     610 FORMAT(' IS ANOTHER TYPE OF TRANSFORM REQUIRED?'/
583       /' TYPE IN YES OR NO')
584     620 FORMAT(' SOURCE',T14,'*D.F* SUM OF S.D* MEAN SQUARE* VAR.RAT*',
585       /' PROB',T63,1H*)
586     621 FORMAT(' BET. TREATS *',I3,1H*, F10.6,1H*,F11.7,1X,1H*,F8.3,1X,1
587       /,F8.5,1H*)
588     622 FORMAT(' BET ANIMALS*',T18,1H*,T29,1H*,T42,1H*,T52,1H*,T61,1H* )
589     623 FORMAT(' (WIT.TREATS)*',I3,1H*, F10.6,1H*,F11.7,1X,1H*,F8.3,1X,1
590       /,F8.5,1H*)
591     624 FORMAT(' BET. ANIMALS*',I3,1H*,F10.6,1H*,F11.7,1X,1H*,F8.3,1X,1H*
592       /F8.5,1H*)
593     625 FORMAT(' RESIDUAL     *',I3,1H*,F10.6,1H*,F11.7,1X,1H*,T52,1H*,T6
594       /1H*)
```

FIGURE A.12

PROGRAMME RIA (Lines 595–608)

```
595    626 FORMAT(' TOTAL          *',I3,1H*,F10.6,1H*,T42,1H*,T52,1H*,T61,1H*)
596    627 FORMAT(' RESIDUAL     *',T18,1H*,T29,1H*,T42,1H*,T52,1H*,T61,1H*)
597    628 FORMAT(' (REG.+UNK.)*',I3,1H*,T29,1H*,F11.7,1X,T52,1H*,T61,1H*)
598    635 FORMAT(' IS ANOTHER PAIR OF TREATMENT GROUPS ',
599       /'TO BE COMPARED'/' IN SAME ASSAY?'/' TYPE IN YES OR NO')
600    700 FORMAT(///' FIRST TREATMENT GROUP'/)
601    701 FORMAT(' THE HORMONE CONC. IN ',A4, 'ANIMAL IS ',F7.2)
602    702 FORMAT(' THE MEAN CONC. IN THIS TREATMENT GROUP IS ',F5.2,/
603       / ' THE UPPER 95% CONFIDENCE LIMIT IS ',F5.2/' THE LOWER 95%',
604       /' CONFIDENCE LIMIT IS,',F5.2)
605    703 FORMAT(///' SECOND TREATMENT GROUP'/)
606    710 FORMAT(//////////)
607    709 FORMAT(///)
608        END
```

FIGURE A.13

SUBROUTINE FTEST (Lines 1–54)

```
1         SUBROUTINE FTEST(F,DF1,DF2,PROB)
2         INTEGER*4  DF1,DF2
3   C ... CACM 346 ..........................................................
4         REAL*8  IMFLPB,PROB
5         REAL*8 F1,F2,F,FG,FT,CBRF,THETA,STH,CTH,STS,CTS,X,XX,VP,A,B,GAMMA
6   C
7         IF((DF1.LT.1).OR.(DF2.LT.1).OR.(F.LT.-1D-06)) GOTO 47
8         IF(F.LT.1D-06) GOTO 48
9   C
10        MAXN=500
11        F1=DF1
12        F2=DF2
13        FT=0D0
14        X=F2/(F2+F1*F)
15        VP=F1+F2-2D0
16  C
17        IF(((DF1/2)*2.EQ.DF1).AND.(DF1.LE.MAXN)) GOTO 10
18        IF(((DF2/2)*2.EQ.DF2).AND.(DF2.LE.MAXN)) GOTO 20
19        IF((DF1+DF2).LE.MAXN)  GOTO 30
20  C
21        F1=2D0/9D0/F1
22        F2=2D0/9D0/F2
23        CBRF=F**(.333333333333D-12)
24        FG=-((1D0-F2)*CBRF+F1-1D0)/SQRT(F2*CBRF*CBRF+F1)
25        FT=IMFLPB(FG,0D0,1D0)
26        GOTO 49
27  C
28     10 XX=1D0-X
29        DO 1 J=1,DF1,2
30        I=DF1-J+1
31        IF(I.EQ.DF1) GOTO 1
32        VP=VP-2D0
33        FT=XX*VP/I*(1D0+FT)
34      1 CONTINUE
35        FT=(X**(5D-01*F2))*(1D0+FT)
36        GOTO 49
37  C
38     20 DO 2 J=1,DF2,2
39        I=DF2-J+1
40        IF(I.EQ.DF2) GOTO 2
41        VP=VP-2D0
42        FT=X*VP/I*(1D0+FT)
43      2 CONTINUE
44        FT=1D0-(1D0-X)**(5D-01*F1)*(1D0+FT)
45        GOTO 49
46  C
47     30 THETA=DATAN(SQRT(F1*F/F2))
48        STH=DSIN(THETA)
49        CTH=DCOS(THETA)
50        STS=STH*STH
51        CTS=CTH*CTH
52        A=0D0
53        B=0D0
54  C
```

FIGURE A.14

SUBROUTINE FTEST (Lines 55–95)

```
      IF(DF2.EQ.1) GO TO 31
      DO 3 J=1,DF2,2
      I=DF2-J-1
      IF(I.LT.2) GOTO 33
      A=CTS*(I-1)/I*(1D0+A)
    3 CONTINUE
   33 A=STH*CTH*(1D0+A)
   31 A=THETA+A
C
      IF(DF1.EQ.1) GO TO 32
      DO 4 J=1,DF1,2
      I=DF1-J-1
      IF(I.LT.2) GOTO 41
      VP=VP-2D0
      B=STS*VP/I*(1D0+B)
    4 CONTINUE
   41 CONTINUE
      GAMMA=1D0
      IF(DF2.EQ.1) GOTO 43
      ID=(DF2-1)/2
      DO 5 IX=1,ID
      GAMMA=IX*GAMMA/(IX-5D-01)
    5 CONTINUE
   43 CONTINUE
      B=GAMMA*STH*(CTH**DF2)*(1D0+B)
   32 FT=1D0+.6366197723675D-12*(B-A)
C ... CONSTANT FACTOR IS 2/PI
      GOTO 49
C
   46 PROB=0D0
      GOTO 50
   47 WRITE(6,600)
  600 FORMAT('0',5X,'****ERROR IN F TEST****')
      GOTO 50
   48 PROB=1D0
      GOTO 50
   49 CONTINUE
      IF(FT.LT.-1D-06) GOTO 46
      PROB=FT
   50 RETURN
      END
```

FIGURE A.15

Subroutine DOUBLE

```
1 C  THIS SUBROUTINE CONVERTS F RATIO AND PROBABILITY INTO DOUBLE
2 C  PRECISION AND CALLS FTEST SUBROUTINE
3       SUBROUTINE DOUBLE(F,N1,N2,PROB)
4       DOUBLE PRECISION DF, DPROB
5       DF=F
6       CALL FTEST (DF,N1,N2, DPROB)
7       PROB=DPROB
8       RETURN
9       END
```

Subroutine UNCONC.
(Parameter M controls type of transform, if M equals 3, i.e. the logit cpm - log dose transform is selected, then the transformed x value has to be converted back to the untransformed form — Line 7.)

```
1 C  THIS SUBROUTINE CALCULATES CONCENTRATION IN UNKNOWN XU GIVEN MEA
2 C  CPM VALUE,YUM, AND PARAMETERS A,B,M
3       SUBROUTINE UNCONC(XU,YUM,A,B,M)
4       XU=(YUM-A)/B
5       IF(M.EQ.3)XU =EXP(XU)
6       RETURN
7       END
```

Subroutine CONLIM

```
1 C       THIS SUBROUTINE  CALCULATES THE 95%  CONFIDENCE LIMITS
2 C       FOR AN UNKNOWN GROUP OR ANIMAL USING A SIMPLIFICATION OF
3 C       FIELLERS THEOREM
4       SUBROUTINE CONLIM(NU,G,B,YUM,SXX,ANSW,T,YBAR,M,UCLW)
5       XU=(YUM-YBAR)/B
6       VII=(1.0/G)+(1.0/NU)
7       VARXU=(ANSW/(B*B))*(VII+(XU*XU/SXX))
8       SDXU=SQRT(VARXU)
9       UCLW=T*SDXU
10      IF(M.EQ.3)UCLW=EXP(UCLW)
11      RETURN
12      END
```

FIGURE A.16

The User logs in and gives the command to run programme R.I.A. He is asked to type in the number of concentration groups in the standard (calibration) curve. He selects to do this interactively by typing in CONSOLE then the figure. He is then asked to type in the concentration value at each point on the standard curve.

```
?// LOGIN BBSL10
PASSWORD?
 12.27 15/06/76 ETU = 992747 FFS = 024 UFS = 276
?// GROUP BBSA17
?// RUN RIA(P),,,,700
?// H

02 BBSL10:BBSA17.RIA    ,7000 BBSL10 09 11 12:27:43 15/06/76 021800

02  TYPE IN THE NUMBER OF CONCENTRATIONS IN STANDARD CURVE
02?*DS01 IS CONSOLE

02?*DS01 6

02  NOW TYPE IN THE VALUES OF THESE CONCENTRATIONS- X VALUES
02  IN ASCENDING ORDER PLEASE
02?*DS01 0

02?*DS01 5

02?*DS01 10

02?*DS01 20

02?*DS01 40

02?*DS01 80
```

FIGURE A.17

For each of these concentration values the user is asked to type in the number of cpm observations followed by the cpm numbers.

```
02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 3

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 7813

02?*DS01 7575

02?*DS01 7753

02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 3

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 7299

02?*DS01 7092

02?*DS01 7134

02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 3

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 6623

02?*DS01 6757

02?*DS01 6944

02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 3

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 6536

02?*DS01 6410

02?*DS01 6289

02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 5413

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 5495

02?*DS01 5291

02?*DS01 5434

02  TYPE IN THE NO OF CPM READINGS IN NEXT CONCENTRATION GROUP
02?*DS01 3

02  NOW TYPE IN THE CPM READINGS - Y VALUES - IN THIS GROUP
02?*DS01 3745

02?*DS01 3509
REPEAT
02?*DS01 3509

02?*DS01 3624
```

FIGURE A.18

A table of input values is printed out and then the user is asked to select the linearising transform he desires to try.

In this case the logit cpm–log dose transform is selected. The predicted cpm values from the regression table do not appear to correspond closely to the mean of the actual cpm values.

FIGURE A.18

```
02  X VALUES                                                          *
02       0.0     5.0     10.0     20.0     40.0     80.0 *******      *
02  ************************************************************************
02  Y VALUES                                                          *
02   7813.0  7299.0  6623.0  6536.0  5495.0  3745.0 *******          *
02   7575.0  7092.0  6757.0  6410.0  5291.0  3509.0 *******          *

02   7753.0  7134.0  6944.0  6289.0  5434.0  3624.0 *******          *
02  ************************************************************************
02  K VALUES                                                          *
02        3       3       3       3       3       3       0          *
02  ************************************************************************
02  Y MEAN                                                            *

02   7713.7  7175.0  6774.7  6411.7  5406.7  3626.0     0.0          *
02  ************************************************************************
02  VARIANCE OF Y WITHIN EACH GROUP                                   *
02  15352.0 12000.0 25992.0 15288.0 10960.0 13928.0     0.0          *
02  TRANSFORM REQUIRED ?
02  TYPE IN 1 FOR LOG Y V. X
02  TYPE IN 2 FOR Y/YO V. X

02  TYPE IN 3 FOR LOGIT(Y/YO) V. LOG X
02?*DS01 3

02  TRANSFORMED X VALUES                                             *
02      2.3     3.0     3.7     4.4 ******* ******* *******          *
02  ************************************************************************
02  TRANSFORMED Y VALUES                                             *
02   1.8038  1.7138  0.9069 -0.0580 ******* ******* *******          *
02   1.9549  1.5927  0.7811 -0.1809 ******* ******* *******          *
02   2.1997  1.4849  0.8686 -0.1209 ******* ******* *******          *

02  ************************************************************************
02  MEAN OF Y TRANSFORMED                                             *
02   1.9861  1.5971  0.8522 -0.1199  0.0000  0.0000  0.0000          *
02  ************************************************************************
02  VARIANCE OF Y TRANSFORMED                                         *
02   0.0399  0.0131  0.0042  0.0038  0.0000  0.0000  0.0000          *
02  ************************************************************************

02  Y PREDICTED                                                       *
02   7627.6  2715.3    25.6     0.0     0.0     0.0     0.0          *
02  ************************************************************************
02  UPPER 95% CONFIDENCE LIMITS OF Y PREDICTED                        *
02  12178.5  6945.1  5198.2  6589.5     0.0     0.0     0.0          *
02  LOWER 95% CONFIDENCE LIMITS OF Y PREDICTED                        *
02   3076.8 -1514.6 -5147.0 -6589.5     0.0     0.0     0.0          *

02  ************************************************************************
02
02
02
```

FIGURE A.19

The Hartley test indicates that the data of both the untransformed and transformed data is homoscedastic. The slope and intercept are indicated.

The analysis of variance table comparing variance due to linear regression and variance due to deviations from linearity, together with comparison of variance between and within each concentration group are printed.

The correlation coefficient and Fieller's constant is printed. The user is then asked if he wishes to try another type of linearising transform. An affirmative reply is given and transform type 2 (cpm/cpm at zero concentration) against dose is selected.

FIGURE A.19

```
02
02  HARTLEYS RATIO IS   2.37
02  NO OF GPS(K)=  6  DEGR. OF FREEDOM =   3.00
02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS    8.380

02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC
02  ************************************************************
02  HARTLEYS RATIO FOR TRANSFORMED DATA IS  10.58
02  NO OF GPS(K)=  4  DEGR. OF FREEDOM =   3.00
02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS   15.500
02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC
02  ************************************************************
02      XBAR =     3.342     YBAR =    1.079

02   SXX =       7.2069  SYY =       7.7403SXY=       -7.3435
02  ************************************************************
02  INTERCEPT =  7627.605500   SLOPE =     -1.018955
02  ************************************************************
02
02
02
02

02                          ANOVA  TABLE
02                          ************
02   SOURCE  *D.F*   SSD   *   M.S   *    F    *     P   *
02 ***************************************************************
02 LIN. REGR.*  1*    7.483*    7.483*  491.079*    0.000*
02 DEV.F.LIN.*  2*    0.258*    0.129*    8.454*    0.011*
02 ***************************************************************
02  BETWEEN *  3*    7.740*    2.580*  169.329*    0.000*

02  WITHIN  *  8*    0.122*    0.015*
02 ***************************************************************
02    TOTAL  * 11*    7.862*
02  ************************************************************
02
02
02
02

02 CORRELATION COEFFICIENT =-0.98322
02  ************************************************************
02  THE VALUES OF T FOR   8  DEGREES OF FREEDOM IS   2.306
02  FIELLERS CONSTANT ,G=    0.0108285
02  ************************************************************
02
02
02

02
02  IS ANOTHER TYPE OF TRANSFORM. REQUIRED?
02  TYPE IN YES OR NO
02?*DS01 YES

02  TRANSFORM REQUIRED ?
02  TYPE IN 1 FOR LOG Y V. X
02  TYPE IN 2 FOR Y/Y0 V. X
02  TYPE IN 3 FOR LOGIT(Y/Y0) V. LOG X
02?*DS01 2
```

FIGURE A.20

The transformed cpm values, the predicted cpm values from the regression equation, confidence limits of predicted values, Hartleys test and slope and intercept are printed out. The predicted cpm values correspond much closer to the actual values than the previously selected transform.

```
02  TRANSFORMED X VALUES                                                *
02       0.0      5.0     10.0     20.0     40.0     80.0 *******       *
02  ************************************************************************
02  TRANSFORMED Y VALUES                                                *
02   1.0129   0.9462   0.8586   0.8473   0.7124   0.4855 *******       *
02   0.9820   0.9194   0.8760   0.8310   0.6859   0.4549 *******       *
02   1.0051   0.9249   0.9002   0.8153   0.7045   0.4698 *******       *
02  ************************************************************************
02  MEAN OF Y TRANSFORMED                                               *

02   1.0000   0.9302   0.8783   0.8312   0.7009   0.4701   0.0000       *
02  ************************************************************************
02  VARIANCE OF Y TRANSFORMED                                           *
02   0.0003   0.0002   0.0004   0.0003   0.0002   0.0002   0.0000       *
02  ************************************************************************
02  Y PREDICTED                                                         *
02   7444.1   7200.3   6956.6   6469.0   5493.9   3543.7      0.0       *
02  ************************************************************************
02  UPPER 95% CONFIDENCE LIMITS OF Y PREDICTED                          *

02   7532.2   7280.9   7030.6   6534.6   5566.1   3685.6      0.0       *
02  LOWER 95% CONFIDENCE LIMITS OF Y PREDICTED                          *
02   7356.0   7119.3   6882.5   6403.4   5421.7   3401.8      0.0       *
02  ************************************************************************
02
02
02
02
02  HARTLEYS RATIO IS    2.37
02  NO OF GPS(K)=   6   DEGR. OF FREEDOM =    3.00

02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS     8.380
02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC
02  ************************************************************************
02  HARTLEYS RATIO FOR TRANSFORMED DATA IS    2.37
02  NO OF GPS(K)=   6   DEGR. OF FREEDOM =    3.00
02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS     8.380
02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC
02  ************************************************************************
02      XBAR =     25.833      YBAR =     0.802
02   SXX =    13562.5120  SYY =        0.5481SXY=       -85.7229

02  ************************************************************************
02  INTERCEPT =  7444.101600    SLOPE =      -0.006321
02  ************************************************************************
02
02
02
```

FIGURE A.21

The analysis of variance table, the correlation coefficient, Fieller's constant are printed out. In all cases they indicate that this transform fits the data much better than the logit transform, but the transform of log cpm against dose has not been tried and this transform is therefore selected in answer to the response on the bottom of the page.

