MORPHOLOGICAL AND METABOLIC INVESTIGATIONS ON THE ISOLATED PANCREATIC ISLETS OF TWO TELEOST SPECIES, NORMAL AND OBESE-HYPERGLYCEMIC MICE

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GENERAL INTRODUCTION

The description of the pancreatic islet tissue (1) followed by the findings (2) of their relative importance in diabetes mellitus resulted in both morphological and physiological studies (3, 4) which paved the way ultimately to the isolation of insulin by Banting and Best (5). Later on, the studies (6, 7) concerned with identification of different cell types within the pancreatic islet tissue over a number of years led to the present well-established fact that the beta cells of the islet tissue produce insulin (8) and that this hormone or a precursor is ordinarily stored in the form of beta granules (9). Insulin hormone has been extensively studied and a great deal is known about its structure and about some of the effects which it produces; reviewed recently by Randle (10). The subject of the pancreatic islet and insulin has grown widely and extensively but the major problems of detailed and precise mechanism of action of the hormone in physical and chemical terms, of the chemical and physical changes involved in the secretion of insulin and its physiological role in quantitative terms remains largely unsolved (10). Also, as Brolin (11) in 1964 pointed out an increasing knowledge of islet structure undoubtedly deserves our attention, as there is great controversy regarding the cellular composition of the pancreatic islet tissue.

One of the many important questions confronting the workers associated with diabetes research is: How do the pancreatic islets work? We do not know yet the precise mechanism of a number of functions which the islet tissue perform, including the secretion of insulin in normal and diabetic condition. Although more than 45 years have elapsed since the discovery of insulin, the etiology and pathogenesis of diabetes by pathological, clinical and metabolic studies in man as well as in experimental animals has not yet been fully elucidated. Partly for this

reason, there has been a growing interest during the last few years in the using of a variety of other experimental animals besides the conventional laboratory animals for experimental islet research.

The mammalian islet tissue comprises only 1% of the total pancreatic mass and is embedded in exocrine tissue and it becomes difficult to perform direct biochemical analysis on it. This may explain why so little is known as yet of its metabolic activities. However, in some teleost fish, the endocrine pancreatic tissue may be concentrated in the abdominal cavity into one or two sizeable structures known as the 'principal islets' (12 - 17). Due to this unique anatomical feature the bony fish, i.e. Cottus scorpius (17) offer unequalled possibilities for chemical and metabolic studies of pure islet tissue (16 - 23). The teleost islet tissue contains a type of insulin which does not seem to differ obviously from ordinary mammalian insulin either in its effect on man or in other hormonal and antigenic properties (24, 25). The islet tissue apparently shows the same main characteristics as regards different types of cells and physiological behaviour in mammals (17, 13). This suggests that it may be possible to draw conclusions about the conditions in human islet tissue from experimental results obtained from these poikilotherm animals to as great an extent as is the case of homotherm laboratory animals.

It was recently reported (26) that the principal islets of Tilapia mossambica, a freshwater teleost, are composed of beta cells only. This teleost, hence, seemed to be of great interest and importance due to the fact that several biochemical and metabolic problems associated with the islet tissue could thus be studied on a handsome population of pure beta cells.

Of particular interest, from the etiological point of view, are the mice with hereditary obese-hyperglycemic syndrome (27). Obese-

hyperglycemic mice present a unique array of altered metabolic patterns (28, 29) and are characterised by a recessive gene (30). There is histological evidence (31, 32) of an extremely high functional state of the beta cells compared with their lean mates together with a considerable increase of 'insulin like' activity in the plasma (33).

A technique to isolate intact islets from the fresh mammalian pancreas was recently described (34) and this proved to be very useful in obtaining relatively large amounts of metabolically active islet tissue for varying analytical purposes (35 - 39). The application of this technique to obese-hyperglycemic mice makes it possible to get islet tissue not only in relatively large amounts, but also a fairly pure population of islet beta cells, as it has already been shown that in the islet tissue of this animal the contribution of alpha cells is less than 10% (40). Analyses of the mechanisms associated with insulin synthesis and secretion could thus be carried out on such isolated pancreatic islets.

The aims of this comparative investigation of the etiology of diabetes were two-fold:

A. The examination of the principal islets of Cottus scorpius and Tilapia mossambica, parallel to the pancreatic islets of mice, by modern differential histological and histochemical techniques as well as by electron microscopy. This was to establish the characterisation and nature of varied types of cells present in the islet tissue. Also, the incubation of the principal islets of Cottus scorpius with certain hypoglycemic and hyperglycemic agents, added to the incubation media to study the behaviour of islet cells, particularly the beta cells, under these influences. Some useful information might be obtained which could prove a basis for further study for understanding the process of insulin synthesis/storage or secretion.

B. The study of the regulation of insulin secretory mechanism by determining quantitatively glucose-6-phosphatase enzyme, thought to be involved in glucose metabolism of the islet tissue. It was proposed,

therefore, first to accomplish Hellerstrom's technique (34), isolate the pancreatic islets from normal and obese-hyperglycemic mice and then to study the properties of glucose-6-phosphatase enzyme in the isolated islets. If present, the activity of glucose-6-phosphatase was to be measured in different functional states of insulin secretion. Moreover, as nucleic acids must play a role somehow or another in the process of insulin synthesis/secretion, it was planned to determine quantitatively, by microchemical procedure, the amount of ribose-nucleic acid in the isolated pancreatic islets of both fish and mice in normal as well as experimental conditions. It was also hoped to localize at the ultra-structural level glucose-6-phosphatase enzyme within the islet cells in order to correlate the specific enzyme activity to a particular cell type in the islet.

It was planned to develop a technique of water soluble autoradiography at the electron microscope level. By applying this technique, it was hoped to study the mechanism of action of alloxan on the islet beta cells by following the course of labelled ^{14}C-alloxan in mouse pancreatic islets.

In vitro and metabolic studies on the principal islets of Cottus scorpius were performed at the Wellcome Laboratory of Pharmacology, Gatty Marine Laboratory, University of St. Andrews, Scotland, while investigations on micro-dissected pancreatic islets of mice and on the principal islets of Tilapia mossambica were carried out at the University of Aston in Birmingham.

SECTION I. REVIEW OF STUDIES ON PANCREATIC ISLETS

A. Morphological studies

The discovery in 1889 that total pancreatectomy in dogs caused polyurea and glycosuria (41), positively established the relation between pancreas and diabetes mellitus. Earlier the pancreatic islets had been identified within the pancreas. The first indication of this organ is found in Langerhan's work (1) when he observed intensely yellow areas - irregularly distributed in the fresh pancreas of rabbit - composed of small regular polygonal translucent cells. He called these areas 'groups of cells' and thought that perhaps they had certain connections with the nervous system. Other workers described such structures in the pancreas as 'points folliculaires' (42) or considered them as small lymphatic glands of somewhat special nature (43) or hardly even mentioned them (44). No precise function was attributed to these areas, or if it was, without any definite evidence.

It was Laguesse (45) who in 1893 became convinced that all the various names which had been given to these certain structures referred to the same object described by Langerhans. He called them 'islands of Langerhans' and suggested that these constituted the organ of internal pancreatic secretion involved in carbohydrate metabolism. Schultz's experimental studies (46) proved non-involvement of acinar tissue in the development of diabetes. Later on many workers, including Dewitt (4) furnished the confirmatory evidence that the islets of Langerhans produce internal secretion for the control of carbohydrate metabolism.

In the meantime, increasing evidence of a close relationship between morphologic alterations of the islets - diminution of the islets, atrophy of the islets and vacuolization in the islet cells - and diabetes was provided (2, 47). In the ensuing years, a number of workers arrived at the conclusion that islets of Langerhans constitute an important

factor in the etiology of human diabetes, though it was pointed out (48) that, as some diabetics showed apparently intact islet tissue, it may not be the sole organ related to diabetes.

Investigations, hence, started to study the structure of islets of Langerhans in detail. Basically the study can be classified under four areas:

1. Different cell types of islet tissue and their microscopic structure.
2. Islet histogenesis and embryogenesis of islet cells.
3. Comparative structure of islet tissue in various animals.
4. Islet cytological changes in diabetes.

1. Different cell types of islet tissue and their microscopic structure

A number of characteristic microscopic features of islet tissue which are still claimed as differentiating it from acinar tissue were postulated as early as 1900 (49). These include the absence of zymogen granules, islet tissue being composed of polygonal club-shaped epithelial cells adjacent to the coiled blood capillaries and small size of nucleoli of islet cells as compared to those of acinar cells.

Differentiation of two cell types was first observed by Schulze (49) in the islets of guinea pig. The further elaboration of fixation and staining procedures resulted in identification of two separate granulocytes in the islets of Langerhans (6, 7). Lane (7), in 1907, described the differences in solubility of islet cell granules of guinea pig. The cells which seemed to be dissolved in dichromate $HgCl_2$ fixative were called alpha cells, while those in 70% alcohol were known as beta cells. Benseley (6), in 1911, was able to stain simultaneously both cell types in a single preparation of guinea pig islet tissue. In addition to alpha and beta cells, he also identified an agranular or 'clear' cell which he believed to be a precursor of the alpha cell.

In the pancreatic islets of Rana temporia, as many as five types of cells were observed (50), all of which were considered to be interchangeable. Later on, three granulated cells were demonstrated (51) in the islets of a teleost, Neomanis griseus. The third cell, less granular and clearer than alpha and beta cells, was called a gamma cell and was thought to be the precursor of the other two cell types. On the other hand, studies on the islets of Triton claimed the presence of only one cell type (52).

Bloom, in 1931, described an additional cell type in human islet (53). This cell type was thought to contain granules and was termed the D-cell. It was shown, after Heidenhaim Mallory Azan stain, to contain pale blue cytoplasm filled with a mass of closely packed bright granules in contrast to the fiery red tint of the beta granules and the orange of the alpha granules. Later on, Thomas made a comprehensive study (54) of normal pancreases of 41 mammals and snakes and concluded that besides alpha and beta cells, D-cell is a constant cellular variety possibly in all vertebrates.

Thomas (54) also found a new cell type described as a specific and unique cell in the pancreatic islets of opossum. It was characterised as having large secretory granules (larger than alpha granules) and staining intensely with the trichrome method he used, and was called the 'E' cell.

Another type of cell was found by Mankowski (55) distributed in the parenchyma of guinea pig pancreas cells with somewhat pyknotic nuclei which contain sparse granules which stain with safranin.

Ferner, in 1952, studying the islet tissue of horse, demonstrated a pigmented cell and called it the 'X' cell (56).

It has also been demonstrated that in the dog pancreas a cell is present both within the islet and exocrine parenchyma which contains large acidophilic granules. Though this cell has frequently been confused with the alpha cell, it was shown to be tinctorially different from it (57).

In 1938, it was claimed that the alpha cells are argentaffin (58) but later on it was demonstrated (59) that these cells are entirely different from the argentaffin cells of the intestinal tract. It was also shown (60) that silver staining was restricted to some but not all alpha cells. Depending upon argyrophilic properties after silver staining, when using a refined modification of the alcoholic silver nitrate method of Davenport in rat pancreas, it was suggested, in 1960, that the pancreatic alpha cells should be divided into two distinct groups (61). The argyrophil and non-argyrophil cells were classified as A_1 and A_2 cell types respectively.