```
02
02                          ANOVA  TABLE
02                          ***********
02   SOURCE  *D.F*   SSD   *   M.S   *    F    *     P   *

02 ***************************************************
02 LIN. REGR.*  1*    0.542*    0.542* 2067.833*    0.000*
02 DEV.F.LIN.*  4*    0.006*    0.002*    5.973*    0.007*
02 ***************************************************
02   BETWEEN *  5*    0.548*    0.110*  418.344*    0.000*
02   WITHIN  * 12*    0.003*    0.000*
02 ***************************************************
02    TOTAL  * 17*    0.551*
02  ***************************************************
02

02
02
02
02 CORRELATION COEFFICIENT =-0.99427
02  ***************************************************
02  THE VALUES OF T FOR  12  DEGREES OF FREEDOM IS   2.179
02  FIELLERS CONSTANT ,G=    0.0022961
02  ***************************************************
02
02
02
02
02  IS ANOTHER TYPE OF TRANSFORM REQUIRED?

02  TYPE IN YES OR NO
02?*DS01 YES

02  TRANSFORM REQUIRED ?
02  TYPE IN 1 FOR LOG Y V. X
02  TYPE IN 2 FOR Y/Y0 V. X
02  TYPE IN 3 FOR LOGIT(Y/Y0) V. LOG X
02?*DS01 1
```

FIGURE A.22

The predicted cpm values seem to correspond to the observed values even more closely than the previously selected transform.

```
02  TRANSFORMED X VALUES                                                *
02      0.0     5.0    10.0    20.0    40.0    80.0 *******            *
02  ************************************************************
02  TRANSFORMED Y VALUES                                                *
02   8.9635  8.8955  8.7983  8.7851  8.6116  8.2282 *******            *
02   8.9326  8.8667  8.8183  8.7656  8.5738  8.1631 *******            *
02   8.9558  8.8726  8.8456  8.7466  8.6004  8.1953 *******            *
02  ************************************************************
02  MEAN OF Y TRANSFORMED                                               *
02   8.9507  8.8783  8.8207  8.7657  8.5953  8.1955  0.0000            *

02  ************************************************************
02  VARIANCE OF Y TRANSFORMED                                           *
02   0.0004  0.0004  0.0008  0.0005  0.0006  0.0012  0.0000            *
02  ************************************************************
02  Y PREDICTED                                                         *
02   7608.3  7268.6  6944.1  6338.0  5279.7  3663.9     0.0            *
02  ************************************************************
02  UPPER 95% CONFIDENCE LIMITS OF Y PREDICTED                          *
02   7609.3  7269.6  6945.1  6339.0  5280.8  3664.9     0.0            *
02  LOWER 95% CONFIDENCE LIMITS OF Y PREDICTED                          *

02   7607.3  7267.6  6943.1  6336.9  5278.7  3662.8     0.0            *
02  ************************************************************
02
02
02
02
02  HARTLEYS RATIO IS    2.37
02  NO OF GPS(K)=  6  DEGR. OF FREEDOM =    3.00
02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS     8.380
02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC

02  ************************************************************
02  HARTLEYS RATIO FOR TRANSFORMED DATA IS    3.12
02  NO OF GPS(K)=  6  DEGR. OF FREEDOM =    3.00
02  THE 5% HARTLEY RATIO FOR THIS DEGREE OF FREEDOM IS     8.380
02  HARTLEYS TEST SHOWS THAT THE DATA IS HOMOSCEDASTIC
02  ************************************************************
02      XBAR =    25.833     YBAR =     8.701
02   SXX =    13562.5120  SYY =        1.1370SXY=      -123.8796
02  ************************************************************
02  INTERCEPT =  7608.273400    SLOPE =      -0.009134

02  ************************************************************
02
02
02
```

FIGURE A.23

The analysis of variance table correlation coefficient and Fieller's constant all indicate that this last selected transform (log cpm against dose) is marginally better than (cpm / cpm at zero concentration) against dose and very much better than logit (cpm/cpm at zero concentration) against log dose. A negative reply is therefore given to the question "Is another type of transform required?"

```
02
02                          ANOVA  TABLE
02                          ***********
02   SOURCE  *D.F*   SSD    *   M.S   *    F    *     P   *
02 ***********************************************************
02 LIN. REGR.*  1*    1.132*    1.132* 2418.092*    0.000*

02 DEV.F.LIN.*  4*    0.005*    0.001*    2.912*    0.068*
02 ***********************************************************
02   BETWEEN *  5*    1.137*    0.227*  485.948*    0.000*
02   WITHIN  * 12*    0.006*    0.000*
02 ***********************************************************
02    TOTAL  * 17*    1.143*
02  ***********************************************************
02
02
02


02
02 CORRELATION COEFFICIENT =-0.99760
02  ***********************************************************
02  THE VALUES OF T FOR  12  DEGREES OF FREEDOM IS   2.179
02  FIELLERS CONSTANT ,G=    0.0019635
02  ***********************************************************
02
02
02
02


02  IS ANOTHER TYPE OF TRANSFORM REQUIRED?
02  TYPE IN YES OR NO
02?*DS01 NO
```

FIGURE A.24

The number of animals in the first treatment group is typed in together with an identifying code number for each animal and the cpm observations.

```
02  TYPE IN NUMBER OF ANIMALS IN FIRST TREATMENT GROUP
02?*DS01 6

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 80

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 80   ANIMAL
02?*DS01 1485

02?*DS01 1416

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 81

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 81   ANIMAL
02?*DS01 3734

02?*DS01 2924

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 83

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 83   ANIMAL
02?*DS01 3355

02?*DS01 3984

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 82

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 82   ANIMAL
02?*DS01 4785
```

FIGURE A.25

The data for the first treatment group are continued and then data for the second treatment group is typed in.

```
02?*DS01 3356
02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 84

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 84   ANIMAL
02?*DS01 6098

02?*DS01 6536

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 85

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 85   ANIMAL
02?*DS01 2398

02?*DS01 2200

02  TYPE IN NUMBER OF ANIMALS IN SECOND TREATMENT GROUP
02?*DS01 6

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 90

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 90   ANIMAL
02?*DS01 6098

02?*DS01 6173

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 91

02?*DS01 3

02  TYPE IN THE CPM OBSERVATIONS FOR 91   ANIMAL
02?*DS01 5744

02?*DS01 6029

02?*DS01 6098
```

FIGURE A.26

The data for the second treatment group is continued.

```
02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 92

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 92   ANIMAL
02?*DS01 6536

02?*DS01 6538

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 93

02?*DS01 3

02  TYPE IN THE CPM OBSERVATIONS FOR 93   ANIMAL
02?*DS01 5348

02?*DS01 5050

02?*DS01 5587

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 94

02?*DS01 2

02  TYPE IN THE CPM OBSERVATIONS FOR 94   ANIMAL
02?*DS01 5263

02?*DS01 4926

02  TYPE IN CODE NUMBER OF THIS ANIMAL
02  THEN NUMBER OF OBSERVATIONS ON IT
02?*DS01 95

02?*DS01 3

02  TYPE IN THE CPM OBSERVATIONS FOR 95   ANIMAL
02?*DS01 5618

02?*DS01 6173

02?*DS01 6495
```

FIGURE A.27

A hierarchical analysis of variance table is printed out, which indicates a very high degree of statistical significance between the two treatment groups $P = 0.00003$.

A table of the concentration for each individual animal is printed together with the mean for each group. Upper and lower 95% confidence limits for each treatment group are given.

The user is then asked whether another pair of treatment groups are to be compared in the same assay. In this case he answers no and the programme run is terminated.

FIGURE A.27

```
02  SOURCE       *D.F* SUM OF S.D* MEAN SQUARE* VAR.RAT* PROB      *
02  ************************************************************
02  BET. TREATS *  1*  1.585693*  1.5856934 *  35.073 * 0.00003*
02  BET ANIMALS*    *          *            *        *         *
02  (WIT.TREATS)* 10*  1.275635*  0.1275635 *   2.821 * 0.03423*
02  ************************************************************
02  BET. ANIMALS* 11*  2.861328*  0.2601207 *   5.753 * 0.00118*
02  RESIDUAL    * 15*  1.215088*  0.0810058 *         *         *

02  ************************************************************
02  RESIDUAL   *    *          *            *        *         *
02  (REG.+UNK.)* 27*           *  0.0452112 *        *         *
02  ************************************************************
02  TOTAL      * 26*  4.076416*            *        *         *
02
02
02

02  FIRST TREATMENT GROUP
02
02  THE HORMONE CONC. IN 80  ANIMAL IS  101.55
02  THE HORMONE CONC. IN 81  ANIMAL IS  102.17
02  THE HORMONE CONC. IN 83  ANIMAL IS   80.24
02  THE HORMONE CONC. IN 82  ANIMAL IS   70.19
02  THE HORMONE CONC. IN 84  ANIMAL IS   20.43
02  THE HORMONE CONC. IN 85  ANIMAL IS  131.12
02  THE MEAN CONC. IN THIS TREATMENT GROUP IS 84.28

02  THE UPPER 95% CONFIDENCE LIMIT IS *****
02  THE LOWER 95% CONFIDENCE LIMIT IS,54.42
02
02
02
02  SECOND TREATMENT GROUP
02
02  THE HORMONE CONC. IN 90  ANIMAL IS   32.29

02  THE HORMONE CONC. IN 91  ANIMAL IS   28.95
02  THE HORMONE CONC. IN 92  ANIMAL IS   16.61
02  THE HORMONE CONC. IN 93  ANIMAL IS   39.09
02  THE HORMONE CONC. IN 94  ANIMAL IS   43.97
02  THE HORMONE CONC. IN 95  ANIMAL IS   24.47
02  THE MEAN CONC. IN THIS TREATMENT GROUP IS 30.89
02  THE UPPER 95% CONFIDENCE LIMIT IS 47.71
02  THE LOWER 95% CONFIDENCE LIMIT IS,14.06

02  IS ANOTHER PAIR OF TREATMENT GROUPS TO BE COMPARED
02  IN SAME ASSAY?
02  TYPE IN YES OR NO
02?*DS01 NO
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The Effects of Subtotal and Total Isletectomy in the River Lamprey, *Lampetra Fluviatilis*

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Measurements on the islet tissue of the lamprey indicate that in relation to body weight the total volume is similar to that of the hagfish, *Myxine glutinosa,* and to the ratio of islet volume to body weight derived for human islet tissue. Separate estimates for the volumes of cranial and caudal islet tissue show that in *Lampetra fluviatilis,* the latter is approximately twice the volume of the cranial component, whereas in the nonparasitic species *L. planeri,* the two regions are similar in size. Both subtotal and total extirpation of the islet tissue has been carried out in *L. fluviatilis,* the former operation involving removal of either the caudal or cranial islet regions. Blood glucose levels remain substantially unaffected in subtotal isletectomy, but in total extirpation of the islet tissue these rose to a mean level of 283 mg/100 ml compared with a control value of 52 mg/100 ml. Liver glycogen values were in general too variable to permit definite conclusions, but significant decreases were noted in animals subjected to caudal isletectomy. Comparisons of the glucose tolerance curves for intact and totally isletectomised animals indicate that the operated animals have completely lost the ability to regulate blood glucose levels and that the return toward baseline values after 24 hr can probably be accounted for by urine losses. Measurements of nuclear diameters of islet cells in glucose loaded animals show that after a single glucose injection of 100 mg the volume of the nucleus still remains much larger than those of the controls, 24 hr after the injection. Similar increases in nuclear volumes were observed in animals receiving repeated injections over periods of 8–11 days. In partial isletectomy there was compensatory hypertrophy of the nuclei of the remaining islet cells which tended to be more marked in the cranial region after removal of the caudal lobe. Vacuolisation and marked degranulation of islet cells has been observed only in the cranial region of caudally isletectomised animals. These observations are interpreted as an indication of the continuing physiological demand for insulin in the fasting migratory stage of the river lamprey and the retention of an important role for this hormone in carbohydrate metabolism.

Physiological, cytological, and histochemical studies on the islet tissue and its role in carbohydrate metabolism have disclosed many parallels between conditions in the two groups of extant cyclostomes, the lampreys and hagfishes. In both groups, the islet parenchyma is believed to consist wholly or mainly of β cells, together with some undifferentiated elements, and up to the present time no cells with the histochemical or cytological characteristics of A or D cells have been identified (Winbladh, 1966; Barrington, 1972; Falmer and Patent, 1972; Östberg, Van Noorden, and Pearse, 1975).

In the lamprey and hagfish, the islet tissue tends to form follicular structures with the β cells surrounding a central lumen, and in both *Myxine glutinosa* and *Lampetra fluviatilis* these follicles may be enlarged to form tumour-like cysts, occupying a major part of the islet organ (Falkmer *et al.,* 1973; Hardisty, 1975). In both groups of cyclostomes, extracts of the islet tissue exhibit insulin-like activity and react with antisera to mammalian insulins (Falkmer *et al.,* 1973; Van Noorden and Pearse, 1974). Selective necrosis of the β cells accompanied by hyperglycaemia has been reported after alloxan treatment

both in *Myxine glutinosa* and *L. fluviatilis* (Falkmen and Winbladh, 1964; Winbladh Biuw, 1970). In *L. fluviatilis,* antisera raised against lamprey insulin extracts have been shown to result in raised glycaemia levels (Plisetskaya and Leibush, 1972) and a depletion of liver glycogen. The failure to observe similar hyperglycaemic responses in *Myxine* (Falkmer and Matty, 1966) may be due to the use of antisera to mammalian insulin. Although it has been shown that hagfish and mammalian insulins have some immunological properties in common, permitting the visualisation of hagfish β cells by immunofluorescence using anti-human insulin serum (Falkmer *et al.,* 1974; Östberg *et al.,* 1975), it has, nevertheless, recently been shown that there are so many structural differences between the hagfish insulin molecule and that of pig insulin (Peterson *et al.,* 1974) that it is conceivable that endogenous insulin production in the hagfish may not readily be neutralised by antisera against ox insulin (Falkmer and Wilson, 1967).

In *Myxine,* the islet tissue forms a discrete organ situated at the point where the bile duct enters the intestine, and isletectomy has been carried out without being followed by an elevation in blood sugar levels (Schirner, 1963; Falkmer and Matty, 1966). Hyperglycaemia has been observed in the larval lamprey after cauterisation of the follicles of Langerhans, (Barrington, 1942), but perhaps because of technical difficulties, isletectomy does not appear to have been attempted at the adult stage. During and after metamorphosis, the lamprey islet tissue develops in separate caudal and cranial lobes; the latter forming a distinct nodule dorsal to the gut at the point where the foregut joins the intestine and immediately adjacent to the anterior wall of the pericardium on the left side of the body (Barrington, 1972). The caudal component is embedded in the dorsal surface of the liver over the region where the intestine is united with this organ.