The literature concerning silver impregnation properties of the alpha cells and their classification is very confusing and reflects much disagreement among the present day workers. Fujita, in 1964, while examining the pancreas of a holocephalon fish, Chimaera monstrosa, clearly showed that the argyrophil cells of this fish corresponded neither to all the alpha cells nor to groups of them, but to D-cells which were tinged blue in azan and green in Masson-Goldner staining (61a). He concluded (61a) that the argyrophil A_1 cells reported (61) in the pancreatic islets of a number of species are, in fact, identical to the islet D-cells of Bloom (53). Later on many workers, including Epple (62), arrived at the same conclusion.

Epple pointed out (62) that some argyrophil cells in the pancreatic islets of urodele amphibians did not stain blue with azan and did not, therefore, correspond with the D-cells of Bloom (53). Whether these cells represent a type of D-cell or are functionally independent cell types is not clear. Epple showed (62) these cells to be pseudo-isocyanin metachromatic and aldehyde-fushcin positive like beta cells and called them amphicil cells.

2. Islet histogenesis and embryogenesis of islet cells

The balancement theory (45) that islets arise from exocrine lobules and that throughout life exocrine tissue could be transformed into islet tissue and vice versa is no longer accepted. The islet

tissues appear to develop from two sources. The earliest islets formed originate from the primitive pancreatic cords while the islets that are formed later and even after birth arise from the ducts and centroacinar cells (63). As early as 1911, Benseley (6) formed the opinion that islet and acinar tissue stemmed from a common source in the undifferentiated cells of the pancreatic cords and that the transition between the two did not occur. This view has recently been endorsed and further extended to that such undifferentiated cells continue to be formative of islet tissue in the adult pancreas (57) and also to that centroacinar and acinar cells themselves are a possible source of islet tissue (64). Reports concerning the embryogenesis of islet tissue as studied with an electron microscope are very few. These observations, made on embryonic islet tissue of mice (64), rat (65) and chick (66), however, tend to confirm the origin of islet cells from primordial duct epithelium as suggested by classical embryologists.

The literature concerning the cytogenesis of the islet cells is very confusing and reflects much disagreement about the origin of the alpha, beta and D cells. Some recent findings (67) now indicate that 'muddy cell' is a precursor of the mature islet cell and that alpha, beta and delta cells originate separately and do not represent different phases of the function of maturity of one cell type. However, electron microscopic studies could not detect (63) the granulated cell corresponding to the delta cell of Bloom's original description (53). It was also argued (68) that in the principal islets of teleost fish both alpha and beta cells may be transformed into a third type of cell, the so-called delta cell. Delta cells of human pancreatic islets were also thought to be the degenerate alpha or beta cells (69). This report also described the alpha cells changing into beta cells through 'transformation zone' (69).

Though it has not been possible to ascertain actually the sequence of events in the cytodifferentiation of alpha, beta or delta cells

the very few reports available (64, 66) indicate the development of ribosomes followed by that of the Golgi apparatus, and its enlargement and development of membranes of ergastoplasm.

3. Comparative structure of islet tissue

The presence of at least two distinct and independent cell types, the alpha and beta cells, in the pancreatic islets of animals ranging from cyclostomes to mammals has been established (17, 23, 66, 70 - 77, 86, 108, 115 - 119). There is also a general agreement of the existence of the D-cell in most of the species, particularly mammalian (74 - 77). Other differentiations, involving subclassifications of peripheral and central cells, made in studies of islets of fish (71, 72), urodeles (62, 73, 78), reptiles (79), birds (80, 81), many mammalian species including mouse (82), horse (81) and guinea pig (81) remain a matter of much controversy. The relationship of these islet cell types and also of the D-cell type to the originally described alpha and beta cells is uncertain.

The high magnification and resolution which can be obtained with electron microscopes has made it possible to observe the internal structure of the cell. The application of this tool has been made to the pancreatic islet tissue and certain morphologic features which could be neither visualised nor anticipated by light microscopic studies, have been demonstrated and established in different cell types of this tissue. Classical studies in this connection include those carried out by Lacy and his coworkers (75 - 77) in 1957 and by Bencosme and Pease (83) in 1958, describing the ultramicroscopic morphology of the alpha, beta and delta cells in the islets of various species including rat, rabbit, guinea pig, dog and cat. Since then, such studies have been extended, though limited incase of the pancreatic islets of non-mammalian species and are briefly reviewed here.

i. Cyclostomes

The pancreatic islet tissue of cyclostomes is thought to be the most primitive in the whole vertebrate phyla. The endocrine pancreas, represented mainly by the glandular tissue around the anterior part of the intestine of the lamprey was claimed (84) to contain both alpha and beta cells. Later on, in 1945, the presence of these cell types, however, was doubted (85). Very recently, ultrastructural studies on the pancreatic islet tissue of the lamprey revealed (70) the occurrence of three parenchymal cell types: granular beta cells, vesicular cells representing probably delta cells described in the cat (83) and agranular cells which may represent undifferentiated reserve cells. Also, a complete lack of alpha cells in the pancreatic islet was reported (70).

Of the myxinoidea, the islet tissue of Myxine glutinosa has been studied thoroughly (86, 87) but without definite conclusions. Electron microscopical observations (87) have confirmed the presence of granular beta cell types (86) in the islet tissue of Myxine glutinosa. It was shown (86) that the argyrophil alpha cells are present in the islet tissue but the later studies did not disclose any typical alpha cells (87); on the contrary, these alpha cells were considered to be identical to the agranular cells when observed by the electron microscope (87). The nature of these cells is not clear (87).

ii. Fish

Very few studies have been done on the islet tissue of fish. In selachians the islet organ is formed exclusively in immediate relation to the ducts, and real islets of Langerhans do not exist (88). Both the alpha and the beta cells have been demonstrated histochemically (88). In a holocephalian fish, Chimaera monstrosa, the endocrine pancreas consists of large islets the position of which has been recognised (89) as a transitional type between the

teleostean and elasmobranchial islet system. The cellular elements of the islet system were shown (89) to be the beta cells, the alpha cells and cells of an unknown type - called X-cells, along with a small number of D-cells, demonstrated as argyrophil cells. The nature and function of both D-cells and X-cells remains unknown (89).

Morphological studies by light microscopy in the principal islets of a number of teleost fish, e.g. toadfish (23), Lebistes reticulatus (90), carp (91), tunny fish (92), catfish (93) and bull-head fish (94) have confirmed the presence of granular cells corresponding to mammalian alpha and beta cells. Falkmer (17) observed two types of alpha cells in the principal islets of Cottus scorpius. In teleost fish agranular cells of undetermined nature have also been described (17, 23, 90, 92, 93).

Controversy exists in the identification of the various granular and agranular cells in the principal islet at the electron microscope level (71, 72, 91, 95). The identification of granular alpha and beta cells in the principal islets of bull-head fish was found (72) to be different from that in the carp and a number of salt water bony fish (91). The agranular cells were considered (71) as probably corresponding to delta cells of cat (83) or gamma cells of guinea pig (77). The nature of clear or agranular cells remains unknown. Whether these cells correspond to the delta cell of mammals (83), to the young immature forms of the granular cells (71), or whether it is a secretory stage of the beta cell (95)is not clear.

Histological observations on the principal islet of Tilapia mossambica (26) concluded the presence of only one cell type - beta cell. Electron microscopic study on the islet tissue of this teleost species has so far not been attempted.

iii. Amphibians

Earlier studies on the islet histology of amphibians indicated the presence of both alpha and beta cell types in anurans (96) but showed complete lack of alpha cell in urodeles (52). In Rana temporia, five different cell types were observed (50), all of which were thought to be interchangeable (50) and were believed to represent merely different phases of one secretory cycle (97). Typical alpha and beta cells were, however, reported (98) in the islet tissue of a species of Salamandridae. Even recent observations on urodele amphibians are in striking contrast. Extensive histological investigations (99) by Miller and his co-workers in 1959 confirmed the absence of alpha cells in as many as 16 species belonging to Salamandridae, Amphiumidae and Plethodontidae. They also reported the occasional presence of 'clear' cells, without granules, and believed this to be the transitional stage between the beta cell and exocrine tissue (99). Later on, different types of alpha cells were demonstrated in a number of urodeles (100, 101). The islet tissue of Xenopus laevis was shown (62) to contain three types of cells in contrast to a report that the tissue possesses only the beta cells (102). Similarly three and four types of cells at least have been demonstrated in the pancreatic islets of toad Bufo bufo (103) and Rana catesbiana (78) respectively. It is only very recently that D-cells have been demonstrated (62) in the pancreatic islets of a number of species belonging to family Salamandridae and Amphiumidae.

Electron microscopy (73, 74) of the pancreatic islet of a Salamander and a Newt confirmed the presence of alpha, beta and probably delta cells.

iv. Reptiles

Very few studies have been done on the islet tissue of reptiles. Histological observations on the islet tissue of lizards and snakes

(99, 104 - 107) and turtles (99) have shown it to be composed of at least two well differentiated granular cells - alpha and beta cell. It was also argued that these two cell types are but different secretory phases of a single type of cell (105). Considerable difference in the pancreatic islet morphology of reptiles of different orders has been observed (99). A relatively large number of alpha cells were found in the islets of lizards and snakes (99) and were considered responsible for a large content of glucagon. In snakes, the alpha cells have been demonstrated (79) to be classified as A_1 and A_2 cells.

Very recently the pancreatic islets of lizard, Eumeces fasciatus, were examined by an electron microscope (108) which confirmed the previous investigations on tortoise (109) showing the presence of alpha and beta cells only. No description of pancreatic islets of alligators or crocodiles has appeared so far.

v. Birds

Lacy (110) described alpha and beta cells in the pancreatic islets of chicken, which was confirmed later on by Sato et al. (74). The sub-classification of alpha cell into A_1 and A_2 cell types as demonstrated histologically (111) in the pancreatic islets of chicken and duck, however, remains to be established. Delta cells have also been reported in the pancreatic islets of sparrow (80).

vi. Mammals

In contrast to very little information concerning islet cytology in non-mammals particularly lower vertebrates, there is relatively much published work available in the case of the mammalian species. The presence of alpha and beta cells (74, 76, 77, 83, 110, 115, 119) and of delta cells (113, 123) to a great extent has been well established in mammalian pancreatic islets.

The identification of islet cells was accomplished in a number of species by utilizing serial sections of the islets (76, 77, 83) and a difference in the ultrastructure of the secretory granule

was indicated. Two granular cell types with ultrastructure features well distinguished from the beta cells were observed in the pancreatic islets of obese-hyperglycemic mouse (82) and identified as A_1 and A_2 cell types. One of them was provided with complete cell membrane while the other appeared to be arranged in syncitium (82).

Extensive electron microscopic studies on the pancreatic islets have been carried out in rat (65, 74, 77, 113), guinea pig (74, 77, 83), rabbit (77, 114, 115), mouse (82, 115), cat (74, 83, 114), dog (74, 77, 116), and man (110, 117 - 119). Other mammals studied for the ultrastructure of pancreatic islets include hamster (114), pig (74), horse (120) and monkey (121, 122).

3 (b) Comparative Ultrastructure of Islet Cells.

The electron microscopic observations on the pancreatic islets of a number of species have shown that the islet cells, particularly alpha and beta cells display certain characteristic features (76, 77, 82, 83, 113). Continuous plasma membranes surround individual cells and the nuclei of various cell types, which are mostly spherical but sometimes ovoid as in rabbit islets (77, 114, 115) and usually show double membranes.

The cytoplasm of the beta cells contain many secretory granules, consisting of a central electron dense material contained within a smooth surfaced sac, and separated from it by a clear space (76, 77, 83). Marked species variation occurs in the beta granule morphology. Mostly beta granules are round and homogenous as in rat and rabbit (65, 74, 77, 113 - 115) but sometimes the granules are disc shaped, made up of rectangular crystalline plates as in dog (74, 77, 116), disc, lens or spindle shaped composed of dense prismatic structures as in cat (74, 83, 114), angular crystals as in chicken (74, 110), oval shaped as in hag-fish (85) or round irregular bar shaped as in man (110, 117 - 119). The granular core of the beta cells is of irregular opacity in most of the species and there is general belief that the overall density is less than that of the alpha cells (74, 76, 77, 113 - 119) with the

exception of mouse (82) and man (119) where the densities of alpha and beta granules are approximately equal.

The beta cells have been shown (77, 83) to have more mitochondria, better developed endoplasmic reticulum and more RNA particles than the alpha cells. It has been pointed out that the fine structure of the beta cells within a single islet may vary with the degree of activity of these cells (82).

The alpha cells have been characterised by the presence of numerous secretory granules of more electron density than the beta granules (74, 76, 77, 113 - 119). These granules appear uniformly dense and homogenous structures, round to ovoid in shape. Species differences in alpha granule morphology have not been observed (74, 75, 77). The cytoplasm of alpha cells contain small numbers of ovoid, elongated or rod shaped mitochondria in most of the species including rabbit, dog (77, 75) and teleost fish (72, 95) and usually do not show any significant difference in the appearance and relative localisation from those present in the beta cells. The Golgi apparatus is prominent in both types of cells in most of the species but in lesser proportion in the alpha cells than in the beta cells (74). Golgi apparatus is usually formed by vacuoles, saccules with frequently dilated poles, and vesicles, some of which are filled with a moderately homogenous material (72).

Most recent studies (123) suggested the presence of three sub-types of alpha cells, based upon the size of the secretory granules and their relationship to the encompassing membrane sacs. Whether these sub-types represent different stages in the life cycle of the alpha cells or are distinct cell types is not known.

The C cells, observed in the guinea pig (54) have been characterised by the absence of distinct secretory granules and relatively few organelles in the cytoplasm (77, 123). These were termed as agranular cells in a number of species (95, 87, 71) but were also suggested to represent degranulated alpha cells (75, 77) and sometimes have been regarded as beta or 'clear' cells (74) in some other species.

The electron microscopic observations in the pancreatic islets of rat (113), guinea pig (123), rabbit, dog and opposum (75) have characterised the D-cells as the cells containing a homogenous population of secretory granules with low electron opacity and a concentration of the granules at one side of the cell.

Recent studies with electron microscope (75) have confirmed the presence of the 'X' cell in the pancreatic islets, observed by light microscope in the uncinate process of dog pancreas (57). The presence of secretory granules of low electron opacity appearing as kidney shaped, U-shaped, triangular, rectangular or round granules within the individual cells characterises these unique cells (75).

4. Islet cytological changes in diabetes

In vivo studies have established that administration of a variety of chemical agents, e.g. alloxan (124 - 126), a number of hormones, e.g. growth hormone, cortisone and adrenocorticotrophin (116, 127, 128), hypoglycemic agents, e.g. tolbutamide (129 - 131) in single or repeated doses produces certain cytological changes in the pancreatic islets of a number of mammalian species and these changes have been considered to be associated in some way or another with hyperglycemia or hypoglycemia produced by these agents.

Selected necrosis of the pancreatic beta cells following the administration of alloxan have been demonstrated in teleostean species including catfish (93), toadfish (94) and Cottus scorpius (17) and degenerative morphological changes in the beta cells have been described. The pancreatic beta cell cytology in fish (71), lizard (108) and rabbit (124 - 126) following the administration of alloxan has also been studied with the electron microscope. Alloxan has been seen to produce a vacuolization of the cytoplasm and clear signs of nuclear necrosis (71, 108, 124). In the beta cells of alloxan treated rats the secretory granules were apparently well preserved (132). In alloxanised lizards the granule membranes in the beta cells were disrupted (108).

Prolonged treatment with growth hormone in dogs can eventually result in severe irreversible diabetes concomitant with progressive dysfunction of the beta cells (127). Earlier studies (133, 134) found out that the administration of anterior pituitary extracts caused partial or complete degranulation of the beta cells of the pancreatic islets, the later stages of which were associated with the development of hydropic degeneration of the beta cells. Electron microscopically it was observed that after the administration of growth hormone, the beta cell degranulation is characterised by loss of the membranous sacs and their contained bars (116) as well as visualization of glycogen as aggregates of electron-dense granules in the cytoplasm. Frequently mitochondria in some of these beta cells have been observed to become swollen and vacuolated and eventually disintegrated (116).

Cortisone administration causes pancreatic changes like those seen after growth hormone in a variety of animal species (128). It was shown that the administration of cortisone or hydrocortisone for up to four months produces hypertrophy of the pancreatic islets and beta cell degranulation as well as vacuolization of beta cells (135). Lazarus and co-workers studied with the electron microscope the progressive changes in the beta cells after prolonged treatment with steroids in rabbits (128) and demonstrated the loss of electron-dense material from the cytoplasmic sacs very shortly after the appearance of hypoglycemia. During prolonged treatment and in the presence of severe hyperglycemia the glycogenization of the beta cells was observed to be very intense (128).

It has been demonstrated unequivocally that the prolonged administration of sulfonylurea derivatives produces degranulation of the pancreatic beta cells as in rabbits (130), rats (129) and guinea pigs (131). The time of onset of degranulation in different species varies considerably. Histological changes in the pancreas after administration of tolbutamide closely resemble those after glucose (76). Electron microscopic studies demonstrated that the beta cells of rats treated with sulfonylurea derivatives for several weeks showed increased activity as

evidenced by hyperplasia of the Golgi apparatus and enlargement of the mitochondria (129, 130). The electron microscope observations in rats following the administration of tolbutamide indicated that the same morphologic mechanism of beta cell secretion occurs as is found in normal beta cells and in beta cells stimulated by hyperglycemia (129). It is generally agreed that the morphologic effects of chronic administration of sulfonylurea derivatives is a reversible process and despite degranulation no degenerative changes of beta cells occur (76, 129 - 131). It has also been clarified by electron microscopic studies that degranulation of the beta cells after sulfonylurea administration is a primary effect of these drugs and not secondary to any hypoglycemia which may be induced (130).

B. Metabolic Studies

It has been well established that the major function of the pancreatic beta cell is to synthesize, store and release insulin. In relation to these activities of the beta cells, with particular reference to the factors controlling the insulin secretion, current knowledge is briefly reviewed here.

1. Insulin Synthesis

Insulin is a protein which is presumably synthesized relatively rapidly, is readily isolated and purified and whose structure is known (10). Its biosynthesis is regarded as following the generally accepted mechanism for the synthesis of any protein involving polyribosomes (23, 136). Electron microscopic observations have revealed certain changes within the ergastoplasm during synthesis of insulin, showing thereby that ergastoplasm is the locus of formation of beta granules (76, 110, 129, 130). This is also supported by the findings that microsomal fraction is the primary site of insulin synthesis when incorporation of tritiated amino acids *in vivo* and *in vitro* was studied in fish islet tissue (136).

Studies of insulin biosynthesis have been made by incubating mammalian pancreas (137) or fish islets (136, 138) in the presence of

radioactive amino acids in vitro, followed by subsequent isolation of of labelled insulin. Incorporation of amino acids into insulin corresponding to the structure of the molecule was shown in rat pancreas (137) but was found to be lacking in methylthiouracil-treated rats. In these experiments (139) the incorporation of ^{14}C-tryosine specific activity in the A chain was considerably less than in the B chain. The findings indicated that insulin was not synthesised entirely from free amino acids and it was suggested, therefore, that it forms at the expense of additions to, and rebuilding of, their precursors possibly at the macromolecular level (139). Uneven labelling of insulin was also noted in goosefish islet tissue incubated in vitro (138).

A unique problem in the biosynthesis of insulin is the manner in which the two polypeptide chains connected by disulfide bridge are made. Current evidence (138) favours the concept that two insulin chains may be synthesised independently and combined by the enzymatic oxidation of the sulphydryl group (140). Glutathione-insulin transhydrogenase found in fish islets (136) may serve as insulin zipase as suggested by Lazarow (140).

2. Insulin Storage

Within the beta cell insulin appears to be located in granules that either contain or consist of stored insulin (9, 76, 77, 110, 114, 117). The fact that degranulation occurs after hyperglycemia (8), steroids (128), growth hormone (116, 127, 133) and sulfonylureas (129 - 131) with a corresponding decrease of extractable insulin from the pancreas (9, 31) supports this view. Secretion granules containing stored insulin have been isolated from fish islet tissue by differential centrifugation (140). The membranous sac surrounding the granule is probably lipo- and glycoprotein in nature since it can be completely dissolved with saponin or deoxycholate (136).

Since insulin forms insoluble complexes with zinc it was suggested that it is stored in the beta granules in this form (141).

3. Insulin Secretion

Lacy and co-workers (76, 110, 129) have described the sequence of events occurring during the release of secretory granules from the pancreatic beta cells following its stimulation by tolbutamide administration, studied with the electron microscope. The process, termed emicytosis, involves margination of the beta granule, followed by fusion of the sac enclosing the granule with the plasma membrane of the beta cell which results in rupturing of the sac and release of granule contents directly into the extra-cellular space (110, 129). The cytoplasm between the two granules liberated close to each other appears as a 'bulge' at the surface of the cell, forming a microvillus (110, 129).

Support for this type of mechanism of release of beta granules has been demonstrated by electron microscopic studies in mammalic pancreatic islets, following hyperglycemia (76) produced by the administration of glucose and following treatment with tolbutamide (76, 110, 129 - 131), growth hormone (116) and cortisone (128). Whether the pancreatic beta cells of man or teleost fish secrete the granules by this method is unknown. It has been repeatedly emphasized (76, 110, 129, 130) that before liberated insulin reaches the circulation it must traverse several membranes. The significance of changes in these membranes in the regulation of insulin release is currently unknown.

4. Regulation of Insulin Secretion

i. Stimulatory factors of insulin secretion

Glucose has been observed to stimulate insulin secretion by many workers over a long period of time. Earlier studies, using isolated perfused pancreas *in vitro* (142) as well as *in vivo* (143) or perfused pancreas *in situ* (144) demonstrated a prompt hypoglycemia in response to a variety of sugars, suggesting stimulation of insulin secretion. Increased plasma insulin activity was measured in these experiments (142, 144) as well as in man (145) and in dog, corresponding to the increased concentration of glucose (144).

Development of specific and precise assays for insulin and better *in vitro* preparation of perfused pancreas helped Grodsky and co-workers (146, 147) to demonstrate that insulin secretion is directly and continuously stimulated as a function of glucose concentration and is not simply a threshold effect in which insulin is secreted completely at a critical glucose concentration. A relatively simple method, involving incubation of peices of pancreas *in vitro*, was developed (148) and a study of the effects of various factors on insulin release, again in conjunction with biological or immunological methods of insulin assay, was made (148, 149). Refined techniques using perfused pancreas (150) and isolated pancreatic islets (151) have recently been used to study the regatory factors of insulin secretion *in vitro*.