MATERIALS AND METHODS

River lampreys were caught below the weir at Tewkesbury on the River Severn, or were obtained from the screens of the Power Stations at Berkeley or

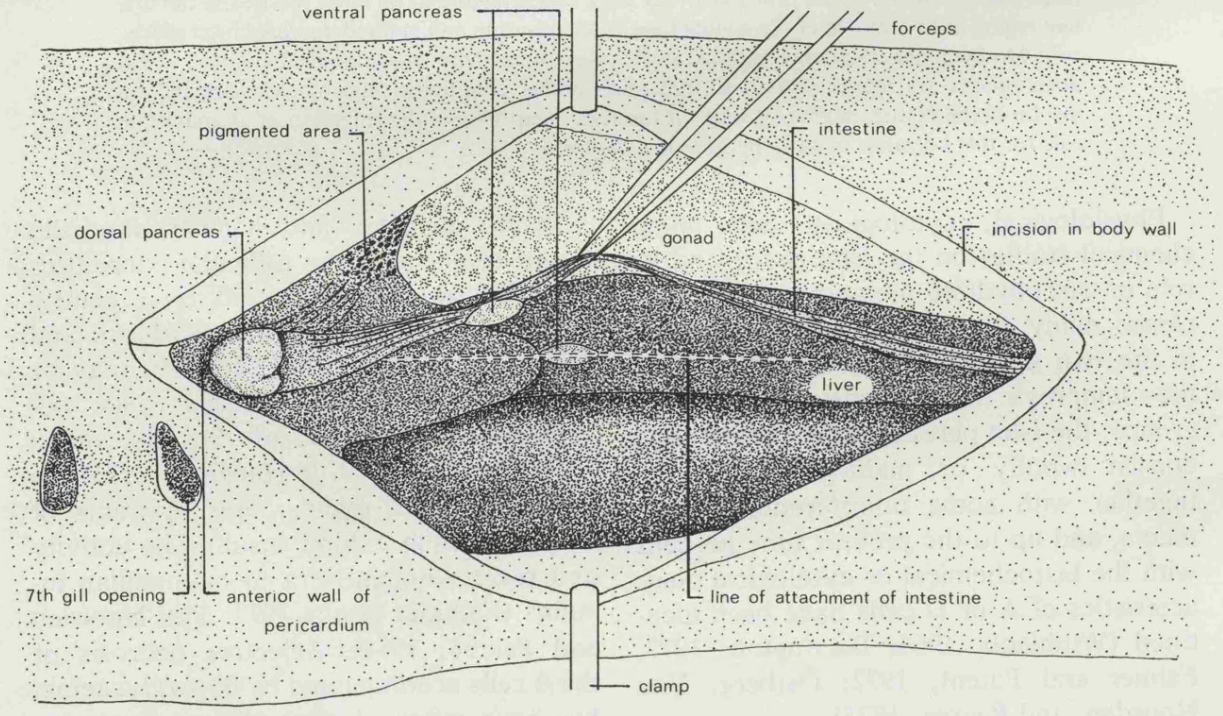

FIG. 1. Exposure of the islet tissue in the course of isletectomy as seen from the left side of the lamprey.

Oldbury-on-Severn in the course of their upstream spawning migration, which generally reaches its peak in the period between October and December (Hardisty and Potter, 1971). After transport to the laboratory, they were kept in large wire mesh live boxes, either in a lake or in a rectangular concrete basin, provided with a recirculation and filtration unit. Experimental animals were transferred to fibre glass tanks at least 10 days before experimentation and these were held in a cold room at 9–10°. All tap water used was dechlorinated by continuous aeration and the tanks were provided with Eheim pump filters.

Isletectomy

Prior to operation the lampreys were immersed for 5 min in a 0.1% solution of MS 222 and where necessary, anaesthesia was maintained in the course of operation, by pipetting the solution on to the gill openings. After the animal had been laid in the operating dish on its right side, covered with a damp cloth, kept cool by ice, an incision was made on the left side of the body starting directly in front of, and some 3–6 mm above, the last gill opening and extending horizontally backward for a distance of about 2 cm in the liver region (Fig. 1). The skin and muscle of the body wall were then cut through to expose the body cavity. On the anterior wall of the pericardium, the cranial pancreas may be identified as a slight swelling on the gut, at the point where the latter passes through the pericardial wall. This was then held in the forceps and trimmed away from the anterior end of the intestine and adjacent tissues using a fine pair of iridectomy scissors. To expose the caudal pancreas, the intestine was separated from the liver by cutting through its point of attachment to the dorsal surface. The caudal pancreas, partially embedded in the surface of the liver, could then be identified by its paler colour and was destroyed by electrocautery. The incision in the body was closed with a continuous silk suture and the animal returned to the tank. In the winter, mortality from the operation was very low and the animals were killed after periods varying from 2 to 3 wk, but there is no reason to doubt that they would survive for longer periods, especially if fungus infection is successfully controlled. There are indications that, as with hypophysectomy, mortality is higher in late winter or early spring and at these periods the majority of isletectomised animals were killed a week after operation.

Glucose Loading

In our experience, the hyperglycaemic response of lampreys to injection techniques is less marked when they have been previously anaesthetised with MS 222 (Sandoz) immediately before injection. Other investigations have shown that this reagent may safely be used in experiments on carbohydrate metabolism in fish, where concentrations are controlled and anaesthetisation is carried out within a brief and standardised time before sampling (Black and Connor, 1964; Crowley and Berinati, 1972).

In glucose loading experiments, the lampreys, after prior anaesthetisation for 5 min in a 0.1% solution of MS 222, were injected intraperitoneally with 100 mg of glucose as 0.2 ml of a 50% solution. For a 50-g lamprey this approximates to a dosage of 2 g/kg of body wt. Groups of animals were killed and blood samples were taken at various intervals between 2 and 24 hr after injection. Throughout the course of the experiments, control samples were taken from animals receiving an injection of 0.2 ml of 8.125% sodium chloride solution, which was equiosmotic with the 50% glucose solution. In long-term glucose loading, animals were injected twice daily with 100 mg of glucose for periods of 8–11 days.

Glucose Analysis

Blood samples were taken by cutting off the tip of the tail after prior anaesthetisation in 0.1% MS 222 for 5 min. Glucose analyses were carried out by the Glucose Oxidase-Perid method (Boehringer). Blood samples, collected in a centrifuge tube, were spun at 800*g*, and 50 μl of the supernatant was deproteinised by the addition of uranyl acetate solution to make up the volume to 500 μl. This was then centrifuged at 800*g* for 5 min and 100 μl of the supernatant assayed in accordance with the manufacturer's instructions. The method is capable of an accuracy of $\pm$ 3%. Since aliquots of the plasma sample were required for insulin radioimmunoassays, duplicate glucose analyses were not routinely carried out. At the end of the 24-hr glucose loading experiments, samples of the aquarium water were assayed for glucose and the total volume of water measured, to enable estimates to be made of urinary glucose excretion.

Liver Glycogen

Glycogen was determined by a modification of the method of Krebs *et al.* (1963). Approximately 500 mg of the liver tissue was washed for 3 min in ice cold lamprey Ringer and then homogenised in sufficient acetate buffer (pH 4.5) to give a homogenate of 0.11 mg/ml. 0.5 ml standard glucose solution was added to 4 ml of acetate buffer. The following procedure was employed in duplicate for both standards and homogenates:

0.5 ml of the solution was incubated with 0.5 ml amyloglucosidase (1 mg/ml in acetate buffer, pH 4.5) at 55° for 2 hr. 0.2 ml of 0.3 *M* perchloric acid was added, which was neutralised after 5 min by adding 1 ml of 0.6 *M* potassium dihydrogen phosphate (pH 7.0). The neutralised solution was then centrifuged at 700*g* for 4 min before removal of the supernatant for assay by the glucose oxidase method.

Histological Techniques

For routine examination, the whole of the body region containing the islet tissue was fixed for 24 hr in Bouin's solution and after paraffin embedding, sections were stained by the aldehyde–fuchsin technique (Gomori, 1950) in the modification of Cameron and Steele (1959), or by Pollak's (1944) trichrome technique. In early stages of the work, the effectiveness of the surgical techniques was checked by examining serial sections of partially or totally isletectomised animals.

Estimates of Islet Tissue Volumes

These were made by serially sectioning the pericardial and liver region, subsequently tracing the outline of the islet tissue at intervals of five or ten sections, depending on the size of the animal. Cut-outs of these areas on drawing paper were then measured on an automatic area meter (Hayashi-Denko, model AAM-5)[1] which is estimated to have an accuracy of ± 1%. The total volume was then estimated, taking into account the magnification factors and the appropriate intervals between successive tracings.

Measurement of Nuclear Diameters

Measurements of islet cell nuclei were made with a Filar ocular micrometer[2]. The nuclei were measured at random over the area enclosed by grid lines superimposed on the microscope field. Fifty nuclei were measured in each animal and the results expressed as the mean of the shorter and longer diameters. No attempt was made to differentiate between the cells of the "light" and "dark" cords.

RESULTS

Islet Tissue Volumes

In both estimates of islet tissue volumes for adults of *L. fluviatilis*, the caudal component was considerably larger than the cranial region, although the ratio of the two volumes varied from 1.9 in one animal to 6.1 in the other (Table 1). In the case of the individual with the high caudal/cranial ratio, the cranial tissue was necrotic and the larger volume of the caudal region may therefore be the result of compensatory hypertrophy. The single estimate for the macrophthalmia stage (a period in the life cycle after the completion of the external metamorphic changes, but before the onset of downstream migration and feeding) shows that, even at this period, the caudal islet tissue is larger than the cranial component. On the other hand, in the two adult brook lampreys *L. planeri*, (a species which does not feed after metamorphosis), there is little, if any difference in the volumes of the two regions. This may reflect the general atrophy which overtakes the gut of the nonparasitic species during the onset of sexual maturation following almost immediately upon metamorphosis.

Blood Glucose Concentrations in Normal and Isletectomised Animals

Extensive data is available on glycaemia levels in river lampreys under both normal and experimental regimes (Bentley and Follett, 1965b; Rothwell and Fielding, 1970; Plisetskaya and Kuz'mina, 1971; Larsen, 1973). Considerable variability has been recorded in normal animals, attributable to differences in temperature, the conditions under which the animals have been maintained, or the techniques employed in blood sampling and analysis. No significant seasonal differences were found by Leibson and Plisetskaya (1968), but Bentley and Follett (1965a) reported that blood sugar values in March were lower than in the preceding autumn. On the other hand, higher levels were observed by Larsen (1973) in sexually mature animals in the spring.

Our own records for the 1973/74 season, based on 127 animals of both sexes, gave a mean value of 51.9 ± 1.5 mg/100 ml glucose over the autumn and winter periods, with no detectable upward or downward trends throughout this time. The mean value for 76 males (49.8 ± 1.9 mg/100 ml) was below that of the 51 females (55.2 ± 2.5 mg/100 ml) but the difference is of only a low order of statistical significance.

[1] U.K. Agents: Scientific Dimensions Ltd., Ford House, 54 High Street, Fordingbridge, Hampshire SP6 1AX.

[2] American Optical Company, Buffalo, New York, USA.

TABLE 1
VOLUMES OF CRANIAL AND CAUDAL ISLET TISSUE IN *Lampetra fluviatilis* AND *L. planeri*

Species and stage	Body wt (g)	Volumes of islet tissue mm³			Ratio Caudal Cranial	Volume of islet tissue mm³ 100 g body wt
		Cranial	Caudal	Total		
Lampetra fluviatilis						
macrophthalmia	1.4	0.02	0.03	0.05	1.5	3.6
adults	50.0	0.32	1.96	2.28	6.1	4.6
	33.5	0.48	0.09	1.38	1.9	4.1
Lampetra planeri						
adults	2.7	0.07	0.07	0.14	1.0	5.2
	5.5	0.09	0.11	0.20	1.2	3.6

Blood glucose values are available for 22 lampreys of both sexes which had undergone total isletectomy and were sampled at periods from 1 to 3 wk after operation (Table 2). Although there was a wide range of blood sugar levels, there were no indications that values increased or decreased with the lapse of time between operation and blood sampling. In over half the experimental animals the blood glucose concentration exceeded 300 mg/100 ml and the mean was 283 ± 17.1 mg/100 ml with a range from 72 to 383 mg/100 ml.

In subtotal isletectomy, removal of cranial islet tissue was attempted in 14 animals and in this group (Table 2), values varied from 21 to 69 mg/100 ml with a mean of 42.9 ± 4.2 mg/100 ml. Although this is lower than the control value, the difference is not statistically significant. After caudal isletectomy, the range and mean values were rather higher than in the cranially isletectomised lampreys (32–85 mg/100 ml and 51.8 ± 5.4 mg/100 ml), but neither the differences between the means of the two operated groups nor the comparison with the control group is significant.

Liver Glycogen

Although sex differences in liver glycogen levels do not appear to have been reported previously, in our data for the 1973/74 season the mean value for 21 females was 141 ± 16.1 mg/100 g compared with a value of 261 ± 20.8 mg/100 g for 17 males. In the experimental series, only males were present in adequate and consistent numbers in the various groups and for this reason the data on liver glycogen in Table 2 refers only to male lampreys. Mean values for all groups, except the caudally isletectomised animals are very similar, but in these the mean is signifi-

TABLE 2
BLOOD GLUCOSE AND LIVER GLYCOGEN CONCENTRATIONS AFTER SUBTOTAL AND TOTAL ISLETECTOMY

	Mean blood glucose mg/100 ml + SE		Mean liver glycogen mg/100 g + SE (males only)	
	(*N*)		(*N*)	
Intact controls	127	52.0 ± 1.5	17	261.1 ± 20.8
Cranial Isletectomy	14	42.9 ± 4.2	9	274.9 ± 27.8
Caudal Isletectomy	12	51.8 ± 5.4	7	59.1 ± 12.8
Total Isletectomy	22	283.0 ± 17.1	12	260.2 ± 40.3

cantly low compared with either the controls or the cranial and total isletectomised groups.

Glucose Loading in Intact and Isletectomised Animals

In glucose loading tests on 77 intact lampreys (Fig. 2), maximum blood glucose values were usually recorded within 2 hr of an injection of 100 mg glucose and at this point, individual animals showed considerable variability in blood sugar levels. At 24 hr values were however, much more uniform and approached normal control levels. Analyses of the water in which the test animals had been kept, showed that maximum glucose losses occurred within the first 2 hr, but over the whole 24-hr period these losses accounted for only about 0.5% of the injected glucose.

Similar glucose loading experiments were carried out on 24 lampreys that had been completely isletectomised a week previously. Groups were sampled at intervals of 2, 8, and 24 hr after the initial injection. At 2 hr individual values varied from 762 to 1650 mg/100 ml and in four cases exceeded 1000 mg/100 ml. Over the remaining period the decline in glycaemia levels appears to have been approximately linear, approaching initial baseline levels and becoming more uniform at 24 hr. Analyses of the water at the end of the 24-hr period indicated losses of about 60% of the injected glucose, but because of the possibility of bacterial breakdown, this is likely to be a conservative figure.

Measurements of Nuclear Diameters

In lampreys subjected to cranial isletectomy, a relatively small but significant increase ($P < .02$) in nuclear diameters was

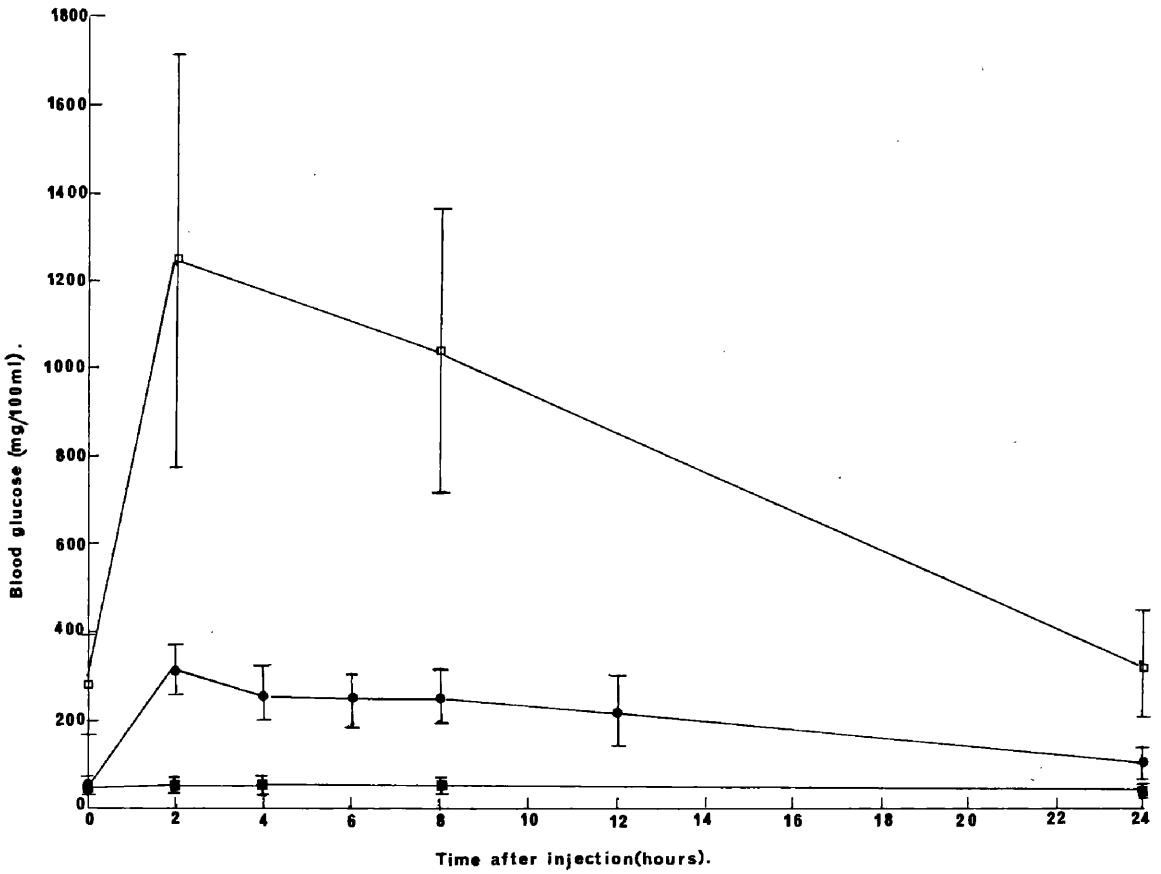

FIG. 2. Changes over 24 hr in blood sugar levels of totally isletectomised river lampreys (-□-□-) after glucose loading, compared with intact glucose loaded animals (-●-●-) and saline (8.125%) injected controls (-■-■-). The vertical bars represent standard errors of the means.