All of these studies have confirmed that the normal stimulus for insulin secretion is the concentration of glucose perfusing the pancreatic islets (146 - 148).

Besides sugars, a large number of factors have been reported to stimulate or to influence the stimulation of insulin secretion by glucose (146 - 160). *In vitro* studies (146, 149) showed that growth hormone neither stimulated insulin secretion at low glucose concentration nor inhibited at high concentration of glucose. On the other hand, it was found, using a ligated rat pancreas preparation *in vitro* (152) that bovine growth hormone (100 µg/ml) when added to preincubation medium containing glucose (4 mg/ml), caused a significant increase in the release of insulin, although no effect was obtained at a glucose level of 1 mg/ml. A number of other hormones were employed to observe the stimulation of insulin secretion and these include glucagon (148, 151, 153 - 155), epinephrine (149, 154), cortisone and ACTH (155) and insulin (153).

In vivo studies in man as well as in experimental animals showed tolbutamide and other sulfonylurea derivatives to enhance insulin secretion (156, 157) but *in vitro* studies (148, 157) indicated that they accelerated insulin secretion at low glucose concentration but not at high glucose concentration. Also, specific degranulation of beta cells was not

observed (158) after incubating rat islets with glucose or with tolbutamide.

Insulin secretion has also been shown to increase when incubated in vitro with Adenosine 3-5-phosphate (155) or Adenosine triphosphate (159). Citrate and β-hydroxybutyrate were found to stimulate the release of insulin in vitro (160). On the other hand these were found to be without effect in another study in vitro (149).

Reports about the possible role of the nervous system in insulin secretion have appeared (161).

(ii) Specific structural action of glucose on pancreatic islet tissue

Studies designed to determine whether sugars other than glucose are capable of stimulating insulin secretion have yielded conflicting results. Mannose was found not to induce the release of pancreatic insulin in earlier studies (143, 145) but later studies showed it to be active (147), or almost as effective (149) as glucose in stimulating insulin release in vitro. Moreover, comparison of metabolism of different hexoses in fish islets showed (22) that glucose and mannose were utilised at approximately the same rate. Recently measurement of plasma insulin in normal and hyper-insulin responding subjects showed (154) mannose to be an effective stimulator of insulin secretion in man.

Fructose was found to be utilised by fish islets (22, 162) at a much slower rate than glucose. Metabolism of galactose has been shown (162) not to occur at all. In in vitro studies fructose was only partially (147, 157) or insignificantly (149) effective in stimulating the insulin release.

The isometric structure of glucose at carbon atom 1 - 3 apparently is not required to stimulate insulin secretion since in vitro studies with pieces of pancreas (149) or perfused pancreas (147) or isolated pancreatic islets (151) have shown that D-Galactose (147-149), xylose and L-arabinose (147) are ineffective although they are structurally identical to glucose in this portion of the molecule. In vitro experiments also indicated that insulin release was not accelerated by

3-O-methyl-D-Glucose, D-fructose, D-ribose, D-2-deoxyglucose, N-acetyl glucosamine (147, 163).

(iii) Glucose uptake by pancreatic islet tissue

Pancreatic islets do not appear to have a barrier to the entrance of glucose. This has been shown in fish islets (162) where glucose freely diffused resulting in a rapid equilibrium of extracellular and intracellular glucose similar to that seen in the liver. Free diffusion into the mammalian islets is also indicated by the failure of phlorrhizin (149) or high concentration of non-metabolized sugars (147) to inhibit glucose action in the pancreatic islets. The observation that phlorrhizin (a known inhibitor of membrane transport for sugars in mammalian cells) does not prevent glucose from accelerating insulin release at high or low glucose concentrations suggests that the beta cells, like the liver cells, may be freely permeable to the sugars (149).

(iv) Product of glucose metabolism as a regulatory factor

Most of the sugars that were found not to stimulate insulin secretion have been shown to be non-metabolized whereas those which stimulated or seemed to stimulate insulin secretion were readily metabolized in the pancreatic islets of fish as well as mammals (22, 147, 149, 151, 162, 163). It was discovered that glucose stimulation of insulin secretion is blocked by inhibitors of its metabolism e.g. D-mannoheptulose (163, 164), glucosamine (165) and 2-deoxyglucose (166). On the other hand, the inhibitory effect of 2-deoxyglucose on insulin release induced by glucose did not occur at all (149).

D-mannoheptulose has been found to suppress the stimulating effect of a high glucose concentration on insulin secretion in vivo (164) as well as in pieces of rabbit pancreas incubated in vitro (163). Recent studies using isolated pancreatic islets (151) and pancreatic islets cultured in vitro (167) have confirmed these findings. Mannoheptulose has also been shown to inhibit phosphorylation of both glucose and mannose as well as glucokinase and hexokinases in rat liver slices (168). Mannose is known to be phosphorylated by both glucokinase and hexokinases of animal tissues and has been shown to be readily metabolised beyond the

hexose phosphate stage (149, 168). It has been demonstrated in toadfish that those sugars which stimulate insulin secretion are capable of being phosphorylated by the pancreatic islets (169).

Most of these studies, hence, raised the indication that glucose must be metabolised to be effective and that the stimulatory effects of glucose are in some way related to their phosphorylation and further metabolism. Extensive studies on toadfish islets also confirmed the possibility that insulin release mechanism could be modulated by one of the metabolic products of glucose which appear in increasing amounts as a consequence of increased rate of glucose utilisation (162). The possible pathway of glucose metabolism in the pancreatic islets which ultimately provides the substrate or co-factor that triggers insulin release is unknown (147, 149, 151, 162, 163).

(v) Glucose metabolism within the pancreatic islet tissue

Histochemical staining techniques were applied to the pancreatic islets to study the activities of certain enzymes involved in the metabolic pathways of glucose. Lazurus' histochemical investigation demonstrated, in addition to certain phosphatases (170, 171, 172), the presence of some of the enzymes in the beta cells, involved in the oxidative pathways (173). Later on, a number of similar studies were carried out in the pancreatic islets of a variety of vertebrates, including man (174, 175), normal and obese-hyperglycemic mice (176, 177), KK-mouse (178), rabbit (179 - 182), rat (179, 180), dog, horse, chicken, duck and teleost fish (180). Isolated pancreatic islets of guinea pig have also been used to study some of the hydrolytic and oxidative enzymes (183).

Lazarus also studied phosphatases by histochemical procedures in rabbit pancreatic islets following treatment with alloxan (184, 185), cortisone (185, 186), and tolbutamide (184, 186). Similar histochemical studies were also carried out by other workers on the pancreatic islets from rats and rabbits after treatment with alloxan (180 - 182) and cortisone (179) and in rabbits after 8-OH (181). Enzymatic studies

have been made on rat pancreatic islets, after treatment with tolbutamide, synthaline (180) and growth hormone (179), as well as of hypophysectomized animals (179). Other such studies include those in white mouse after injection of dextrose and tolbutamide (187) and in guinea pig after synthaline injections (180).

Lacy, in 1962, was the first to determine quantitatively the enzymatic activities in the pancreatic islets (188). By utilizing Lowry's technique, he recognized and dissected islet samples in microgram range from the unstained frozen-dried sections and then applied micro-enzymatic procedures to such samples. Lacy and co-workers extended such studies to islet samples obtained from rabbit (189, 190), rat (190, 191), obese-hyperglycemic mice (192) and man (190). They also measured these enzymes in glucose-injected rabbits (189) and in tolbutamide-treated rats (191). Lazarow and co-workers, using the same technique, determined lactic and malic dehydrogenases (LDH and MDH) in microdissected islets of normal as well as alloxan-treated rats (162).

Hellerstrom, in 1964, refined the freehand microdissection technique (34) which enabled him to obtain fresh and larger amounts of pure islet tissue than Lacy's method. Since then he and his co-workers have determined a number of enzymes in the isolated pancreatic islets by micro-chemical (35 - 38) or electrophoretic studies (39) in mouse, obese-hyperglycemic mouse and in normal as well as in cortisone-treated rats.

The only quantitative enzymatic study done so far on fish islets is that of Lazarow and co-workers. They have determined phosphatases (162, 193) and a number of enzymes involved in hexose monophosphate (HMP), glycolytic, and tricarboxylic acid cycle (TCA) pathways and have recently published their comprehensive work (23, 162).

Nothing has appeared so far about the quantitative study of glucose-6-phosphatase in the pancreatic islets of mammals.

(a) Glycolytic pathway enzymes in pancreatic islet tissue

Significant hexokinase activity phosphorylating hexoses, e.g. glucose fructose and mannose has been demonstrated (194) in homogenates of toadfish

islets. The affinity for the hexose kinase activity for glucose was smaller than that of high affinity animal enzymes such as rat brain or adipose tissue hexokinase, but greater than that of low-affinity enzyme probably characteristic of rat liver. It was concluded (194) that increases and decreases in glucose metabolism by islet tissue in the presence of increasing and decreasing glucose concentrations are more likely to be the result of changes in the amount of glucose offered for phosphorylation within the cells.

Once phosphorylated glucose readily enters the glycolytic pathways in the pancreatic islets and is eventually converted into lactic acid the percentage yield of which varied from 23 to 90% in teleost fish islets (122). The enzyme catalyzing the hydrolysis of glucose-6-phosphate has been studied only in toadfish islets where its presence has been denied (162, 193). The level of phosphogluco-isomerase was found to be higher in microdissected islets than in exocrine pancreas (190).

Histochemical studies revealed a very weak reaction for 3-phosphoglyceraldehyde in human pancreatic islets (175). The activity of aldolase and 3-phosphoglyceraldehyde dehydrogenase was found to be lower in the principal islets than most of the other tissues of toadfish (162). It was concluded that of the enzymes of glycolysis studied in islet tissue, aldolase would be more likely to limit this metabolic pathway (162).

LDH activity was observed principally in the duct epithelium, exocrine pancreas and alpha cells with a scant staining of beta cells in rabbit pancreas (173). Histochemical studies on pancreases of rat, rabbit (179), normal and obese hyperglycemic mice (176), KK mouse (181) and of man (175) have confirmed weak reaction for LDH activity in islet beta cells but demonstrated a pronounced reaction for this enzyme in A_2 cells of rat, rabbit (179) and guinea pig islets (183). Quantitative measurement of this enzyme in microdissected islets of rabbit (188-190) and rat (190, 194) showed it to be one third of that in exocrine pancreas. Another similar study showed it to be five times less in islets than in exocrine pancreas (191). The enzymatic activity in islets of non-

injected animals was not found to be significantly different from those treated with tolbutamide or saline (191). On the other hand, a significant gradual increase was found (194) in microdissected islets of rat twelve hours after alloxan administration. It was shown that from 72 hours onwards LDH activity started to decline, but even at 168 hours after alloxan administration, the enzymatic activity was still 78% higher than that of controls (162, 194). Some of these studies (190, 194) suggested that islet tissues are metabolically more equipped for glucose metabolism.

Experiments performed in the presence and absence of oxygen showed inhibition of insulin release by anoxia (149, 196) and it was argued that probably glycolytic pathways do not provide the signal for insulin secretion.

(b) The Pentose 'shunt' enzymes in pancreatic islet tissue

A large number of studies have demonstrated the presence of both the enzymes and metabolic functions of the hexose monophosphate shunt in the pancreatic islet tissue (22, 23, 162, 173 - 176, 179 - 181, 188 - 192, 197, 198).

Glucose-6-phosphate dehydrogenase (G-6 PDH) is concerned with conversion of G-6-P into 6-phosphoglucomutase which is the first step in glucose breakdown via HMP shunt. Consequently the high activity of G-6- PDH has been interpreted as indicating the existence of an actively operating HMP 'shunt'. High activity of this enzyme was observed in the pancreatic beta cells of a number of species including rabbit (173, 181), rat (180), mouse (176, 180), guinea pig, dog, chicken (180) and man (175). Another histochemical study on pancreases of rat and rabbit (179) found (179) a less striking difference in the activity of this enzyme between islets and exocrine pancreas. In human pancreatic islets, a weaker and less pronounced reaction for G-6 PDH was observed, as compared to relatively strong reaction in duct epithelium (173, 174). Moderate reaction for this enzyme has also been observed in alpha cells of mouse and rabbit as compared to negative reaction in islets of chicken (180 - 181). The distribution of this enzyme probably varied considerably according to animal species (174, 180).

Quantitative microchemical studies on microdissected islets of rabbits showed that the level of G-6 PDH activity in them is not significantly different from that in exocrine pancreas (188, 189). Similar results were obtained in toad fish islets (23, 162) as well as in human islet tumors (190). The pancreatic islets of normal and obese-hyperglycemic mice, however, were found to exhibit a much higher activity as compared to exocrine pancreas (192). In contrast to findings in rabbit (188,189), this enzyme was found to be much more active in islets than in exocrine pancreas of rats (191).

Another enzyme, 6-phosphogluconic dehydrogenase (6-PGDH), involved in HMP 'shunt' has also been studied in the pancreatic islets of a number of species both by histochemical (173 - 176, 179, 181) and microchemical procedures (23, 162, 188 - 192). A high activity of this enzyme was demonstrated in the islets of human pancreas (175). By microchemical methods, similar activities of this enzyme were found in islets and exocrine pancreas (190 - 191), though another study recorded a significant higher 6-PGDH activity in the pancreatic islets of rabbit (188).

A decrease was observed in G-6PDH activity in the pancreatic islets after the administration of alloxan, dithiozone (181) and cortisone as well as after hypophysectomy (179), though another histochemical study demonstrated the enzymatic activity even at 18 hours after alloxan administration (197). On the other hand, a minor increase was recorded in rabbit islets after seven weeks of growth hormone treatment (179). No difference in G-6 PDH activity was found by microchemical study in micro-dissected islets of normal and tolbutamide treated rats (191). Similar results were obtained from a study on untreated, glucose injected and starved obese hyperglycemic mice (192). On the other hand, repeated glucose infusion to rabbits for 8 days resulted in an increased activity of G-6PDH of both the pancreatic islets and exocrine pancreas (189).

Study of oxidation of specifically labelled glucose to CO_2 by teleost fish islets (22, 23, 162, 198, 199) as well as by human islet tumors (199) has also presented strong evidence for the existence of

an active 'shunt' pathway in pancreatic islets. There are, however, certain reports (149, 198), which argue against this view. P-phenylenediamine and phenanzine methosulphate have been shown (149) to inhibit insulin secretion *in vitro*. Also, it was found out that the oxidation of glucose-1-^{14}C in fish islets was not enhanced by increasing glucose concentration of the medium (198).

(c) Tricarboxylic acid cycle (TCA) enzymes in pancreatic islet tissue

Of all the enzymes involved in the TCA cycle, only succinic-dehydrogenase (SDH) and malic-dehydrogenase (MDH) enzymatic activities have been studied in the pancreatic islets. There is a report of the presence of alpha-ketoglutarate oxidase in toadfish islets (23, 162).

Intense reaction for SDH activity was demonstrated in pancreatic islets as well as in duct epithelium of rabbit (173, 180, 181) and mouse pancreases (176, 180), though earlier, one of these studies (176) had reported a weak reaction in islets. The enzyme activity has also been found to be weak (179) or moderate in islets (174) and more marked in duct epithelium and exocrine pancreas of rat, rabbit, guinea pig and man (180). This was confirmed by another histochemical study on isolated pancreatic islets of guinea pig (183). No difference in reaction for SDH activity was observed in pancreatic islets of normal and obese-hyperglycemic mice (176). SDH activity has been measured by microchemical procedure only in toadfish islets (23, 162) and was found to be among the lowest of the toadfish tissues.

A weak reaction for MDH activity was observed histochemically in pancreatic islets of rabbit (179) and man (175). Quantitative determination of this enzyme revealed a much higher activity, three or four times greater in beta cells of microdissected islets of rabbit as compared to exocrine pancreas (188 - 190). This was, later on, confirmed by the findings in rat (162, 190, 191), normal and obese-hyperglycemic mice (192), duck (200) and man (190). Though MDH activity in toadfish islets was found to be relatively low in comparison with other tissues (23, 162), it seemed to be concentrated within beta cells

and it was suggested that MDH may play a special role in the beta cell metabolism.

After glucose administration, no change (189) or very slight decrease (192) in MDH activity of both islets and exocrine pancreas was found. On the other hand, a minor increase of the enzyme activity in pancreatic islets was reported in starved mice (192). Cortisone, growth hormone and hypophysectomy treatment in rabbits failed to induce any changes in MDH activity of islets when observed histochemically (179). Quantitative determination of MDH activity in microdissected islets of rat showed a progressive marked decrease in the enzyme activity following alloxan administration, and it was concluded that the decrease in the enzyme activity occurred prior to complete disappearance of beta cells (162).

d Phosphatases in pancreatic islet tissue

Though non-specific acid phosphatase has been demonstrated in islets of a number of species by histochemical staining (179, 180, 185, 186) the studies failed to show any obvious correlation between the islet phosphatase activity at PH 5.0 - 5.4 and the functional state of beta cells (185, 186). Activity was greatest at the secretory poles of exocrine pancreatic cells and at the vascular poles of islet cells (186). The enzyme has also been shown by microchemical method to be significantly higher in islets than that of the exocrine pancreas of obese-hyperglycemic mice (35). The enzymatic activity seemed (35) to have two PH optima (3.5 and 5.3). There was no pronounced change in acid phosphatase activity of islets after 7 days starvation (35). The relative activities of different molecular forms within this group of enzymes has been measured after separation by the disc electrophoresis and it was suggested that a real difference between the endocrine and exocrine pancreas with regard to the distribution of enzyme activity between different molecular varieties exists (39).

An attempt has recently been made to localise acid phosphatase at the ultrastructural level in endocrine pancreas of normal (201) and tolbutamide treated rabbits (202), and the relation of this enzyme in secretory mechanism of beta cells has been discussed (202).

A very low activity of alkaline phosphatase, as measured at PH 9.1 in both islets and exocrine pancreas has been demonstrated (35) thus confirming the earlier histochemical findings (178 - 180, 185) of a weak reaction for this enzyme in the pancreatic islets of a number of animal species.

Histochemical staining methods revealed enzymatic cleavage of ATP in the pancreatic islets of rats (179). ATPase is in high concentration in the membrane surrounding insulin granule (203), suggesting a role in the storage or secretion of insulin. The enzyme has also been recorded in islet tissue of cortisone-treated rabbits (179, 203). By microchemical methods, sulphydryl-dependent ATPase with a PH maximum activity of about 9.1 was measured in exocrine and endocrine pancreas of obese-hyperglycemic mice (35). Steroid treatment significantly decreased the activity of ATPase, as did alloxan, while starvation did not bring about any changes of the enzyme (35). Quantitative studies failed to find any difference in the enzyme activity between the islets of normal and obese-hyperglycemic mice (35, 36) but it has been shown to be elevated in the islets of obese-hyperglycemic mice (179). The level of this enzyme, hence, may (171, 203) or may not (35, 36) correlate with the functional activity of beta cells. This enzyme has also been localized at E.M. level in the islets of rabbit (204).

Histochemical staining reactions for enzymatic splitting of Adenosine 5-monophosphate (AMP.5) in the islets revealed intensification of activity after cortisone treatment suggesting that a 5-nucleotidase might be concerned with the synthesis and/or release of insulin from the beta cells (179). Microchemical methods characterized the apparent 5-nucleotidase activity and confirmed the stimulation of AMP.5 - splitting

activity by cortisone treatment (36).

Pronounced activity of another enzyme, G-6-pase was demonstrated in islets of guinea pig, rabbit and dog pancreas (184, 186) and it was suggested that this enzyme might play an important role in controlling the insulin output by the beta cells. The significance of glucose production via G-6-pase is uncertain. Presence of this enzyme has been both confirmed (177, 205) and denied (206) as a result of histochemical studies. It has not been detected in fish islets by microchemical assay (193). Histochemical staining of G-6-pase showed an intense enzyme activity in the islet tissue of the obese-hyperglycemic mice, while only a weak reaction appeared in the islets of lean littermates (35, 177). On the other hand, a decrease of islet G-6-pase-like activity was observed histochemically in rabbit islets after administration of tolbutamide (186).

SECTION II. MORPHOLOGICAL INVESTIGATIONS ON THE PANCREATIC ISLETS OF FISH

Chapter 1. INTRODUCTION

As compared to the mammalian pancreatic islet, relatively few histological and histochemical investigations have been performed on principal islets of teleosts with modern differential staining procedures (17, 58, 72, 92, 93). Principal islets of most of the teleosts have been shown to contain two major granular cell types, alpha and beta cells depending on their respective staining properties. The possibility that there are three kinds of cells has also been proposed (51, 207). In some bony fish, cells of agranular nature have been observed (17, 92, 93, 208). On the other hand, principal islets of Tilapia mossambica have been recently reported to consist of only one cell type, i.e. beta cells (26). Alpha cells in principal islets of Cottus scorpius have been classified into A_1 and A_2 cells (17, 209). There appears to be some controversy regarding the classification of cell types in principal islets of teleosts and the findings are somewhat tentative (72, 91, 94, 207 - 209).

There are some difficulties in the identification of various granular and agranular cells in principal islets even at the electron microscope level (71, 72, 91, 94, 119). Also, the improved image quality provided by thermostable embedments (210) has suggested that further descriptive work on cytology of normal pancreatic islets of a different animal would be profitable. The principal islets of Tilapia mossambica have previously not been studied with electron microscopy.

Moreover, study of structural changes in the secretory cells, under the influence of certain hypoglycemic or hyperglycemic agents, may enable the mode of insulin storage or secretion from the beta cells to be understood, especially when studied with electron microscopy (114, 129). No attempt has previously been made to study the cytological

changes in cellular structure of principal islets of any teleost fish incubated *in vitro*. In *in vitro* experiments, islet viability has been demonstrated physiologically (22).

It was, therefore, proposed to carry out histological and histochemical investigations to study the principal islets of *Cottus scorpius* and *Tilapia mossambica*, and to know their cellular composition; to establish the characterization of different types of cells applying electron microscopy; and to incubate the principal islets of *Cottus scorpius* with diabetogenic agents e.g. alloxan, with hypoglycemic agents, e.g. tolbutamide, with certain hormones, e.g. growth hormone and adrenocorticosteroids, in order to study their effect on the structure of islet cells as observed by light microscopy and, if possible, by electron microscopy as well.