TABLE 3
CHANGES IN NUCLEAR DIAMETER OF ISLET CELLS AFTER GLUCOSE LOADING AND PARTIAL ISLETECTOMY[a]

Treatment	Number of nuclei measured	Mean nuclear diameter + SE (μm)
Isletectomy		
Caudal	100	7.02 ± 0.05
Control	150	6.31 ± 0.04
Cranial	150	6.53 ± 0.04
Control	100	6.36 ± 0.06
Glucose Loading		
Single injection after 24 hr	250	6.81 ± 0.03
Twice daily injections 8–11 days	150	6.71 ± 0.05
Controls	150	6.31 ± 0.04

[a] Differences in means: Caudal Isletectomy/Control $P < .001$. Cranial Isletectomy/Control $P < 0.02$. Cranial Isletectomy/Caudal Isletectomy $P < .001$. Glucose, single injection/Control $P < 0.001$. Glucose daily injections/Control $P < 0.001$.

observed in the remaining caudal tissue (Table 3). A much larger increase however, occurred in animals subjected to a removal of the caudal pancreas when compared with the controls ($P < .001$), and the difference between the means for the two types of operation is also statistically significant ($P < .001$). These indications of differences in the response of cranial and caudal islet tissue to functional demand imposed by partial isletectomy appear to be paralleled by other cytological changes. Thus, in sections stained by the aldehyde–fuchsin technique, degranulation of the aldehydefuchsinophil cords appeared to be more marked in the cranial tissue than in the caudal islets after partial isletectomy. In addition, vacuolisation has been observed in two cases in the cranial islets of caudally isletectomised animals, but has not been seen in the caudal tissue after removal of the cranial component.

Increases in nuclear volume have also been observed in the cranial islet cells 24 hr after a single injection of 100 mg glucose and these increases are almost as great as those observed in the islets after caudal isletectomy (Table 3). In a group of animals subjected during December to a twice daily injection of 100 mg glucose for periods of 8–11 days, the increase in nuclear diameters was slightly less than in the animals receiving only a single glucose injection. Out of a total of seven animals treated in this way, three showed extensive vacuolisation of the islet tissue.

DISCUSSION

Both in absolute volume and in relation to body weight, the islet organ of the two cyclostome groups is remarkably similar (Table 4). In *Myxine*, the total weight of islet tissue dissected from 347 animals with body weights of 40–50 g has been given as 0.76 g (Falkmer and Matty, 1966), representing an average of 2.2 mg per animal. Thus the ratio, islet tissue mg/100 g body wt, must be about 4.8 which is very similar to the figures obtained for *L. fluviatilis* and *L. planeri*. A similar ratio has also been reported for the principal islets of the teleost, *Cottus scorpius* (Falkmer, 1961). These values are much higher than the approximate ratio of 0.8 derived from data on the total volume of the islets in the pancreas of the 480-day rat (Hellman *et al.*,

TABLE 4
RELATIVE WEIGHT OR VOLUME OF ISLET TISSUE IN VARIOUS VERTEBRATE SPECIES

Species	Total islet volume mm³ or weight mg 100 g body wt	Source
Lampetra fluviatilis		
macrophthalmia	3.4	
adults	4.1–4.6	
Lampetra planeri		
adults	3.6–4.9	
Myxine glutinosa	4.8	Falkmer and Matty (1966)
Cottus scorpius	5.0–6.0	Falkmer (1961)
Rat (480 day)	0.8	Hellman *et al.* (1964)
Human (21 yr)	4.8	Ogilvie (1937)

1964), but are similar to the ratio of 4.8 for human islets at the age of 21 (Ogilvie, 1937). Such comparisons are all the more remarkable, bearing in mind differences in metabolic rates and the low carbohydrate content of the cyclostome diet. Since it is likely that the proportion of β cells in the cylostome islet tissue is at least equal to, if not greater than in mammals, it must be presumed, either that the insulin secretory potency of the lamprey and hagfish β cells or the sensitivity of the target organs is lower than in the rat, or alternatively, that this hormone plays a larger part in some aspects of metabolism other than that of carbohydrates. For example, the suggestion has been made that in the lower vertebrates insulin may play a primary role in protein metabolism (Tashima and Cahill, 1968).

Differences in cytological and physiological responses to subtotal isletectomy, affecting either the caudal or cranial region, are readily comprehensible in the context of the relative volumes of the two regions, and there are at present no indications of cytological differences, other than the frequent occurrence of cysts and other lesions in the cranial islets (Hardisty, 1972, 1975).

In his classical studies on the morphology and histology of the lamprey pancreas, Boenig (1929) regarded the islet tissue of the adult lamprey as degenerate and functionless, and more recently Sterba (1955, 1969) tended to discount a significant role for this tissue in carbohydrate metabolism, considering that it might be of more importance in relation to lipid metabolism. Although there is now some evidence for the involvement of insulin in the lipid metabolism of teleosts (Minick and Chavin, 1972; Lewis and Epple, 1972), the role of this hormone in lampreys has not as yet, been thoroughly investigated. On the other hand, the considerable number of physiological studies published during the present decade, point quite unequivocally to the importance of insulin in homeostatic mechanisms controlling blood sugar levels; a point that receives further emphasis from the observations previously described.

The fact that normal or near normal blood glucose levels are apparently maintained in the fasting river lamprey when either the cranial or caudal islet tissue alone is removed, suggests that at this period in the life cycle, the total insulin secreting capacity of the islet tissue may be more than equal to the physiological demands of the tissue. Radioimmunological assays of insulin levels in river lamprey blood (Plisetskaya and Leibush, 1972) have indicated a fall in insulin output throughout the migratory period, and at spawning time in the spring, the val-

ues were only about half those recorded in the autumn. It is therefore quite possible that seasonally dependent variations would be found in the response of lampreys to partial isletectomy. In our experiments, conducted during midwinter, removal of the caudal islet tissue gave a rather higher mean value for blood glucose concentrations compared with the cranially isletectomised group, although the difference in the means is not statistically significant. Such a result would, however, be consistent with the view that the larger caudal islets have a greater insulin secreting capacity than the cranial component; a conclusion supported by the degranulation observed in the latter region after caudal isletectomy and by the more marked increases in nuclear volume in response to this form of subtotal isletectomy. Vacuolisation of β cells after glucose loading has been reported in a number of teleost species (Khanna and Mehrotra, 1969; Khanna and Rekhari, 1972; Bhatt, 1974). It has also been observed in the islet tissue of larval lampreys following glucose injections (Barrington, 1942; Ermisch, 1966), but significantly was not reported by the latter author in the adult stage of either *L. planeri* or *L. fluviatilis,* even when they were subjected to daily glucose injections over periods of up to 11 days. In both ammocoete and adult of *L. planeri,* the terminal phase of serial glucose injection was necrosis, but this was not seen in the adult of *L. fluviatilis*. In our experiments on the river lamprey, necrosis did not occur after daily glucose injections over a period of 8–11 days and vacuolisation was observed in only three out of seven animals. These differences in response to glucose loading between adult and ammocoete, or between species, may reflect differences in the insulin secreting capacity of the β cells or even in the relative volumes of active tissue. On the other hand, Plisetskaya and Leibush (1972) were unable to detect any increase in insulin-like activity in adult lamprey blood after glucose loading, in spite of high levels of glycaemia, although an insulin response was observed in larval lampreys. Nevertheless, their view that the islet tissue of the river lamprey loses its capacity to respond to a glucose load, conflicts with the evidence of regulation in glucose loading experiments and with the degranulation and increases in nuclear volume observed after both subtotal isletectomy or glucose injection. Vacuolisation, associated with raised glycaemia levels, has been observed by us occasionally in animals subjected to surgical stress or heavily infected with fungus. It has also been consistently produced by prolonged hypoxia accompanied by hyperglycaemia (Hardisty *et al.* (in press). Vacuolisation of the α cells of the teleost, *Scorpaena scropha* subjected to hypoxia has been reported (Mosca, 1957), but this was probably a result of fixation artefacts due to the employment of formalin. In similar experiments on *Cottus scorpius,* no hydropic degeneration was observed by Falkmer (1961) although vacuolisation of the type described by Mosca was regularly seen after formalin fixation.

In mammals it is well established that glucose increases insulin synthesis and release, both *in vivo* and *in vitro* (Metz, 1958; Randle, 1966; Hahn *et al.,* 1970; Hermansen *et al.,* 1970; Hellman, 1970) and similar effects have been reported in teleosts (Patent and Foa, 1971; Palmer and Ryman, 1972). In the mouse, this cellular response is associated with changes in the volume of β cells, and increases in nuclear size have been detected within 30 min of dextrose injection (Mohnike and Moritz, 1964). Swelling, both of the whole cell and the nucleus, were noted by Ermisch (1966) in lampreys injected with glucose, during the degranulation phase that precedes vacuolisation and final necrosis. The increases in islet cell nuclear volumes after subtotal isletectomy, and especially after removal of the larger caudal lobe, may

therefore be interpreted as an indication of compensatory hypertrophy. Together with the evidence of degranulation observed in the cranial islets after removal of the caudal lobe, these responses must reflect a physiological demand for insulin by the tissues of the lamprey and emphasise the continued importance of this hormone in the fasting animal.

The large individual variations in blood sugar levels, especially in the early periods after glucose loading, might be attributed to a number of factors, among them the rate of uptake of the injected glucose into the extracellular compartment, the intensity of insulin secretion, metabolism of glucose, the storage of glycogen in the liver, or the rate of renal excretion of glucose. Like the teleosts, the lamprey shows a much lower tolerance to glucose than the mammal and this has been attributed to either low rates of insulin secretion or to a low carbohydrate diet (Ince and Thorpe, 1974). In the intact lamprey, urine losses are not an important factor and both our estimates and those of Bentley and Follett (1965b) put these at about 0.5 or 2.0% of the injected glucose; much lower than the estimates of 10–15% in the hagfish (Falkmer and Matty, 1965).

Assuming that the whole of the injected glucose is distributed initially in the extracellular phase and neglecting any losses in the urine, it may be estimated that in a 50-g lamprey with an extracellular fluid volume of 22% (Morris, 1972) the glucose concentration might be raised by about 900 mg/100 ml above baseline values; a level far in excess of those observed in glucose loaded intact animals. Allowing for a baseline level of about 280 mg/100 ml, this corresponds quite closely to the kind of values obtained in isletectomised animals within 2 hr of injection. In view of the extent of glucose losses to the ambient water and the absence of any indication of increased liver glycogen concentrations, it is reasonable to suppose that the totally isletectomised lampreys have substantially lost their normal capacity to regulate blood sugar concentrations. In the larval lamprey, the kidney threshold for glucose has been given as about 80 mg/100 ml (Morris and Islam, 1969) but the lower rates of glucose losses in the intact glucose loaded river lamprey indicate much higher threshold values at this stage of the life cycle. Indeed, the fact that totally isletectomised lampreys were able to maintain blood sugar levels of around 300 mg/100 ml for periods of up to 3 wk after operation suggests that threshold values for glucose probably approximate to this figure. However, in both intact or isletectomised lampreys, differences in renal excretion rates may well be an important factor in the variablility of blood sugar values.

In both our own experience and that of other workers, liver glycogen values in river lampreys tend to show extreme variability. While, to some extent this may be due to the techniques involved in sampling and more especially, handling, anaesthetisation, or temperature, a major factor is likely to be the decline in glycogen reserves that normally accompanies the prolonged starvation during the migratory period. Thus, by the time of spawning, liver glycogen reserves have been found to be almost completely depleted (Bentley and Follett, 1965a) and for this reason considerable individual variability would be expected, depending on the period that has elapsed since feeding ceased and on the degree of sexual maturity.

In experiments involving the determination of liver glycogen levels in river lampreys (Bentley and Follett, 1965b; Leibson and Plisetskaya, 1968; Plisetskaya and Leibush, 1972) no attempts appear to have been made to distinguish the sex of the experimental animals. In view of our own findings on sex differences in liver glycogen values, pooling of results from both sexes could give rise to spurious results should there be wide variations in the pro-

portions of the two sexes in various experimental or control groups. Moreover, the situation may be further complicated by sex differences in liver weight relative to body weight and by differences in the liver lipid levels of males and females (Larsen, 1973; Bentley and Follett, 1965a; Moore, 1975).

Previous reports on the effects of exogenous insulin on glycogen levels in the cyclostome liver have been far from convincing, and to some extent this may be attributed to the influence of some of the factors already discussed. Over a short period of 2 hr a rise in liver glycogen values has been recorded in *L. fluviatilis* following injections of mammalin insulin (Bentley and Follett, 1965b) but the opposit result was observed in *Myxine* (Falkmer and Matty, 1966). In experiments on the river lamprey, conducted over 4 successive years, Leibson and Plisetskaya (1968) obtained evidence of a rise in liver glycogen levels in one season only, but a more definite increase in glycogen values was observed in larval lampreys 4 days after insulin injections. The same authors (Plisetskaya and Leibson, 1973), however, detected increased glycogen synthetase activity in the liver of the adult *L. fluviatilis* after the administration of insulin and a decrease in liver glucose was reported after treatment of river lampreys with an anti-lamprey-insulin serum (Plisetskaya and Leibush, 1972).

With the exception of the caudally isletectomised lampreys, the present series of experiments have given no evidence of changes in glycogen levels following isletectomy and there are no indications that the raised blood sugar levels of the totally isletectomised animals have been maintained at the expense of carbohydrate reserves in the liver. The apparently anomalous values observed in the ventrally isletectomised group clearly require confirmation on larger numbers of animals of both sexes.

The contrast between the hagfish and the lamprey in their response to isletectomy is striking and unexpected, since in many other respects the blood glucose concentrations of both cyclostome groups react in rather similar ways to both stress and exogenous hormonal factors (Bentley and Follett, 1965b; Morris and Islam, 1969; Leibson and Plisetskaya, 1968; Falkmer *et al.*, 1973; Falkmer *et al.*, 1974). Furthermore, in *Myxine,* destruction of the β cells by alloxan also results in hyperglycaemia (Falkmer and Winbladh, 1964) although it is possible that this could be a nonspecific response to the toxicity of the reagent. Only 5% of the alloxan treated hagfish were reported as showing degenerative changes in the β cells, and the hyperglycaemic effects of alloxan are known to be associated with toxic effects on the liver and kidney (Brinn, 1973). Lesions in these organs may occur with dosages that cause little alteration in the β cells (Doerr, 1950).

In *Myxine,* the possibility has been considered that the absence of a hyperglycaemic response to isletectomy might be due to the presence of extra insular β cells, either in the bile duct epithelium or the gut mucosa (Falkmer and Matty, 1966; Falkmer *et al.*, 1974). In the larval lamprey, immunofluorescent studies using anti-insulin sera have shown buds of islet cells associated with the gut or bile duct mucosa, and cells containing insulin were seen occasionally at the base of the mucosa (Van Noorden *et al.*, 1972). However, no insulin containing cells were observed in these situations in the adult lamprey (Van Noorden and Pearse, 1974), although it is known that scattered cords of islet tissue do occur between the sites of the cranial and caudal pancreas, constituting an intermediate islet component. Since the presence of this tissue is apparently unable to prevent the development of a marked degree of hyperglycaemia in the isletectomised lamprey, there must be some doubt whether the ex-

istence of comparatively small volumes of extra insular β cells could explain the absence of hyperglycaemia in the isletectomised hagfish (Östberg *et al.*, 1975).

A recent review of the histophysiology of the vertebrate islet tissue (Epple and Lewis, 1973) has emphasised the dangers of extrapolating from conditions in mammals to those of the lower vertebrates and especially in assuming that pancreatectomy must invariably result in diabetes mellitus; a situation that apparently applies generally only to anurans, reptiles, and mammals. In the chondrichthyes, teleosts, and birds, the outcome of pancreatectomy varies considerably and there appears to be no close correlation between the percentage of β cells in the islet tissue and the development of permanent hyperglycaemia. While it is true that functional cell types other than β cells have not as yet been identified in cyclostome islet tissue, the problem of the light and dark cells in the islet parenchyma of the lamprey is still not satisfactorily resolved, and in *Myxine* a second type of granular cell has recently been observed in electron micrographs (Thomas *et al.*, 1973). Until the functional significance of these cell types has been clarified, it would be unwise to speculate on the reasons for the marked differences between the two groups of cyclostomes in their response to isletectomy.

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The Effects of Hypoxia on Blood Sugar Levels and on the Endocrine Pancreas, Interrenal, and Chromaffin Tissues of the Lamprey, *Lampetra fluviatilis* (L.)

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Hyperglycaemia has been demonstrated in river lampreys *(Lampetra fluviatilis)* subjected to a reduced oxygen tension of 20% air saturation for periods of up to 14 days. Maximum blood sugar values were recorded after 7 days hypoxia (mean 92.1 ± 30.9 mg/100 ml) compared with a mean value of 44.5 ± 2.3 mg/100 ml for controls. Similar tests over 7 days on hypophysectomised animals failed to show significant changes in glycaemia. Marked hyperglycaemia was also observed after injections of adrenalin. No significant changes in liver glycogen concentrations occurred after periods of hypoxia.

After 7 days hypoxia, slight vacuolisation occurred in the islet tissue of only a few of the experimental animals, but at 14 days all showed extensive hydropic degeneration affecting the light cells. Equally severe lesions were also seen in all the hypophysectomised animals after only 7 days hypoxia, but did not appear in any of the control series that had been hypophysectomised 1–2 months previously and maintained under normal oxygen tensions. Vacuolisation also occurred in only a small proportion of the animals subjected to daily glucose loading over a 8–11 day period, but was not seen after shorter periods of adrenalin administration.

Measurements of nuclear diameters of islet cells showed marked increases in nuclear volumes after 24 hr of glucose loading or after adrenalin injections, but not after hypoxia treatment. Aldehyde-fuchsinophil granulation of the dark cords was reduced after glucose loading, but was still retained by the cells of the dark cords after hypoxia treatment. Marked differences have been recorded in the mean nuclear volumes of light and dark cells and, although both respond to glucose loading, the increases in nuclear size tended to be greater in the light cells. Stimulation of mitotic division has been seen in hypoxia, glucose loading, and especially after exogenous adrenalin treatment, but is apparently confined to light cell lobules. The histological observations and nuclear measurements are consistent with the view that both light and dark cells may be regarded as developmental stages in a single β-cell-type.

Increased nuclear volumes were recorded in chromaffin tissue in the earlier stages of hypoxia and after glucose loading, but were not observed in hypophysectomised animals. No nuclear enlargement was seen in the interrenal tissue under hypoxic conditions, but a slight increase in mean volumes occurred after glucose loading. In adrenalin injected animals, nuclear hypertrophy was very pronounced, probably accompanied by hyperplasia.

Although hypophysectomy inhibits the hyperglycaemic and behavioural responses to hypoxia, it does not prevent the hydropic degeneration of the islet tissue. While there is some evidence for a pituitary influence on chromaffin tissue activity, it remains in doubt whether this is mediated through glucocorticoid activity of the interrenal tissue.

In the course of investigations into the relation between hyperglycaemia and histological changes in the islet cell of the river lamprey, *Lampetra fluviatilis*, some studies have been made on the effects of hypoxia. The blood sugar levels of cyclostomes respond to a variety of environmental changes, loosely referred to as stress situations (Morris and Islam, 1969; Leibson and Plisetskaya, 1969; Plisetskaya and Kuz'mina, 1971). These include the transfer of animals to the laboratory, handling, continuous light and injection or surgical procedures. Hyperglycaemia has also been ob-

served following the administration of adrenalin, 8-arginine vasotocin, cortisol (Bentley and Follett, 1965), and mammalian ACTH (Larsen and Rothwell, 1972).

There are now many reports of hyperglycaemia in teleosts under so-called conditions of "asphyxia," usually induced by transferring the animal to a small volume of water or even by exposing them to air for relatively short periods (McCormick and Macleod, 1925; Simpson, 1926; Menton, 1927; Beutler, 1939; Mosca, 1957; Al-Gauhari, 1958; Black and Tredwell, 1967; Chavin and Young, 1970; Plisetskaya and Kuz'mina, 1971). As has been pointed out (Falkmer, 1961), such procedures do not indicate specifically the effects of oxygen depletion, since they inevitably involve general stress reactions that occur when fish are handled or disturbed. The extent of this so-called "asphyxiation hyperglycaemia" appears to be related to the habits and activity of different fish species (Plisetskaya *et al.*, 1971).

Both the Pacific lamprey, *L. tridentata*, and the European river lamprey, *L. fluviatilis*, are able to tolerate very low oxygen tensions, and in the latter species standard oxygen consumption increases down to at least 20% air saturation at 9.5° (Johansen *et al.*, 1973; Claridge and Potter, 1975). Below this level, the animal detaches itself frequently and exhibits violent swimming movements, which are similar to the avoidance responses of teleosts and elasmobranchs (Whitmore, Warren, and Doudoroff, 1960; Randall, 1970) at reduced oxygen tensions just above the lethal limits. In both the adult and larval lamprey, there is a marked increase in ventilatory frequency under hypoxic conditions, and in *L. fluviatilis* this is accompanied by a very small increase in heart rate (Claridge *et al.*, 1975). Because of its relative tolerance of low oxygen tensions, the lamprey offers the opportunity to explore the effects of defined degrees of hypoxia over a protracted period.

In lampreys as in other vertebrates, physiological overloading of the islet tissue may result in vacuolisation or necrosis (Ermisch, 1966). Vacuolisation of islet cells has been described in larval lampreys following glucose injections (Barrington, 1942, Ermisch, 1966), but occurs only occasionally in the adult river lamprey, even after repeated glucose loading (Hardisty, Zelnik, and Moore, 1975). These lesions have also been observed in severe fungal-infected individuals or occasionally after surgical procedures, and have generally been associated with elevated blood-sugar levels. Vacuolisation has also occurred sporadically among "normal" lampreys maintained in the laboratory, or held for periods in live boxes in the River Severn, Vacuolisation of α cells has been reported in the islet tissue of the teleost, *Scorpaena scropha* under conditions of "asphyxial hyperglycaemia," but this has subsequently been interpreted as an artefact of formalin fixation (Falkmer, 1961).

When applied over periods of up to 14 days, hypoxia provides a consistent means of producing hyperglycaemia in the river lamprey, offering an opportunity to study the interrelationship between glycaemia levels and the histological changes in islet cells and other endocrine tissues.

In the islet parenchyma of the lamprey the dominant cell types are the so-called "dark" and "light" cells; the former containing granules which react with aldehyde-fuchsin and stain metachromatically with pseudoisocyanin. Particularly in the larval stage, the islet cells may form follicular structures, which occur less regularly in the adult. Nevertheless, the dark cells occasionally tend to form cords whose cavities may contain blood cells. The light cells, which are also granular, do not react with aldehyde-fuchsin, nor metachromatically with pseudoisocyanin, and typically tend to form solid lobules. While the functional significance of dark and light cells is still uncertain, the balance of opinion favours the view that both should be regarded as stages in the

cycle of β-cell development (Barrington, 1972); a view consistent with the ultrastructural evidence (Titlbach and Kern, 1969). In the course of the present experiments, observations on the different responses of the two cell types to hypoxia and other forms of experimental treatment may throw some light on their functions and interrelationship.

MATERIALS AND METHODS

River lampreys were obtained from Tewkesbury on the River Severn or from the screens of Power Stations at Berkeley and Oldbury on the Severn estuary, in the course of their upstream spawning migration between the months of October–December. A few smaller lampreys have also been obtained in March and these differ to some extent in their physical characteristics from the normal autumn and winter migrants.

Stock animals have been maintained, either in the University lake or in a large outdoor concrete basin provided with a recirculation and filtration unit. Experimental animals were transferred, before use, to fibreglass tanks provided with Eheim filters and stored in a large cold room at 9–10°. All tap water was dechlorinated before use by continuous aeration.

Technique for maintaining hypoxia. During the period of the experiment, lampreys were maintained at an oxygen concentration equivalent to a 20% saturation with air by employing appropriate volumes of compressed air and nitrogen. The compressed air and nitrogen were passed through a series of valves before being bubbled through a mixing jar containing water (Fig. 1). The valves were then adjusted until the required ratio of flowmeter readings was achieved. The mixture was bubbled through dechlorinated water in a large chromatography jar containing the experimental animals. To reduce the pressure required to maintain an adequate flow rate, the air/nitrogen mixture was bubbled through several diffuser stones in series, and the pressure was monitored with a mercury manometer. The concentration of oxygen in the water was checked during the experiments by the Winkler method.

Injection techniques and blood sampling. The general procedure for injection and blood sampling has been described in a previous publication (Hardisty *et al.*, 1975) and involves prior anaesthetisation with MS 222.

In glucose loading experiments, lampreys were injected intraperitoneally with 100 mg of glucose administered as 0.2 ml of a 50% solution, and were killed 24 hr afterwards. At the same time, controls were injected with 0.2 ml of 8.125% sodium chloride solution, equiosmotic with the glucose solution.

In experiments involving exogenous adrenalin administration, the animals were given twice daily intraperitoneal injections of 0.1 mg adrenalin hydrogen tartrate as 0.1 ml of a 1 mg/ml solution. Loss of pigmentation generally occurred within a few hours of injection and became more pronounced with the passage of time. Beyond 3 days, the animals were in poor condition, and the majority were killed after 4 days. Control animals were given the same number of injections of lamprey Ringer of the same volume as the adrenalin injection.

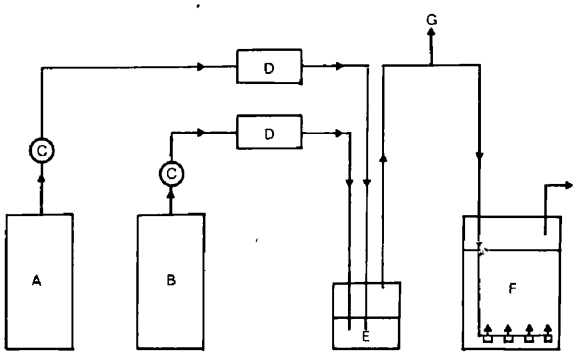

FIG. 1. Apparatus used in the maintenance of hypoxia: A, Nitrogen cylinder; B, Compressed air; C, Valves; D, Flow meters; E, Mixing jar; F, Chromatography jar containing experimental animals; G, Mercury manometer.

Blood samples in all cases were taken after anaesthetisation with MS 222, by cutting off the caudal region behind the cloaca.

Blood glucose and liver glycogen analyses. Glucose analyses were carried out by the glucose oxidase (Boehringer) method, as described in Hardisty *et al.* (1975), and liver glycogen, by the method of Krebs *et al.* (1963), involving incubation of tissue homogenates with amyloglucosidase and subsequent assay by the glucose oxidase procedure.

Hypophysectomy. After prior anaesthetisation, hypophysectomy was carried out, following substantially the procedure described by Larsen (1965), and the extirpation of the adenohypophysis was completed by electrocautery. During the Autumn and Winter, few postoperative mortalities occurred, but owing to a greater incidence of fungal infection, the survival rate was reduced after operations conducted during the Spring.

Histological techniques. After blood and tissue samples had been taken, the whole of the pericardial and liver region of the experimental animals was fixed for 24 hr in Bouin's fluid, and after paraffin embedding, serial sections at 7 μm were cut through the region containing the cranial pancreas and the pronephric funnels. Routinely, sections were stained by Pollack's (1944) trichrome technique and in certain cases by the aldehyde-fuchsin method of Gomori (1950) as modified by Cameron and Steele (1959).

Measurements of nuclear diameters of endocrine tissues. These were made with a Filar ocular micrometer (American Optical Co., Buffalo, New York) and in the

case of islet tissue, the measurements were made at random over the area enclosed by grid lines superimposed on the microscope field. For this tissue, 50 nuclei were measured in each animal, including both light and dark cell types. To assess possible differences in the size of the nuclei in the two cell types and the possibility of a differential response to glucose stimulation, separate measurements were also made on the nuclei of light and dark cells in sections stained by the aldehyde-fuchsin technique, both in control animals and after glucose injection. In the case of interrenal and chromaffin tissues, where variability in nuclear size is less pronounced, measurements were restricted to 20 nuclei in each animal. In all cases, values were expressed as the mean of the longer and shorter nuclear diameters.

Where cell measurements were to be made on experimental animals, it was the normal practice to fix and embed them together with a control group, thus avoiding the possibility of differences due to cell shrinkage during processing.

RESULTS

Blood Sugar Changes

At or below 20% air saturation at 10°, the lampreys became extremely excitable, displaying restless and vigorous swimming movements accompanied by hyperventilation (Claridge and Potter, 1975). Even at saturation values down to 10% there was little or no mortality during a 7-day period, and at 20% saturation the majority of the experimental animals survived a test period of 14 days. Over the first 2–3 days blood sugar analyses showed a small but significant rise compared with the controls, but the increase in glycaemia levels became most marked between 4–7 days, when mean values increased from 75.5 ± 8.0 mg/100 ml to 92.1 ± 6.9 mg/100 ml (Fig. 2). In general, blood sugar values for males tended to be lower than for the females at all time intervals, but because of the small numbers and the variability in individual values, these differences were not statistically significant. A similar (but not significant) sex difference was also present in the control group, where the mean value for 10 females was 47.7 ± 11.9 mg/100 ml compared with 41.5 ± 7.5 mg/100 ml for the same number of males. Since the 14-day hypoxia group consisted entirely of males, the apparent decrease in the mean blood-sugar level (Fig. 2) may therefore be more apparent than real.

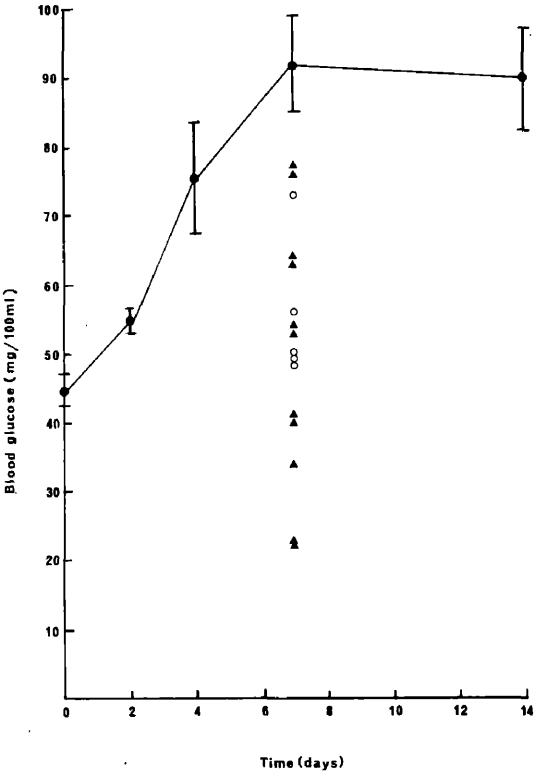

FIG. 2. Blood sugar levels in hypophysectomised and intact lampreys after periods of hypoxia. -●—●, Intact animals. Vertical lines indicate standard errors. ○, Hypophysectomised animals subjected to 7 days hypoxia. ▲, Hypophysectomised animals maintained under normal oxygen tensions. Figures in brackets indicate numbers of animals.

Similar tests were also carried out on five lampreys hypophysectomised 2–3 weeks previously. In their reaction to hypoxia these animals behaved in a manner characteristic of hypophysectomised lampreys, lying for the most part motionless on the bottom of the aquarium and without the rapid increase in ventilatory frequency seen in the intact animals under hypoxia. In this group, the mean blood-sugar level after 7 days was 55.0 ± 10.4 mg/100 ml, with individual values from 48–73 mg/100 ml (Fig. 2). Compared with intact lampreys subjected to hypoxia for the same period, the difference in the means is highly significant ($P > 001$), but the value for the hypophysectomised group is not significantly different from that

of the intact control animals. Hypophysectomy itself has not been shown to result in alterations in glycaemia levels (Larsen, 1973), and the mean value for 12 hypophysectomised lampreys that had been maintained in this laboratory for periods of 1–2 months after operation was 49.8 ± 5.4 mg/100 ml, which is very similar to that of the operated animals after 7 days hypoxia.

Hyperglycaemia was also observed in animals that had received twice daily injections of adrenalin and that were killed 3 hr after the last treatment. The mean value for four lampreys was 198.5 ± 11.8 mg/100 ml compared with 62.8 ± 11.9 mg/100 ml for five controls injected with lamprey Ringer.

Liver Glycogen

Not only are liver glycogen values in the fasting migratory lamprey notoriously variable, but in addition there are marked sex differences; male values being approximately twice those of the female (Hardisty *et al.*, 1975). Because of inadequate numbers of each sex in the separate experimental groups, it has therefore been necessary to pool values for all periods of hypoxia, from 2–14 days. For 18 females the mean value was 123 ± 13.8 mg/100 g, compared to 140.9 ± 12.7 mg/100 g for 18 control animals. In males subjected to hypoxia the corresponding values for 12 animals were 275.1 ± 40.3 mg/100 ml, as against 262.6 ± 20.7 mg/100 ml for 19 controls. For hypophysectomised animals subjected to hypoxia, numbers were small and individual variation too large to make meaningful comparisons, but the range of values fell well within those of intact animals.

Histological Changes in Islet Tissue

The typical appearance of islet tissue stained by the aldehyde-fuchsin technique may be seen in Fig. 11, where there is a marked contrast between the aldehyde-fuchsinophil dark cells and the granular and chromophobic light cells. It may also be noted that although individual lobules may often consist entirely of one or the other of these two cell types, mixed cords do occur in which, however, the dark or light cells are generally aggregated in distinct groups. Secretion via the basal lamina is presumably indicated by the marked reaction to aldehyde-fuchsin of the lobular envelope.