Chapter 2. MATERIALS AND METHODS

i. Animals

a. Fish: (Plates 1 and 2)

Two species - Cottus scorpius and Tilapia mossambica - of teleost fish were used in this study. Cottus scorpius, a marine teleost, is readily obtained from the sea shore in St. Andrews, where investigations on this fish were performed during the summer seasons. The fish were kept in large tanks with continuously running sea water and provided with air pumps. Mostly freshly caught fish were used but sometimes those kept for a couple of days in the aquarium were also used. Asphyxiated fishes were avoided entirely for experiments.

Tilapia mossambica, fresh water teleost, were obtained from the Fisheries Department, Government of Zambia, Zambia during Spring 1966. The fishes were reared in the laboratory from this stock. Adults for breeding were kept in a large tank of fresh-water (Birmingham tap water) at 21° - 24°C and fed daily on maggots (whole) or commercial dried fish food. The young were shaken from the mouth of the adult shortly after the eggs were hatched and subsequently kept in smaller tanks with continuously circulating fresh water maintained at the same temperature as the breeding tanks and regulated by thermostat. Carbon filters were used in the inflow system of water supply to check the variation of chlorine content in the tap water. The water was aerated from a small pump. Commercial baby fish food was given daily to the very young fishes. It was possible to have a large colony of these fishes available for study after a few months. The fishes were kept in different tanks according to their age and body size as it was observed on a number of occasions that baby fish were eaten up by a large-sized fish.

The body weight of Cottus scorpius varied between 80 grams (gms) and 250 gms while that of Tilapia mossambica between 2 gms and

PLATE 1. Cottus scorpius

PLATE 2. Tilapia mossambica

PLATE 3. Adult Obese-hyperglycemic mouse (with a recessive trait for hyperglycemia and obesity from the strain originating at the Roscoe B. Jaakson Memorial Laboratory, Bar Harbor, U.S.A.).

18 gms. Adult fish of both sexes were used in this study. In the case of Tilapia mossambica most of the experiments were performed on the young fishes from 5 to 10 weeks after hatching. At this time they weighed between 0.5 and 3.5 gms.

b. Mammals

Normal albino mice and obese-hyperglycemic mice were used in the present investigation. The former were obtained at the time of experiment from the animal stock of the Departmental Animal House, maintained by a technician. A pair of obese-hyperglycemic mice was requested from the Department of Animal Genetics, University of Edinburgh, Edinburgh during the autumn of 1966 and were bred according to the instructions received from that laboratory. A large colony of such mice was obtained after a few months, (Plate 3).

I am grateful to Dr. D.S. Falconer of the Institute of Animal Genetics, University of Edinburgh, Edinburgh for providing me with a pair of obese-hyperglycemic mice and to the Animal House Technician for breeding them and excellently maintaining their colony.

ii. Preparation of tissue sections for light microscopy

a. Preparation of paraffin sections

Fixation: Fixation of the tissue was carried out generally in Bouin's fluid for about 24 hours. Other particular fixatives for pancreatic islet tissue were the following, depending upon the method of staining procedure applied to the sections afterwards:

Zenker-formol, Helly fluid, Susa, Carnoy's fluid and Lisberg's fixative (211).

Wherever required (after Zenker-formol and Helly's fluid fixation), the tissue was washed for about 24 hours in running tap water before subjecting to dehydration and clearing. Both these fixatives required treatment of the sections with Lugol's iodine in 70% alcohol to remove mercury deposit. Fixed specimens were either transfered to auto-technican (Shandon Scientific Co., London) or proceeded individually for dehydration, clearing and embedding of the tissue material.

Dehydration: 70% Alcohol : 1 change for 2 hours

90% Alcohol : 2 changes for 1½ hour each

Absolute Alcohol : 2 changes for 1½ hour each

Clearing: Benzene and Absolute Alcohol (1:1) : 1 change for ½ hour

Benzene : 1 change for 1 - 1½ hr.

Embedding: Paraffin wax : 2 changes for 2 hours each

The tissue pieces were then placed in a third paraffin bath in the oven for 2 - 4 hours before final block was made. Paraffin wax, melting point 56°C was used and was filtered prior to use. On certain occasions overnight impregnation was carried out and it appeared not to injure the tissue.

In some cases, Dioxan was used for dehydration and clearing but it was found to be unsatisfactory.

Sectioning: The tissue blocks were sectioned at 4 - 6 μ, using a Rotary or Cambridge rocking microtome. A well sharpened knife was used to obtain sections. The knife was sharpened, with microscopic control, on a flat glass or on an Automatic Knife-Sharpener (Shandon Scientific Co., London), using carborundum Alloxite 50 as abrasive, and finished on a fine leather strap.

The sections were picked up on acid-cleaned slides (thickness 0.8 - 1.0 m.m.), coated with adhesive albumen., which were flooded with water and then warmed on a hot plate (40°C) to flatten the sections. The slides were drained of excess fluid and then dried, first on a hot plate at 45°C for 5 - 15 minutes and then in an oven at 45°C for overnight duration.

b. Preparation of frozen sections.

The fresh tissue mounted on a chuck was quenched in isopentane, chilled (-160°C to - 180°C) with liquid nitrogen. Frozen tissue was stored in liquid nitrogen until required for sectioning but was not kept for more than 24 hours. Sections were cut at 6 μ using a freezing microtome (Pearse cryostat). Frozen sections were either picked up from the knife straight on to the cover slip or mounted on glass slides.

They were air dried for 30 minutes to prevent later fixation shrinkage. All sections were post-fixed at room temperature for 3 minutes in 25% Glutaraldehyde (212). Fixation of frozen sections was also attempted in cold Bouin's fluid, 5% formaline and 2% Glutaraldehyde in 0.1 M cacodylate buffer PH 7.0.

iii. Staining Methods

a. Staining Methods for Paraffin sections

Hematoxylin-eosine was used as a routine stain. Following special differential staining procedures were applied to the islet tissue sections:

Aldehyde fuchsin (A.F) method (Gomori 1950) in different modifications (213 - Halmi, 1952; 214 - Gabe, 1953; 215 - Dixon et al. 1960). The sections were counterstained with Ponceaue fuchsin or Ponceaue de-Xylidine or Phloxine.

Heidenhain's Azan (Ferner, 1952).

Periodic Acid Schiff Tricheome Stain (216 - Lazarus, 1958).

Chrome-Alum hematoxylin (Gomori, 1939; Bencosme, 1952) counter-stained with Phloxine, Ponceau fuchsin and Cason's trichrome stain.

Chromotrope-Phosphotungstic acid - trypan blue stain (211 - Lisberg, 1961).

Phosphotungstic acid/Hematoxylin (217 - Levene, 1964).

Tannic acid/Basic fuchsin/Iron hematoxylin/Alcian blue (218 - Monroe and Spector, 1963).

Phloxine/azur/Hematoxylin (219 - Maldonado and Jose, 1967).

Silver impregnation methods as modified by Hellerstrom and Hellman in 1962 (61) and Grimelius in 1964 (220) for argyrophilia.

Pseudo-isocyanin (PIC) method for insulin demonstration, as modified (221) by Coalson in 1966.

Levine and Glenner's method (1958) to demonstrate protein rich in trypto-phan.

For nucleic acids: Methyl-Green-Pyronin Y (Kurnick, 1955).

Gallocyanine-chrome alum method (Einarson, 1951) and Feulgen's method.

The staining properties of islet cells were studied (222) usually by two, three or sometimes four successive steps on the same or adjacent sections: i. Toluidine blue, and for ii. silver impregnation (Toluidine blue removed by 70% alcohol), iii. Pseudo-isocyanin (silver removed by preoxidation), iv. Chrome-Alum Hematoxylin and Phloxine or Aldehyde fuchsin with one or another of the counterstains, though sections treated in this manner were not suitable for photomicrography, they were useful for investigation. References cited above and not occurring in the reference list are found in Pearse, 1960 (223).

b. Frozen section staining.

Post-fixed frozen sections were stained with freshly prepared Aldehyde Fuchsin for 12 - 15 minutes after oxidation with acidified $KMnO_4$ for five minutes (212).

Coalson's Pseudoisocyanin method (221) was also attempted to stain the beta cells in the frozen sections.

iv. Preparation of sections for electron microscopy

Fixation.

The principal islets were fixed in one of the following fixatives: Buffered Osmium tetroxide (OsO_4)(224). Veronal acetate buffer was prepared with sodium barbitone (2.94 gms), sodium acetate (hydrated) (1.94 gms) made up to 100 ml. with distilled water. 5 ml. of this solution was added to 12.5 ml. of OsO_4 solution (2%; obtained by dissolving OsO_4 crystals from ampoules in distilled water), followed by the addition of distilled water (2.5 ml) and 0.1 N-HCl (5 ml). The PH of the fixative was adjusted with HCl to 7.4. Finally, sucrose (0.045 gms/ml fixative) was added to it (224). The fixative was kept in glass, stoppered bottles in the deep freeze and thawed out before use.

Buffered glutaraldehyde (226; personal communication from Prof. S. Falkmer, Umea University, Sweden). 6.25% glutaraldehyde in 0.067 M phosphate buffer, PH 7.4 was used (PH adjusted with N-NaOH). Special care was taken to use the glutaraldehyde (25% stock solution); the PH of which was not less than 3.5 and the solution was water-clear, not straw-coloured.

Also, the stock solution, before using to prepare the final fixative, was shaken with some activated carbon and filtered thoroughly. The fixative was kept in the fridge when not in use.

In many cases post-fixation was carried out. After fixation in glutaraldehyde fixative the tissue material was washed twice in cold tris-maleate buffer (0.1 M buffer in 7.5% sucrose, PH 7.2), was left in the fridge overnight and then re-fixed, as above in 2% buffered OsO_4 for two hours.

Fixation procedure.

After opening the abdominal cavity in the still living fish, a few drops of cold fixative were put in the islets *in situ*, the islets were cut (still *in situ*) and more cold fixative was put in so that the fixative should penetrate inside the rather tough connective tissue capsule, and lastly, the islets were dissected out. In *Tilapia mossambica*, the islets were very small in size, so the whole procedure was carried out under a dissecting microscope. The islets along with the exocrine pancreas, already bathed by the cold fixative, were removed, placed in a petri dish containing the cold fixative, and islets were then isolated from the surrounding tissue. The excised islets were cut into small pin-head sized pieces which were transferred into small, wide-mouth vials containing fresh cold fixative and were kept for fixation.

The islet pieces were fixed in OsO_4 and glutaraldehyde for two and four hours respectively and kept at $4^{\circ}C$ in the fridge during the fixation period. The specimen that had to be left for some time before dehydration and embedding procedures were completed was usually left in 70% ethanol at $4^{\circ}C$ in the fridge.

Dehydration.

The fixed material was washed with veronal acetate buffer (after OsO_4 fixation) or trismaleate buffer (after glutaraldehyde) for about five minutes in cold solutions. It was then dehydrated in ethanol (Analar) series (for 5 minutes each in 50% and 70% ethanol, 10 minutes

in 90% ethanol, and twice for 15 minutes in absolute ethanol). The dehydration period was kept as short as possible to reduce shrinkage to a minimum. The whole procedure was carried out in the same glass stoppered bottles.