Hypoxia

After 2–3 days hypoxia some increase was noted in the vascularity of the tissue, and blood cells appeared in the interlobular areas and within the lumina of the cell cords and follicles. Mitoses were frequent within the light cell lobules.

More obvious changes were seen after 7 days hypoxia and vascularisation became more pronounced (Fig. 3). In many cases, the dark cells tended to be arranged in follicles or cords, the lumina of which were packed with erythrocytes. In the majority of this group there was considerable irregularity particularly of the dark cords, due to separation between the luminal surfaces of adjacent cells, in some cases leading to necrosis (Fig. 4). Crevices also tended to develop towards the centre of light cell lobules. The nuclear configuration resembled that seen in hyperplastic islet tissue (Hardisty, 1976) with a very distinct pattern of chromatin granules and a large, deeply staining central nucleolus. As in the earlier stages of hypoxia, mitoses were frequent but confined to the light cells. Vacuolisation was present in only 3 of the 12 animals examined and was relatively slight, affecting only a small proportion of the light cell cords (Fig. 5). Within this group there appeared to be some correlation between the incidence of vacuolisation and hyperglycaemia; in the three animals showing these lesions, blood sugar values were 175, 150, and 129 mg/100 ml compared with a mean value of 80 ± 4.0 mg/100 ml for 16 animals in which vacuolisation did not develop after 7 days hypoxia.

After 14 days hypoxia, every animal showed vacuolisation and, with one exception, this was so severe that almost all the

light cells were affected; in these areas only the lobular envelope, cell membranes, and nuclei remained intact (Figs. 6, 7). The surviving dark cells retained a highly granular cytoplasm which was intensely aldehyde-fuchsinophil. No mitoses have been observed in this experimental group.

In spite of their failure to develop hyperglycaemia, every one of the hypophysectomised lampreys subjected to only 7 days hypoxia showed "hydropic degeneration," which was even more severe and extensive than in the intact animals after 14 days under similar conditions (Fig. 8). In this group also, the surviving dark cells retained a conspicuously coarse and dense aldehyde-fuchsinophil granulation, and few if any of the light cells escaped vacuolisation. The possibility that these lesions might be attributable to the operation of hypophysectomy itself may be discounted, since no vacuolisation was observed in a control group of hypophysectomised animals maintained under normal laboratory conditions for periods of 1–2 months.

Over a period of several years, hydropic degeneration has occurred "spontaneously" in a small proportion of laboratory-held animals and in others kept for short periods in live boxes in the river. It has also been seen in a number of animals with severe fungus infection. Figure 9 shows extensive vacuolisation of light cells which occurred in only one of a number of animals subjected to continuous light stress where, however, it was not accompanied by hyperglycaemia.

Adrenalin Injections

Under adrenalin administration, few animals survived more than 4 or 5 days, and all showed marked depigmentation. Ventilatory frequencies increased, and the rate of branchial contraction measured 2 hr after the last injection was 162 ± 2.3 per min compared with 88 ± 8.2 for the saline-injected controls.

No marked abnormalities were noted in the islet tissue, other than increased vascularity, but the incidence of mitosis was certainly higher than in any other experimental group.

Glucose Loading

Animals killed 24 hr after a single glucose injection showed marked degranulation, as judged by the diminished intensity of aldehyde-fuchsin staining of the dark cell cords (Figs. 11, 12). Stimulation of cell division was also evident in the light cells.

Among adult lampreys subjected to daily glucose injections over periods varying from 8–11 days, only 3 out of 13 developed vacuolisation. (Hardisty *et al.*, 1975).

Changes in Nuclear Volumes

Islet tissue. In general, the measurements of nuclear diameters of islet cells in Table 1 does not differentiate between cell types, and represents therefore a random mean of both light and dark cells. Separate measurements have, however, been made of the nuclei of both light and dark cells in sections stained by the aldehyde-fuchsin technique in three control animals and four others killed within 24 hr of receiving a single glucose injection of 2 g/kg. Fifty nuclei of each cell type were measured in each animal. These measurements demonstrate the larger size of the nucleus in the light cells, but show that both cell types respond to glucose loading (Table 1). Both the differences in diameters of dark and light cells and the increases after glucose loading are highly significant ($P < 0.001$). Because of these differences, variability in nuclear diameters of islet tissue is very marked, and for this reason the mean values in Table 1 represent 50 nuclei from each animal, compared with 20 measurements for the other endocrine tissues.

Although in most cases, the dark and light cells appear to be present in roughly equal proportions, this is not reflected in the data in Table 1 where the overall mean value for the nuclear diameters of both types measured indiscriminately does not fall, as expected, approximately midway between the values for the light and dark cells recorded

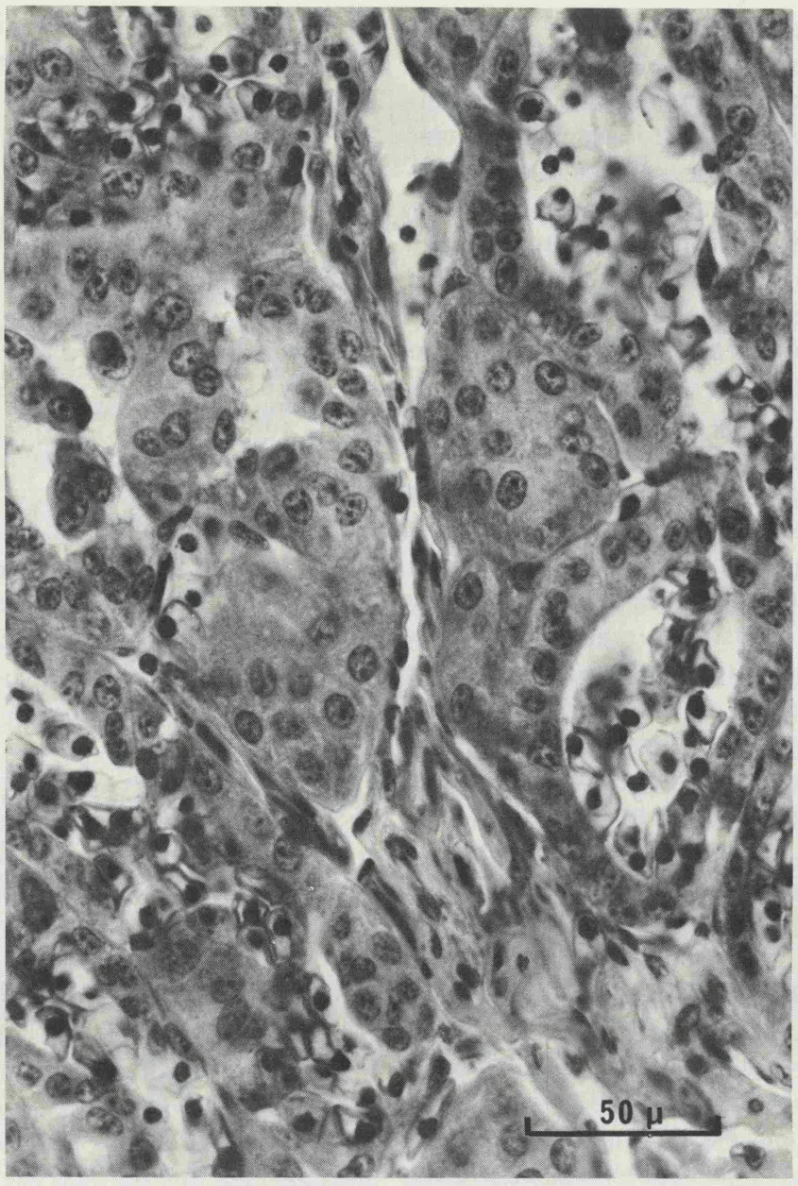

FIG. 3. Seven days hypoxia. Vascularisation and follicle development. Trichrome.

separately. This apparent discrepancy is almost certainly due to the fact that the two sets of measurements include animals not common to both series, and it has been our experience that there are often quite large individual differences between animals which do not appear to be related to body size or sex. This also applies to the hypoxia data in Table 2, which includes experiments conducted at different times and with animals that may represent different populations, showing differences in nuclear volumes. Thus, in interpreting the data in this table, each group, such as the 18-hr and 3-day hypoxia group or the 7- and 14-day hypoxia group, should be compared only with the appropriate controls for that particular group, and comparisons between these groups would be invalid.

In spite of the rise in blood sugar levels, no significant changes occurred in the volume of the nucleus in any of the hypoxia groups, even where extensive vacuolisation had developed. The nuclei of the hypophysec-

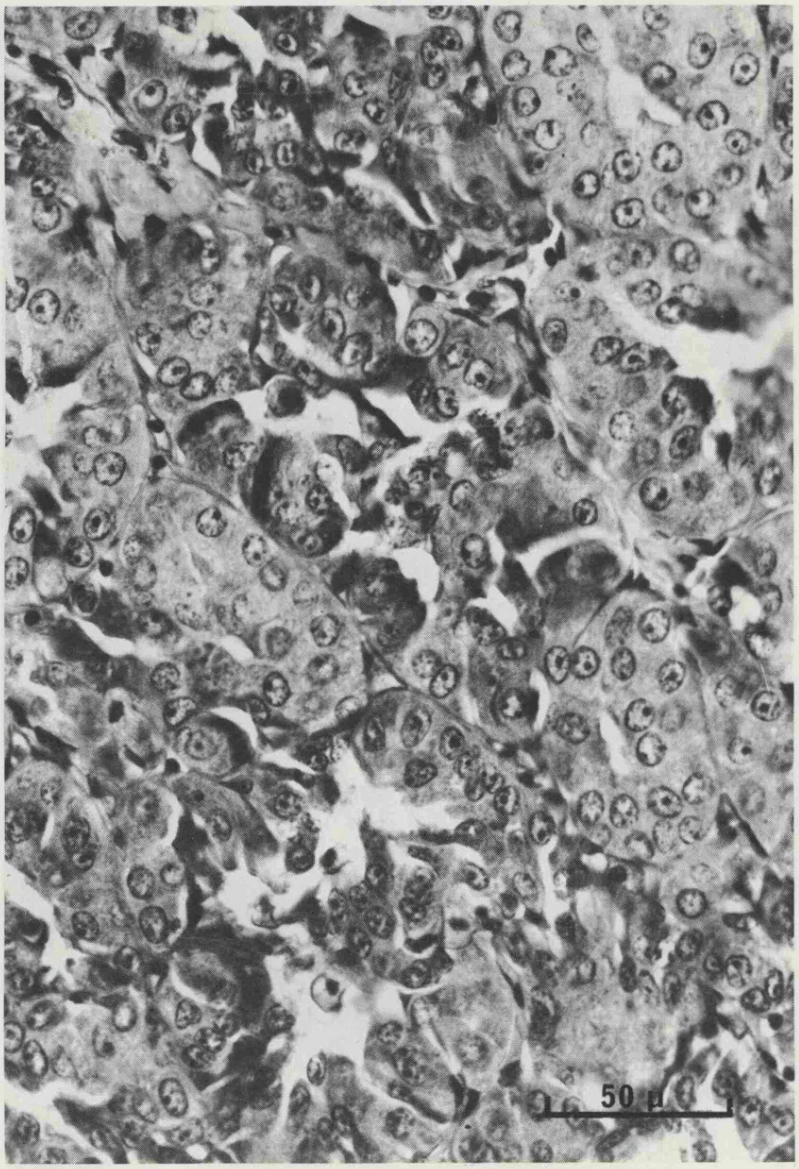

FIG. 4. Seven days hypoxia. Necrotic changes in dark cells. Trichrome.

tomised animals subjected to hypoxia, showed a very small increase in diameter compared with appropriate intact control animals, but this is of only a low order of significance. No significant differences were found between the intact animals and the control hypophysectomised group.

As reported previously (Hardisty *et al.*, 1975), marked increases in nuclear volumes occurred after either a single glucose injection, or after repeated injections over periods of 8–11 days.

In the adrenalin-injected animals the enlargement of the nucleus was almost as great as in glucose-loaded animals.

Interrenal tissue (Fig. 10). No evidence was found for the involvement of this tissue in the hyperglycaemic response to hypoxia and in every group from 18 hr to 14 days, the mean diameters of the interrenal nuclei was almost identical to that of the control group (Table 2). However, in comparison with either intact or hypophysectomised controls, significantly smaller nuclei were ob-

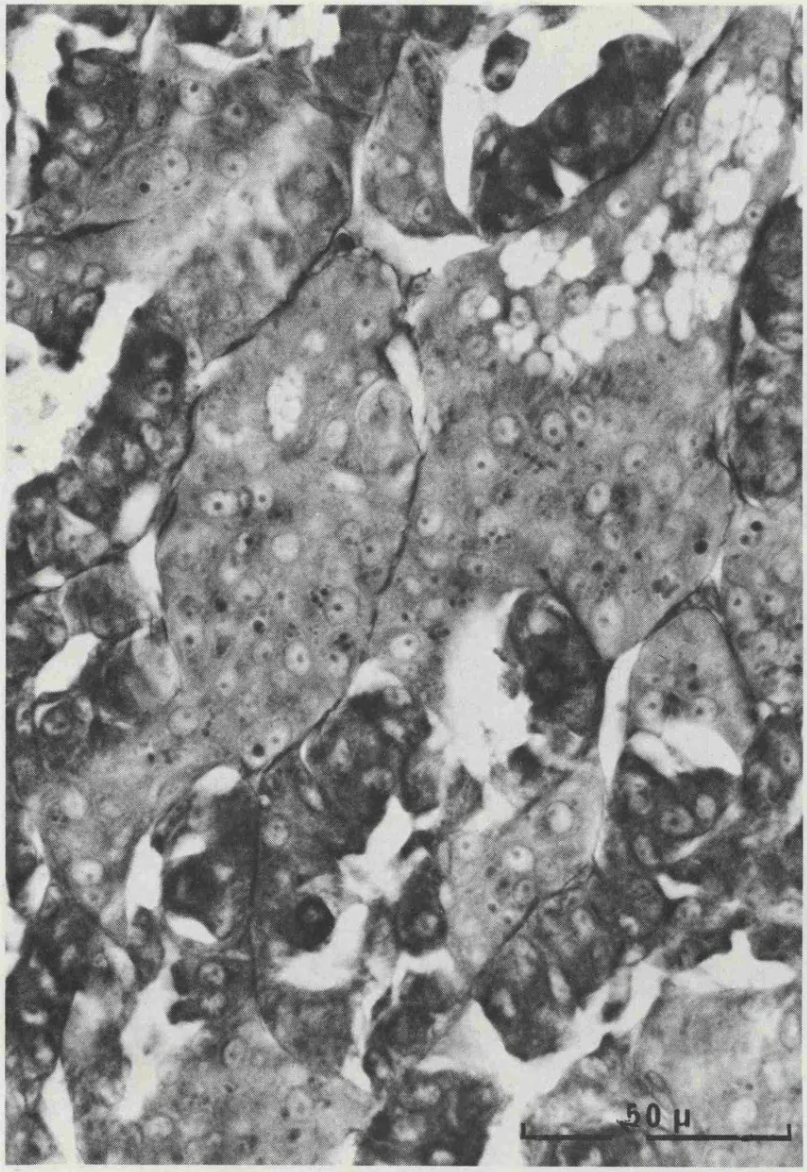

FIG. 5. Seven days hypoxia. Slight vacuolisation of light cells. AF.

served in the hypophysectomised animals subjected to 7 days hypoxia. Glucose loading appeared to result in a small but significant increase in interrenal nuclear volume ($P > 0.001$), but the largest increase was seen in the adrenalin-injected lampreys. This tissue is extremely variable both in volume and distribution, making quantitative comparisons difficult (Hardisty, 1972), but observations on serial sections from a number of adrenalin-treated animals gave distinct indications of hyperplasia.