In some cases, the tissue material was subjected to transitional solvent, 1, 2-epoxy-propane, between ethanol and epoxy resin mixture. The tissue specimen was placed first in the mixture of ethanol and 1, 2 - epoxypropane (1:1), then in epoxypropane and finally in the mixture of 1, 2-epoxypropane and epoxy resin mixture (1:1) for 5 - 10 minutes in each case.

Embedding.

The tissue material was embedded in epoxy resin, Araldite (224). The preparation of the epoxy embedment was carried out in the following way:

Mixture A:	Epon 812	62 ml.
	Dodecenyl succinic anhydride (DDSA)	100 ml.
Mixture B:	Epon 812	100 ml.
	Methyl nadic anhydride (MNA)	89 ml.

Mixtures A and B were taken (7:3) in a dried bottle and 0.15 ml. of Benzyl-dimethylamine, the accelerator, was added, and were mixed thoroughly on a rotary mixer.

For embedding the tissue material in epoxy resin, a gelatin capsule (zero size) or thick sheath of polythene, on one side of which a number of small round grooves (depth 0.3 m.m. approximately, and diameter 0.8 - 0.9 m.m.) with narrow bottoms were made with a drill, was used. The grooves or suitably supported gelatin capsules were filled with a small quantity of the epoxy resin mixture and tissue specimens were transferred rapidly with a minimum adhering liquid into them. The use of polythene sheaths for embedding made the tiny specimens convenient to handle while transferring in the epoxy resin mixture and particularly so when a number of tissue specimens required embedding. After about half

an hour the tissue specimens were transferred into fresh epoxy resin mixture. When they had sunk to the bottom, they were transferred for final blocking into the capsule or groove containing fresh epoxy resin mixture and were left for 48 hours or so in an oven at 10°C to polymerise.

Sectioning:

The gelatin capsules were removed after softening for a few minutes in warm water. The Araldite blocks made in the grooves of the polythene sheath were removed by twisting down the two sides of the sheath. The block, mounted on a chuck, was trimmed with a sharp razor blade to get the right shape of pyramid. The sections were cut with glass knives on either Reichert's ultramicrotome (Reichert, Austria) or LKB 'ultrotome' (LKB-Produkter Ab., Stockholm). Grey to silver (600 - 900 Å) sections were obtained which were floated on water in the trough made up of adhesive tape and sealed with wax to the glass knife. The sections were picked up on specimen grids, naked or coated with a thin film of amorphous carbon. Picking up of the thin section was done by taking the grid in fine tipped forceps and bending its edge slightly so that it could be laid flat upon the surface of the fluid in the trough and after laying it across the section, the grid with the section was pulled away. The sections were thoroughly dried for about 15 minutes avoiding any contamination.

This was followed by an adjacent thick section cut at 1 μ and picked up on a clean, well dried microscope slide. The sections were dried on a hot plate at 45°C.

Staining:

The thin sections were usually stained either with lead citrate (224) for about 10 minutes or with saturated (aqueous) uranyl acetate (224, 226) for about 30 minutes. The sections on the grid were stained by floating them, with the section downwards, on the surface of the staining solution. After the staining was complete according to the schedule, it was washed with distilled water and dried completely before examining in the electron microscope.

The thick sections were studied either after staining or without staining. Unstained sections were examined with a phase microscope. The staining of the thick sections was done by either of the following methods:

Munger's modification (246) of the aldehyde-thionin-hematoxylin-Phloxin B stain of Gomori, 1941.

Toluidine blue (1%) dissolved in 1% borax (226). Sections stained for about two minutes at 85°C.

The stained sections were studied with the light microscope and the photomicrographs taken were matched with the electron micrographs of the thin sections to identify different types of islet cells.

v. In vitro Experiments.

A few experiments were carried out in order to establish the suitable conditions for incubation of principal islets of Cottus scorpius. It included incubation of islets in different incubation media for different periods of time, followed by cryostat sectioning and staining of the islets and careful examination of morphological appearance of the islet cells.

Procedure:

About one ml. of incubation media (Kreb's Ringer Bicarbonate buffer - Umbreit 1949) was pipetted out in 5 ml. conical flasks, completely sterilised, capped with sleeve-type rubber stoppers and which were individually gassed with O_2 : C_2 (95:5) through stainless steel needles. All the flasks were placed in a shaking water bath, the temperature of which was maintained at 37°C (except for the islets incubated in alloxan). Principal islets of Cottus scorpius were dissected out very soon after the abdominal cavity was opened in the still living fish, and were placed in a small dish containing chilled incubation media. One of the two islets is situated in the mesentery near the spleen, the other is located close to the intestinal wall near the pylorus and the bile duct. The connective tissue capsule was carefully removed, making use of a dissecting microscope.

The dissected islets were pooled out and were placed in the conical flasks (two islets in each) containing the incubation media. Two islets were taken out each after one, two and four hours of incubation, frozen on chucks, as described earlier, for cryostat sectioning. The frozen sections of these islets were stained with A.F.(212) or hematoxylin/eosin and were examined very carefully to test their morphological appearance as a convenient reference index for overall tissue viability.

The following incubation media were also tried:

Cottus Ringer (Personal communication from A.J.Matty).

NaCl 7.8 gm/l; KCl 0.18 gm/l; $CaCl_2$ 0.225 gm/l;

$MgCl_2.6H_2O$ 0.204 gm/l; $NaHCO_3$ 0.084 gm/l.

$NaH_2PO_4.2H_2O$ 0.06 gm/l; Glucose 5.0 gm/l.

Marine Teleost Ringer (225)

NaCl 12.0 gm/l; KCl 0.6 gm/l; $CaCl_2$ 0.25 gm/l;

$MgCl_2$ 0.20 gm/l; $NaHCO_3$ 0.068 gm/l

KH_2PO_4 0.068 gm/l; Glucose 0.5 gm/l.

Incubation of islets with different agents:

Once the suitable conditions for incubating the islets were established, experiments were designed to incubate them in different hypoglycemic and hyperglycemic agents. The following pattern was used for these experiments.

Experiment 1, 2. (incubation for one hour)

Flask 1, Flask 2, Flask 3 — six islets pooled out → Fixed for L/M → Bouins fixation (2 islets); Helly fixation (2 islets)

six islets pooled out → Fixed for E/M

Flask 4 - two islets one each fixed for L/M and E/M

Flask 1, 2, 3 contained the required agent or hormone under study while flask 4 contained no agent or hormone and hence islets incubated in it served as controls.

Another two pairs of experiments were done as above but incubating the islets for different periods of time (two each for 2 hours and 4 hours.)

In the case of alloxan, however, the islets were incubated for 5, 30 and 60 minutes during experiments 1 - 2, 3 - 4 and 5 - 6 respectively. Also, all these experiments were carried out inside the cold room (+ 2°C to + 4°C).

Concentrations of the drugs or hormones added in vitro to the incubation media during this study were as follows:

drug/hormone	Concentration in the incubation medium
1. Alloxan	2.5×10^{-4} M
2. Tolbutamide	100 mg/100 ml.
3. Growth hormone	10 mg/100 ml.
4. Hydrocortisone	100 mg/100 ml.

The incubated principal islets were processed for light microscopy or electron microscopy as described in (ii) and (iv) of Chapter 2, (Section II). Paraffin sections of the incubated islets were examined critically after staining with A.F. (214), Periodic acid - Schiff Trichrome (216) and sometimes with pseudo-isocyanin.

vi. Photomicrography

Unstained or stained thin sections were examined with a Superscope (Japan Electron Optics Laboratory Co.Ltd., Japan), and with an electron microscope (A.E.I) and electron micrographs were taken at an original magnification ranging from 1,500 to 20,000 diameter, using Ilford film (black and white) or Ilford plates (special Lantern Contrast) respectively. These were then subsequently enlarged on glossy photographic paper (Ilford4) (Ilfoprint LR3.IP).

Photomicrographs of tissue sections were taken on a microscope (Leitz Wetzlar, Germany) with an Automatic Exposure Unit (Vickers Instruments Ltd., U.K.) using black and white (Adox, K.B.14) or a colour film (Agfacolor C.K). Processing of the colour film and printing was done through the Ilford Company.

vii. Measurement of islet cells and their nuclei

Paraffin sections were used to measure the size of islet cells as well as of their nuclei at random in the islet tissue of both Tilapia mossambica and Cottus scorpius. For this purpose, simple methods involving the eye-piece micrometer and the stage micrometer were used.

Chapter 3. OBSERVATIONS AND RESULTS

i. Morphology of the Pancreas

Pancreatic islets of Cottus scorpius correspond closely to the descriptions of teleost fish given by earlier workers (17, 93). The two principal islets are rounded structures lying in the abdominal viscera. Of these, the 'splenic islet' is situated in the mesentery near the spleen and is always the larger of the two in size, with an approximate diameter of 2 - 3 m.m. Its weight varied between 5 - 8 mg. The smaller one, the 'pyloric islet' (approximate diameter 0.6 - 1.5 m.m.) is located close to the intestinal wall near the pylorus and the bile duct. Its weight varies between 0.4 and 1.0 mg. It was observed that larger fish had somewhat larger islets, both in diameter and weight, than small fish. During this study the largest splenic and coeliac islets from a fish weighed 16.4 mg. and 2.8 mg. respectively.

In Tilapia mossambica, the endocrine pancreas is embedded in the exocrine pancreatic tissue which is diffusely distributed in the liver and mesenteries. It is located between the liver and corpus of the stomach as two, three and sometimes four or five 'principal islets'. The position of the principal islets in the animal body is shown in Plate 4. The principal islets of T. mossambica are rounded or ovoidal bodies lying close to one another, with an average weight of 0.6 mg. It was apparent that proportionally large fish had a somewhat larger amount of islet tissue than smaller fish. As compared to the principal islets of Cottus scorpius, these are very small in size with an approximate diameter of 0.3 - 0.5 m.m. Unlike those of Cottus scorpius, the principal islets in T. mossambica appear as a white-yellowish mass and not as separate discrete bodies. Comparatively smaller ones of the four or five principal islets (approximate diameter 0.18- 0.25 m.m.) in Tilapia mossambica have been referred to as 'secondary' islets.