Chromaffin tissue (Fig. 10). After 18 hr hypoxia treatment, the mean diameters of the chromaffin cells showed a very considerable increase compared with the control group (Table 2), and at 3 days the difference, although reduced, was still significant ($P > 0.001$). On the other hand after 14-days hypoxia treatment, values were almost identical to those of the control group. In both the hypophysectomy control group and also in those subjected to hypoxia, mean chromaffin nuclear diameters were signifi-

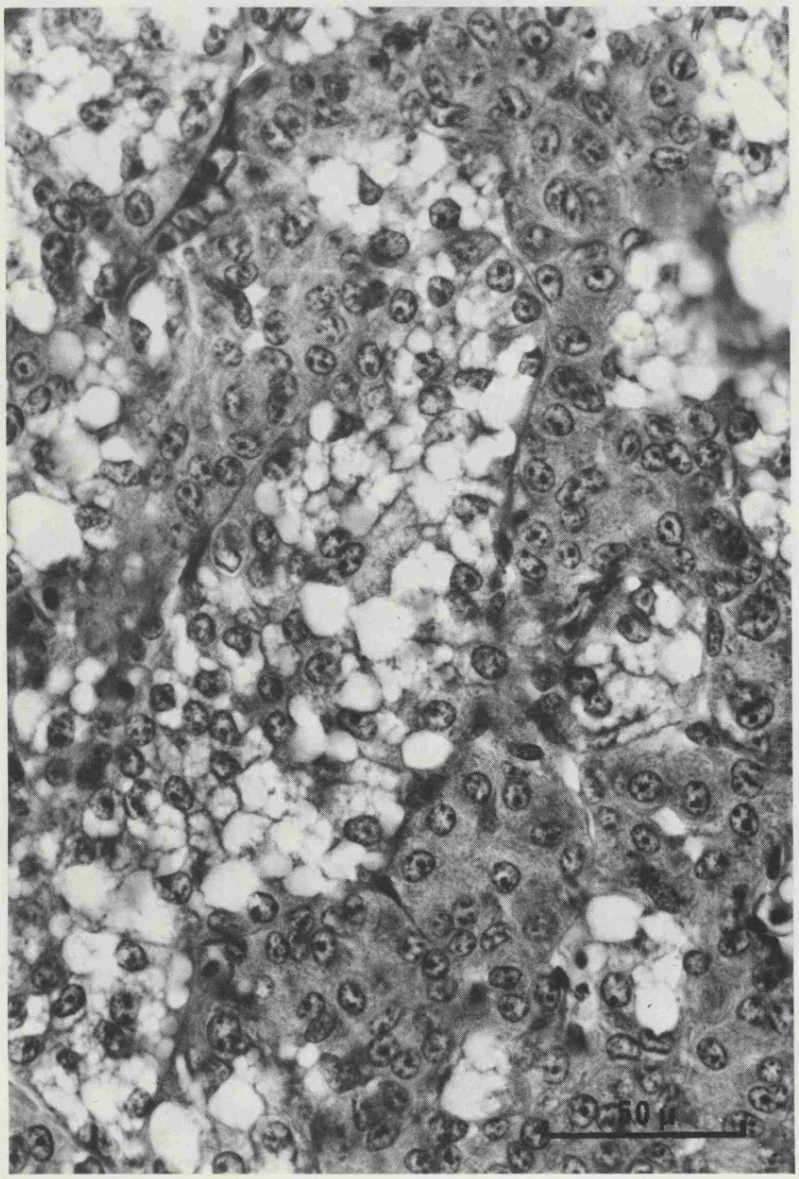

FIG. 6. Fourteen days hypoxia. Extensive hydropic degeneration. Trichrome.

cantly smaller than those of intact controls ($P > 0.001$). Chromaffin nuclei were greatly enlarged after glucose loading and a smaller increase was also seen in the adrenalin-injected group ($P > 0.05$).

DISCUSSION

In teleosts, increased heart rate, intense motor activity and hyperventilation have been reported under catecholamine stimulation (Serfaty *et al.*, 1964; Waitznegger and Serfaty, 1967; Pickford *et al.*, 1971), and similar responses have been observed in lampreys under hypoxic conditions. Involvement of chromaffin tissues is also indicated by the observed increases in nuclear volumes. Determinations of catecholamine levels in lampreys under normal and stress conditions (Bloom *et al.*, 1963; Stabrowsky, 1967; Dahl *et al.*, 1971; Mazeaud, 1969, 1972) have shown increased adrenalin levels after asphyxia and forced swimming. In *L. fluviatilis*, deficient aeration or transport out of water is said to result in elevations in

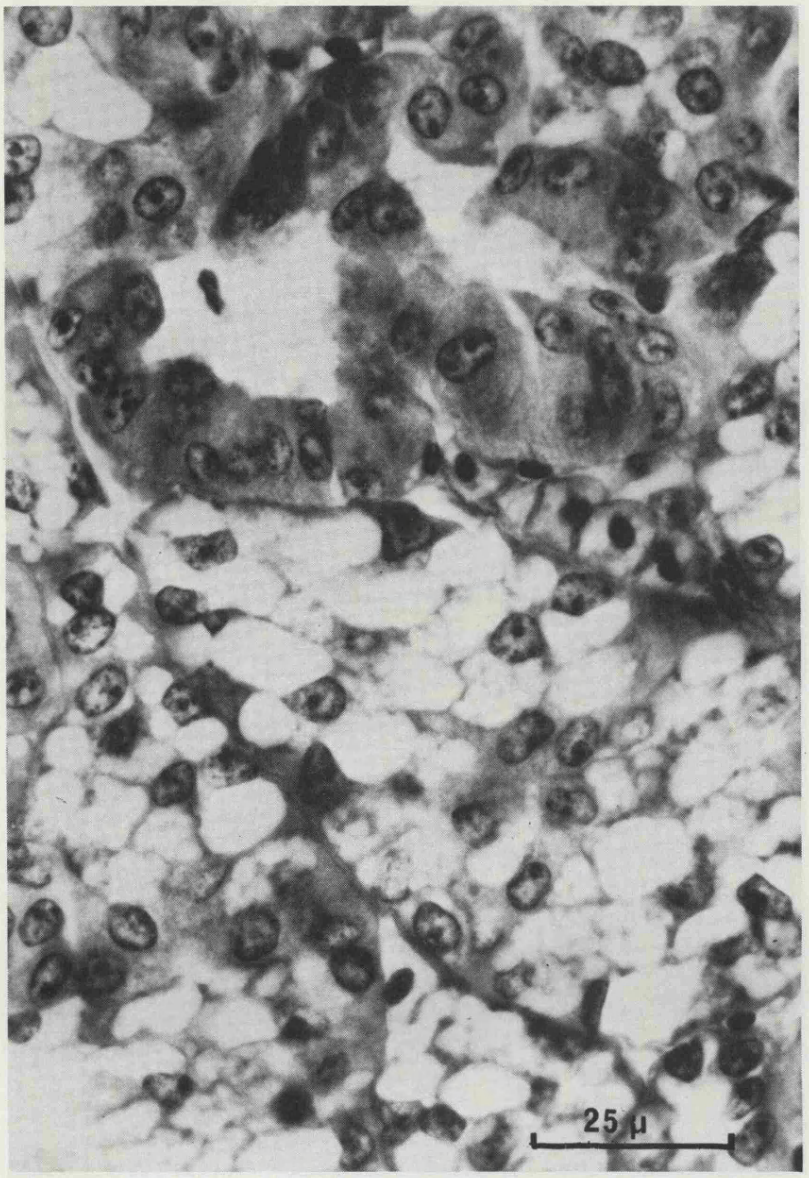

FIG. 7. Fourteen days hypoxia. Vacuolisation affecting light cell lobules. Trichrome.

blood adrenalin concentrations especially in males (Plisetskaya and Prozorovskaya, 1971), but in *Petromyzon marinus* the adrenalin levels of normal females have been reported as being twice as high as in males (Mazeaud, 1969). This latter finding may have some relevance to the trend towards higher blood sugar values in female river lampreys exposed to hypoxia.

It is difficult to decide how far the hyperglycaemic response to hypoxia is a direct result of reduced oxygen tensions, or is an indirect consequence of the hyperactivity associated with this condition. In both lampreys and teleosts, exogenous adrenalin characteristically evokes, a rapid but often transient hyperglycaemia (Young and Chavin, 1963; Mazeaud, 1964; Bentley and Follett, 1965; Banerji and Ghosh, 1973; Thorpe and Ince, 1974), and similarly in the usual "stress" or "asphyxial hyperglycaemia" blood sugar levels show a rapid rise, reaching maximum values within a matter of hours (McCormick and Macleod, 1925; Simpson,

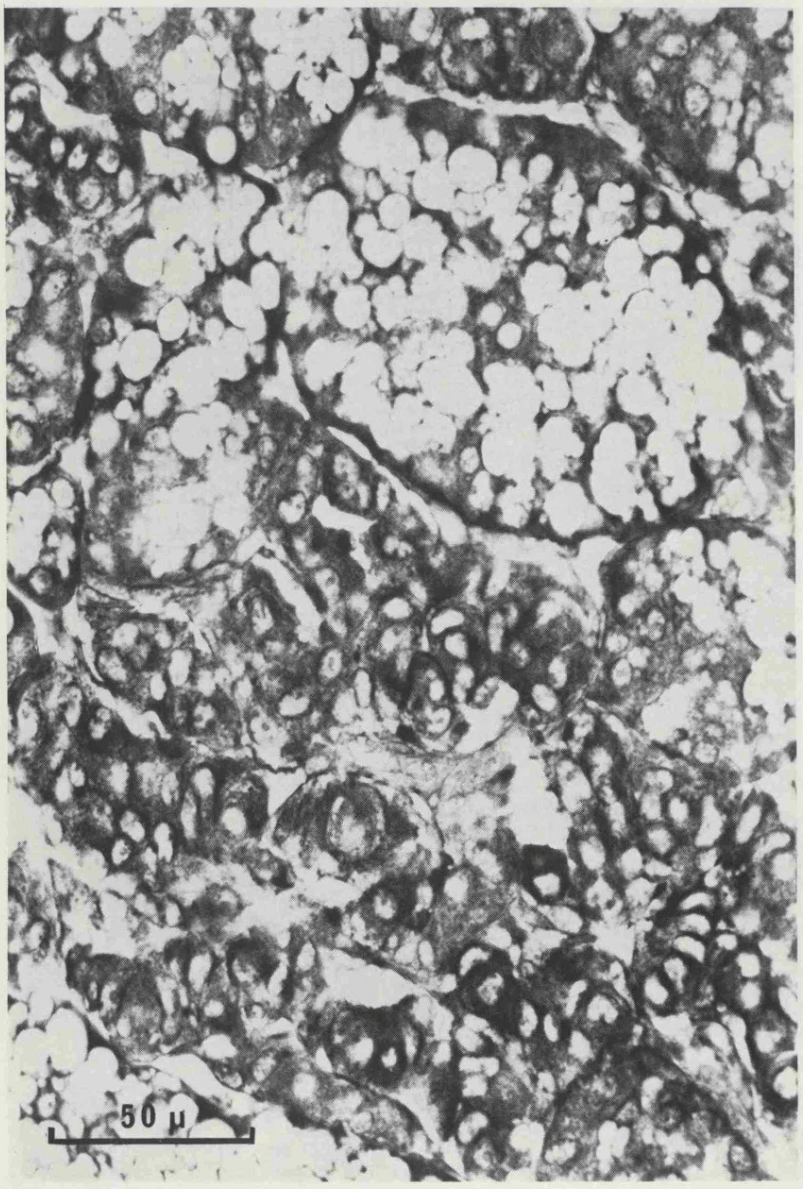

FIG. 8. Extensive hydropic degeneration in light cells of a hypophysectomised animal subjected to 7 days

1926; Menten, 1927; Al-Gauhari, 1958; Black and Tredwell, 1967; Leibson and Plisetskaya, 1969; Chavin and Young, 1970; Plisetskaya *et al.*, 1971; Tandon and Joshi, 1973). Both conditions may be contrasted with the response of the lamprey to hypoxia, in which the hyperglycaemia develops only slowly and takes 7 days to reach maximum values.

Failure to detect changes in liver glycogen in the hypoxia experiments might be due to variability and the continuous decline in liver carbohydrate that characterises the fasting migratory lamprey. On the other hand, since exogenous adrenalin has been found to decrease muscle, but not liver glycogen in the river lamprey (Bentley and Follett, 1965), mobilisation of carbohydrate reserves in the body wall may be the major factor in the maintenance of hyperglycaemia.

Particularly in the absence of enlargement of islet cell nuclei, the role of insulin in hypoxia is difficult to assess. In mammals,

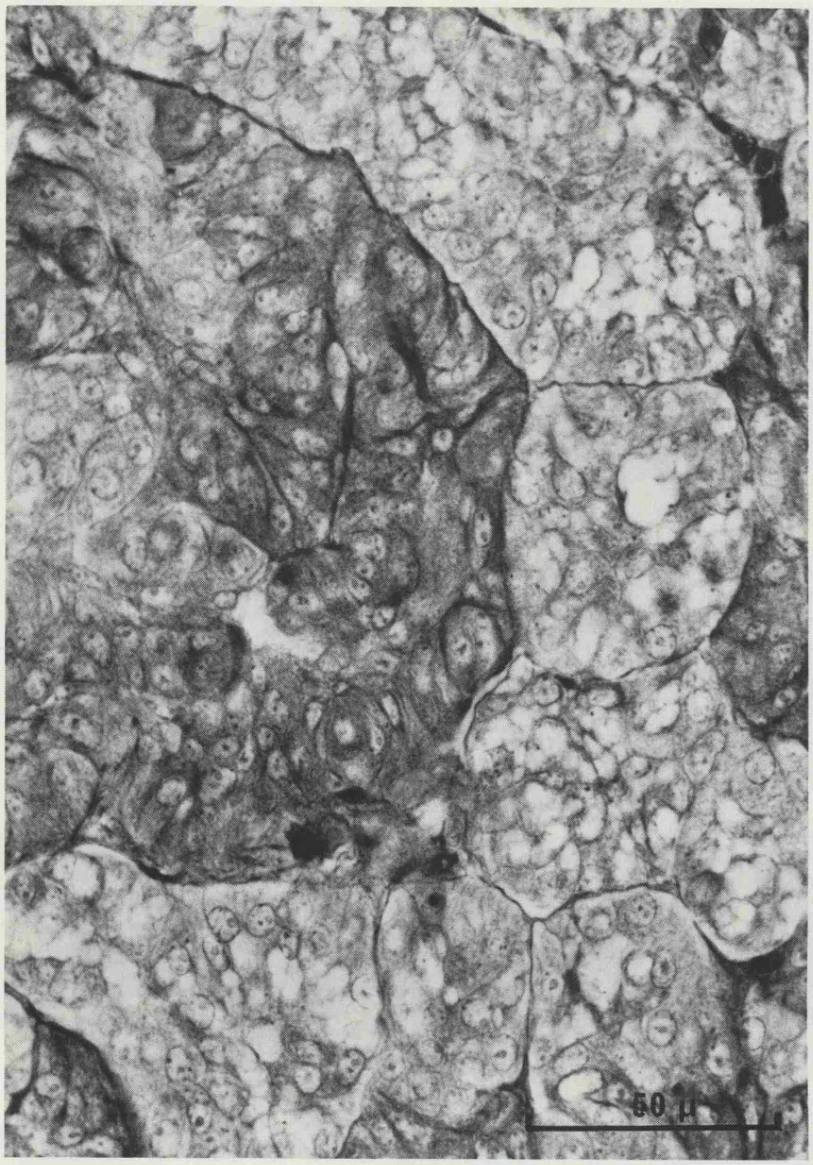

FIG. 9. Vacuolisation in light cell of an animal subjected to light stress for 7 days. AF.

hyperglycaemia is the main factor in the release of insulin from β cells (Ashcroft *et al.*, 1971), but this glucose-stimulated secretion is inhibited by adrenalin *in vivo* and *in vitro* (Howell and Taylor, 1966; Milner and Hales, 1969; Bassett, 1971). Furthermore, in isolated islets and pancreatic slices, anoxia is said to reduce the effects of insulin-secretory agents, including glucose (Hales, 1971). Thus, in addition to any direct effects of adrenalin on gluconeogenesis or glycogenolysis, its inhibitory activity may play a part in the generation or maintenance of hypoxial hyperglycaemia. A role for glucocorticoids in stress hyperglycaemia is well established in teleosts, but the measurements on interrenal nuclei lend no support to the involvement of this tissue in the lamprey.

The retention of normal blood sugar levels by hypophysectomised lampreys subjected to hypoxia is difficult to understand, especially in view of the extensive vacuolisation of islet tissue. In birds and mammals, direct

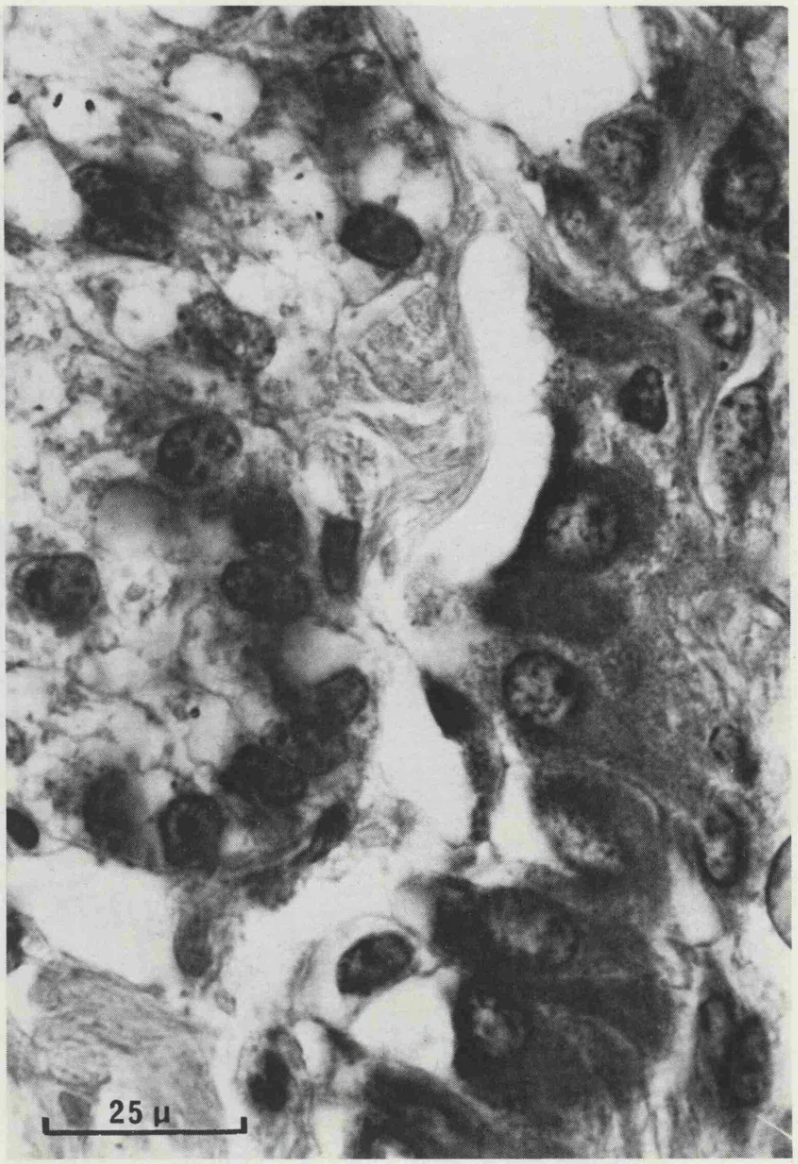

FIG. 10. Surface of aorta with group of interrenal cells (left) and granular chromaffin cells (right). Trichrome.

or indirect pituitary control of adrenalin synthesis via the regulation of phenylethanolamine-*N*-methyl transferase (PNMT) activity is well established (Wurtman and Axelrod, 1965; Pohorecky *et al.*, 1971; Wasserman and Bernard, 1971; Newcomer *et al.*, 1972; Manelli *et al.*, 1973), and in Amphibia also, adrenalin levels are reduced after hypophysectomy (Rapela and Gordon, 1956). Similarly, in teleosts, pituitary stimulation of glycogenolysis through ACTH and glucocorticoids has been reported (Chester Jones *et al.*, 1974), and these hormones are said to cause hypertrophy of chromaffin tissues (Mahon *et al.*, 1962; Olivereau, 1966). However, more recent work has not substantiated these findings in teleost and elasmobranch species (Mazeaud, 1972; Peyrin and Pérès, 1970). After examining PNMT activity and catecholamine levels in the sea lamprey, *P. marinus*, Mazeaud (1972) concluded that the methylation of noradrenalin is independent of corticosteroids, a view supported by a

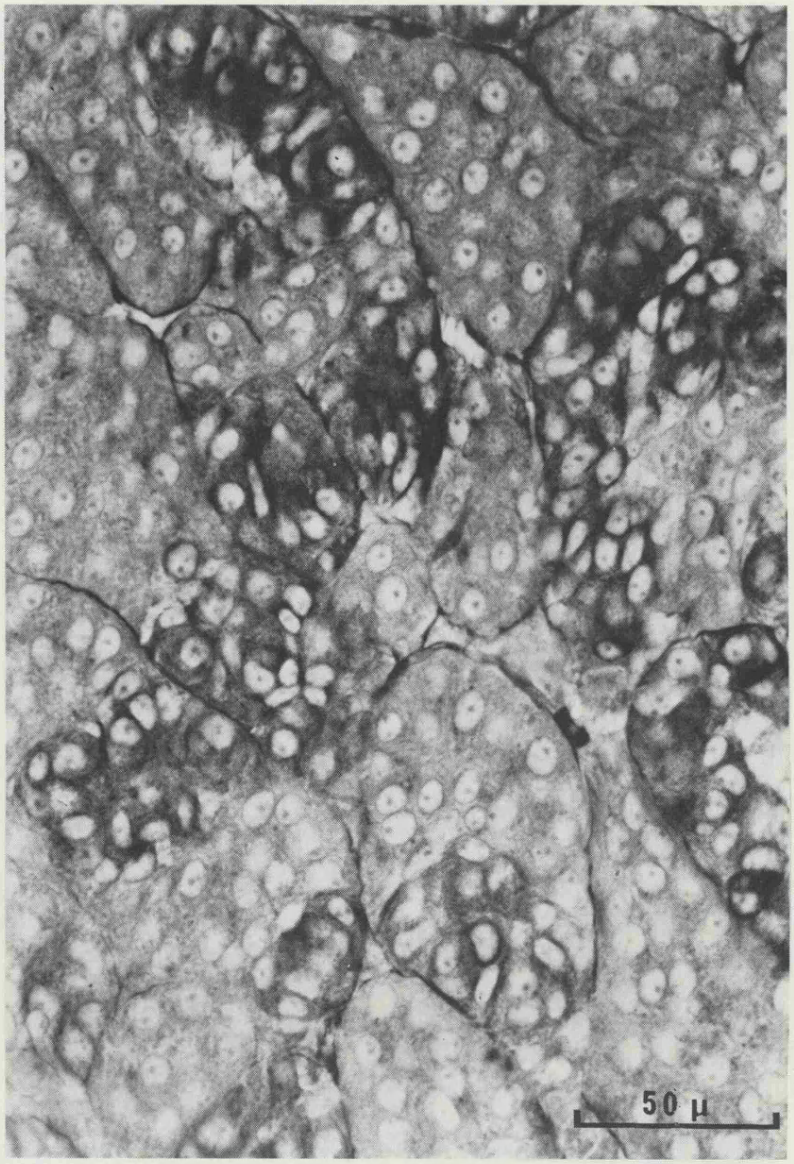

FIG. 11. Islet tissue from normal control animal. AF.

mistaken belief in the separation of chromaffin and interrenal tissues. In fact, in the region of the sinus venosus and Cardinal veins, small groups of both tissues are frequently in direct contact (Fig. 10). While in both the hypophysectomy control group and those subjected to hypoxia there has been some evidence of decreased volumes in chromaffin cell nuclei, there remain considerable difficulties in postulating pituitary control of catecholamine biosynthesis, mediated through ACTH or glucocorticoid activity. In spite of some indications that the lamprey adenohypophysis may produce an ACTH-like factor (Larsen and Rothwell, 1972; Youson, 1973), the homology of the interrenal with the adrenocortical tissue of the gnathostomes still rests mainly on morphological criteria (Hardisty and Baines, 1971; Hardisty, 1972; Youson, 1972) and biochemical evidence for the existence of the usual vertebrate corticosteroids is still lacking (Seiler *et al.*, 1970; Weisbart *et al.*, 1970; Weisbart, 1975).

The highly significant interrenal nuclear hypertrophy, probably accompanied by

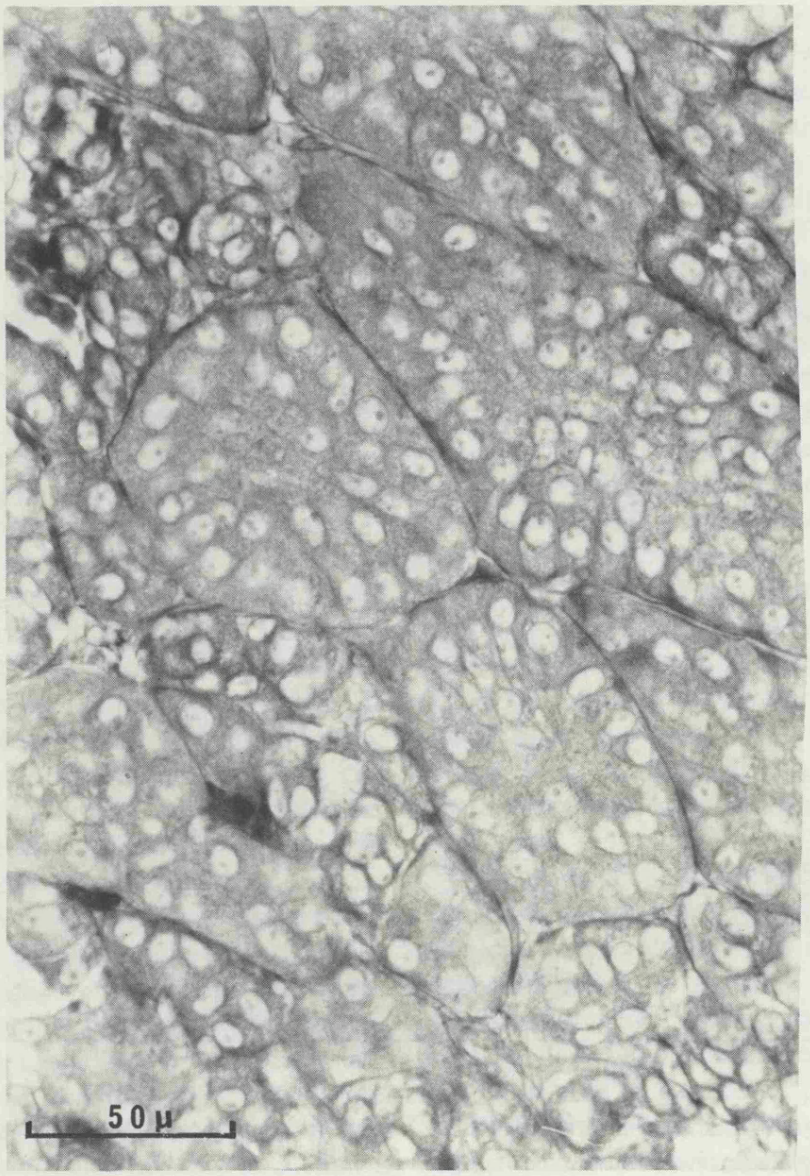

FIG. 12. Degranulation of dark cells after glucose loading. AF.

hyperplasia, following adrenalin administration parallels similar responses in the teleost, *Heteropneustes fossilis* (Yadov *et al.*, 1970). The much smaller increase in interrenal nuclear volumes in glucose-loaded animals and the absence of significant changes in hypoxia appear to discount the possibility that this response to exogenous adrenalin is evoked specifically by hyperglycaemia. Consideration of the close topographical relationship of interrenal and chromaffin tissues raises the possibility of some interaction, other than the stimulation of PNMT activity referred to in previous paragraphs.

Vacuolisation resulting from an overloading of the functional capacity of the β cells has been widely reported in teleosts after glucose loading (Khanna and Mehrotra, 1969; Khanna and Rekhari, 1972) and has been shown to be a temporary and reversible condition (Bhatt, 1974), apparently differing qualitatively from the more severe and extensive hydropic degeneration observed in the present experiments. In mammalian islets also, hydropic degeneration of β cells

TABLE 1

MEAN NUCLEAR DIAMETERS (μm) OF DARK AND LIGHT ISLET CELLS IN NORMAL AND GLUCOSE-LOADED ANIMALS

	N	Dark	N	Light	N	All cell types
Glucose-loaded	4	6.33 ± 0.04	4	6.89 ± 0.04	4	6.81 ± 0.03
Controls	3	5.98 ± 0.04	3	6.44 ± 0.04	3	6.31 ± 0.03

TABLE 2

MEASUREMENT OF NUCLEAR DIAMETERS IN ISLET, INTERRENAL, AND CHROMAFFIN TISSUE IN HYPOXIA AND VARIOUS EXPERIMENTAL TREATMENTS

	Mean nuclear diameters (μm) ± S.E.					
	Number of animals	Islet cells	Number of animals	Interrenal	Number of animals	Chromaffin tissue
Hypoxia						
18 hr	3	6.83 ± 0.05	5	5.79 ± 0.05	5	6.58 ±.0.05
3 days	3	6.71 ± 0.06	4	5.73 ± 0.06	4	6.27 ± 0.07
Controls	3	6.79 ± 0.06	4	5.70 ± 0.06	4	5.82 ± 0.07
7 days	3	6.20 ± 0.05	—	—	—	—
14 days	3	6.33 ± 0.03	4	5.80 ± 0.07	4	6.60 ± 0.02
Controls	3	6.28 ± 0.03	4	5.79 ± 0.06	4	6.65 ± 0.07
Hypophysectomy						
7-day hypoxia	3	6.43 ± 0.04	4	5.32 ± 0.05	4	6.22 ± 0.06
Controls	3	6.34 ± 0.04	4	5.82 ± 0.05	4	6.33 ± 0.06
Unoperated controls	3	6.28 ± 0.03	4	5.79 ± 0.08	4	6.65 ± 0.07
Adrenalin injected	3	6.71 ± 0.05	3	6.32 ± 0.05	3	6.60 ± 0.07
Saline-injected controls	3	6.27 ± 0.05	3	5.66 ± 0.05	3	6.38 ± 0.08
Glucose loading						
24 hr	5	6.81 ± 0.03	4	5.87 + 0.05	4	6.91 + 0.10
Controls	3	6.31 ± 0.03	4	5.60 ± 0.05	4	6.25 ± 0.07

has been regarded as only a transitory lesion, reflecting extensive functional strain in cells that escape necrosis, but are nevertheless affected by hyperglycaemia (Warren *et al.*, 1966). Where these lesions have been observed in other groups of lampreys, for example after repeated glucose loading or subtotal isletectomy (Hardisty *et al.*, 1975), they have been associated with marked increases in nuclear volumes. This contrasts with the situation in hypoxia, where in spite of hyperglycaemia and the development of vacuolisation nuclear volumes remained unchanged. In mammalian β cells, increased nuclear volume has been noted within 30 min of a single dextrose injection, and although some decrease occurred thereafter with the disappearance of Gomori-positive granulation, the enlargement of the nucleus persisted for some hours during the degranulatory and secretory phase (Mohnicke and Moritz, 1964).

Equally paradoxical is the observation of hydropic degeneration in the hypophysectomised lampreys subjected to hypoxia where it was accompanied by normal blood sugar levels. The occurrence of these lesions, in the absence of hyperglycaemia and without the usual indications of islet cell stimulation, casts some doubt on the assumption that vacuolisation represents a simple physiological overloading of the insulin secretory capacity of the cell and is a direct consequence of elevated blood sugar

levels. In this connection, it is significant that both glycogen stores and glycolytic intermediates are reported to accumulate in the β cell after adrenalin inhibition of insulin release (Hellman, 1970). The apparent lack of a cellular response under conditions of hypoxia might be related directly to the reduced oxygen tensions and their effects on cellular metabolism, and in this connection the reports of anoxic inhibition of β-cell activity may be recalled (Hales, 1971).

Cytological observations in the course of these experiments have some relevance to the problem of the functional status of dark and light cells. In the later stages of hypoxia, both in intact and hypophysectomised animals, there was no marked degranulation of the dark cell cords, such as occurs in glucose loading. On the contrary, these cells often showed an abnormally high concentration of coarse aldehyde-fuchsinophil granulation, contrasting with the vacuolated light cell areas. Similar differential lesions have also been seen in glucose loading and in cases of "spontaneously" occurring vacuolisation. These observations would be consistent with the view that both light and dark cells should be regarded as developmental stages of a single functional cell type (Barrington, 1972), and the reaction of the dark cell granules to both aldehyde-fuchsin and pseudoisocyanin techniques points to the latter as being the terminal or storage phase in the β-cell cycle. Moreover, it may be significant that where increased vascularity has been noted in the islet parenchyma, accumulations of erythrocytes appear to be mainly confined to the lumina of the dark cell cords, which would presumably be concerned with insulin release. A similar interpretation is also supported by the nuclear measurements, which show that although both cell types are stimulated by glucose loading, the increase in volume tended to be rather greater in the light cells. If this interpretation is valid, the development of vacuolisation in the light cell lobules suggests that earlier phases in insulin biosynthesis and granule development may be more sensitive than later stages to whatever factors may be immediately involved in the genesis of these lesions. The degranulation of the dark cells under glucose loading and their retention of aldehyde-fuchsinophil granulation under hypoxic conditions would thus represent the influence of factors which respectively stimulate or inhibit insulin release. The importance of distinguishing insulin biosynthesis from insulin release is emphasised by the results of adrenalin injection, which resulted in very marked increases in the nuclear volumes of islet cells. In the mammalian β cells, mitoses increase in frequency after glucose injection (Korcakova, 1971), and in the lamprey increased mitotic frequency has been noted in hypoxia, glucose loading, and above all after exogenous adrenalin, all of which raise blood glucose levels. These divisions have, however, been observed only in the light cells, again pointing to these elements as representing an earlier stage in the β-cell cycle.

The possible ecological effects under field conditions of reduced oxygen tensions in the aetiology of islet cell lesions is difficult to assess. Whether in polluted estuaries or rivers, prolonged exposure to suboptimal, but much less severe, reductions in oxygen tensions would be capable of producing extensive and permanent hydropic degeneration is open to question. Certainly, we have observed a number of examples of these lesions in fungus-infected river lampreys, perhaps due to interference with gill function. The occurrence of "spontaneous" islet cell damage of this type in animals maintained under laboratory conditions, or stored in live boxes in the river, suggests that severe vacuolisation may develop under conditions, which, although unfavourable, are nevertheless compatible with survival.

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