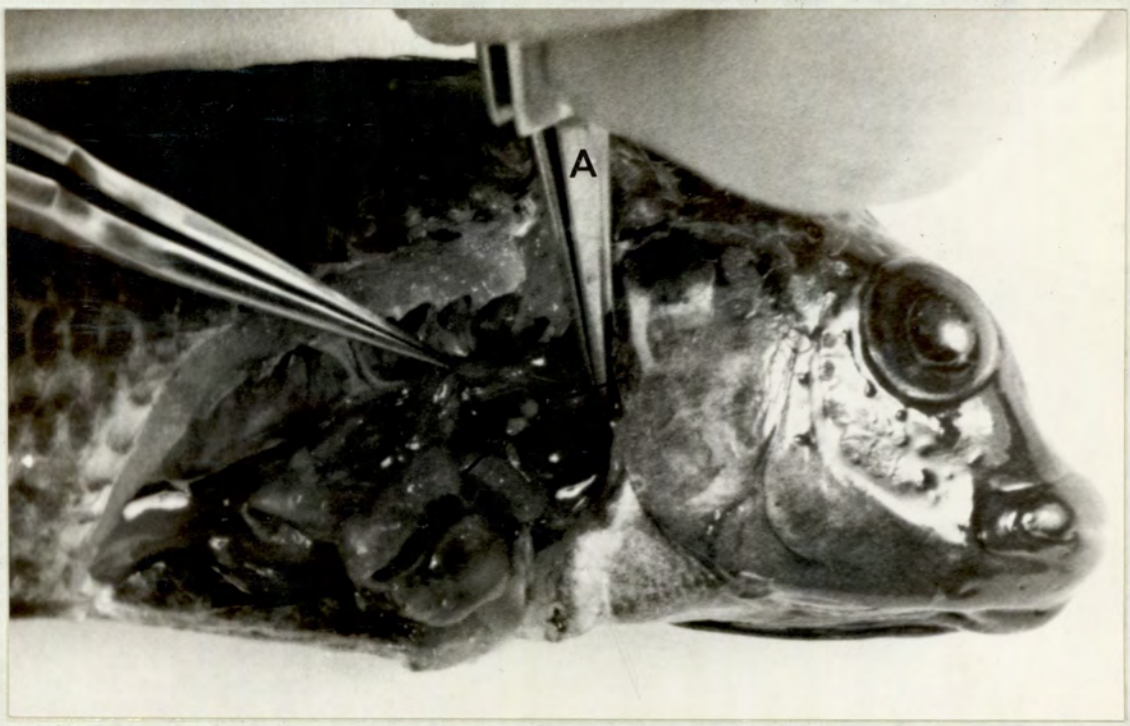


PLATE 4. Tilapia mossambica: Principal islets (arrows) seen lying in the abdominal cavity of the dissected fish (gall bladder lifted up by the forecep l).

The principal islets of Cottus scorpius are surrounded by a thin layer of acinar tissue (Plate 5). On the other hand, those in T. mossambica, as stated earlier, seem to be embedded in the exocrine pancreatic tissue. (Plate 6). In both cases, however, the principal islets are separated from the exocrine pancreatic tissue by a connective tissue capsule, very delicate in the case of T. mossambica. In some cases the connective capsule of two islets was united for a short length without any exocrine pancreatic tissue in between. (Plate 6). The principal islets contain pure endocrine tissue inside the capsule and strands of exocrine pancreatic tissue, as observed in the centre of bullhead fish principal islet (72) were not present. The encapsulating connective tissue can be removed, though not as easily as in the case of Cottus scorpius and enough pure islet tissue can be obtained for microchemical analyses. As reported previously (17), in some cases, small bud-like projections and partly detached minimal islets, outside the capsule enclosing the principal islet may be present in Cottus scorpius.

ii. Light Microscopy

a. Islet histology in Tilapia mossambica.

It was soon found during the preliminary study that formaline and alcohol fixations evoked a severe vacuolisation of the cytoplasm of the cells in the peripheral region, particularly in the case of principal islets of Cottus scorpius. Besides many other fixatives listed under 'Materials and Methods', glutaraldehyde (6.25%) in sea water was tried but complete preservation was not obtained. Out of various modifications of Gomori's aldehyde fuchsin for islet beta cells, those by Gabe (214) and Dixon (215) proved the best. The sections were stained for 2 - 3 minutes in A.F. after oxidation in acidified $KMnO_4$ rather than in Lugol's iodine. Gomori's chrome-hematoxylin did not give good results for differentiation of different cell types. On the other hand, periodic acid Schiff trichrome stain (216) produced comparatively better results, though the distinction between different types of cells with regard to colour of the stains was not clearly defined. Counter-

staining with ponceau-fuchsin proved better than phloxine when used after aldehyde fuchsin.

After staining with a non-specific routine stain, the entire cellular mass of all the islets in Tilapia mossambica gave a uniformly faint reaction (Plate 7); no differentiation of dark and light regions as observed in the principal islet of Cottus scorpius during the present investigation and reported previously (17, 58, 92) was seen in any of the principal islet examined. But, on applying special differential staining methods it was soon found out that the principal islets of Tilapia mossambica contain more than one type of cell diffusely distributed in the islet tissue (Plate 8). This is contrary to the earlier report (26) of only one cell type in the principal islets of this teleost species.

The majority of the islet cells studded with granules in their cytoplasm stained dark purple with aldehyde fuchsin while the rest of the cells took the faintly pink background stain (Plate 8). These cells were present without any definite localisation within the islet cellular mass, though often inside the peripheral rim and in close association with other granular cells. These cells were seldom found singly interspersed but in small groups usually scattered around the capillaries and mostly occupying a greater portion of the islet tissue. Intercellular spaces of any dimension were never observed among these islet cells. The granules of these cells stained steel-blue to black with chrome-hematoxylin, bright red with Mallory azan and faintly violet blue with Maldonado and Jose's modification, but did not show any affinity for phloxine, light green, orange G or aniline blue. The granules of almost all of these cells were metachromatically stained bright red with pseudo-isocyanin (Plate 9). Sometimes the cells appeared very lightly stained, depending upon the degree of granularity and possibly upon the thickness of the tissue section. Beta cell cytoplasmic processes wedged between orthochromatically stained

tissue components were very clearly visible. A striking visual contrast of these cells was provided by the intensity of the orthochromatically stained background. This in one way was a drawback for photographs taken in black and white. Prolonged washing in cold water to remove background stain decreased the intensity of colour in these cells. Also, pseudoisocyanin stained slides could not be kept for longer periods. This staining reaction clearly demonstrated that these cells correspond to insulin producing beta cells of higher vertebrates.

As a rule the shape of these beta cells was observed to be more or less slightly elongated but without any pointed ends (Plate 8). Oval or nearly oval beta cells were to be found as well. These cells were observed to have distinct cell membranes and slightly excentrally located nucleus. Nuclei of these cells were round to oval in shape having a distinct nucleolus and distinct chromatin networks. Measurement of the beta cells showed the short axis to vary from 5.0 to 6.5 μ and the long axis from 10.0 to 12.0 μ . The nuclear dimensions varied from about 3.5 to 4.5 μ.

These pseudo-isocyanin positive cells did not show any reaction for silver impregnation. Silver impregnation methods produced a diffuse dark brown staining of the cytoplasm which was sometimes obtained in the beta cells. This did not occur regularly and seemed to be a false argyrophilia. PAS staining of these cells sometimes showed glycogen to be present, as reported previously in Opsanau tau (23).

Intermingled with groups of pseudo-isocyanin positive cells were found another type of granular cells, the cytoplasm of which took the pinkish red colour of the counterstain (Ponceau fuchsin or ponceau de-xylidine) after application of the aldehyde-fuchsin or chrome-hematoxylin staining procedures (Plate 8 11). The cytoplasm of these cells contained a sparse amount of fairly small barely discernible granules which stained specifically with phosphotungstic acid - hematoxylin and were observed

blue on a mauve cytoplasmic background. Though it was not possible to demonstrate these cells along with beta cells on the same slide by PTAH sequence (217), they were observed to be different from beta cells when an adjacent section was stained with A.F. These granules did not show any reaction for aldehyde-fuchsin or pseudo-isocyanin stains. By applying PAS-trichrome stain the granules of these cells took a deep orange to reddish colour. With chrome-hematoxylin they stained dark red. It was also possible to detect the granules of these cells with phloxine-Azur II-hematoxylin(219) when they stained dark purple. But with this method it was difficult to get contrast between different types of islet cells. The tinctorially staining properties of these cells correspond well to those of mammalian alpha cells.

The alpha cells were generally oval in shape and 5.0 x 7.0 μ in size but some of these cells measured as much as 9.0 x 11.0 μ. These cells were found mostly broader than the beta cells but shorter in length. They were observed to be present singly or in small groups. The nuclei of these cells were nearly spherical in shape and of the same size (3.5 x 4.0 μ) as the beta cell nuclei.

To identify these cells by silver impregnation technique, procedures as modified by Grimelius (220) and by Hellerstrom-Hellman (61) were applied to the tissue sections. It was found that neither of these methods demonstrated any characteristic argyrophil cells in the islet tissue. Careful examination at higher magnification, however, showed that a very faint dark brownish staining of the cytoplasm as well as nuclei could be identified in some of the pseudo-isocyanin negative cells. Under lower magnification this staining appeared similar to (Plate10) false argyrophilia as observed sometimes in the beta cells, but a distinct blackening of the cytoplasm as it should be in a positive argyrophil cell was never observed. From the observations it appeared that all the pseudo-isocyanin positive and aldehyde fuchsin-positive cells were non-argyrophilic and some of the pseudo-isocyanin-negative

and aldehyde-fuchsin negative cells could be argyrophilic.

In association with the alpha and the beta cells another type of cell containing no granules and, in fact very little completely clear cytoplasm at all was observed. (Plate 12). These cells were less abundant and were found mostly at the periphery of the islet tissue. They were occasionally observed in the central region as well. These cells appeared more uniform in shape than the other two cell types. Nearly round in appearance these cells were 6.0 x 8.0 μ in size and with rather indistinct cell membranes. Nucleus of this cell type was always round, smaller in size (3.0 x 3.0 μ) than those of the alpha and beta cells, usually centrally located and containing rather inconspicuous nucleolus.

These cells did not show any positive reaction for any of the special differential staining procedures including silver impregnation methods and generally took a faint background stain on a section demonstrating either alpha or beta or both cell types. However, it was easy to identify them after counterstaining the section with light green when they appeared homogenously translucent green. With Maldonaldo and Jose' staining sequence, the cytoplasm of these cells was observed as light blue. Though less conspicuous in appearance these cells, were nevertheless easy to be located in the cellular mass of islet tissue by their agranular light green cytoplasm, spherical shape and more peripheral position. These cells by their characteristics probably correspond to the 'agranular' (71, 87, 95) or 'clear' cells (74) described in higher vertebrates.

The difference in shape between the alpha and the beta cells was not significant, the beta cells sometimes having comparatively thinner and more elongated appearance. The nuclei were usually oval in both granular cell types and contained 1 - 3 nucleoli.

Histochemical methods for nucleic acid staining applied to the islet tissue sections showed that ribose-nucleic acid (RNA) content in two granular cell types seemed much lower when compared with

exocrine pancreatic cells; the reaction for RNA being only slightly stronger than after the RNA digestion. It was, therefore, very difficult to assess any difference at all between various endocrine cells in their RNA content, as observed by their affinity towards specific histochemical methods for nucleic acids.

Histologically, no difference was found at all in the cellular composition between 'principal' and 'secondary' islets of Tilapia mossambica.

b. Islet histology in Cottus scorpius.

Non-specific staining i.e. hematoxylin-eosin of the principal islets of Cottus scorpius clearly demonstrated that two regions in the islet tissue can easily be distinguished; dark cells in the central region and light cells in the peripheral region. The central dark region, however, contained a number of groups of cells in close association with dark cells, so as to give a patchy appearance (Plate 12). By applying special differential staining techniques it was possible to observe two basic types of granular cells as well as cells devoid of any granules.

Application of aldehyde fuchsin/ponceau fuchsin stain showed the bulk of central dark region to be composed of aldehyde-fuchsin-positive cells as the granules of these cells stained dark purple with this stain, whereas the rest of the cells in the central dark region took the counterstain instead and appeared pinkish red (Plate 12) and hence were aldehyde-fuchsin-negative. Cells of the latter type were observed in the light peripheral region as well. On the other hand, it was also observed that granules of some of these cells sometimes got stained with aldehyde fuchsin. However, when pseudo-isocyanin stain was applied to the tissue section, the granules of all the aldehyde-fuchsin-positive cells of the central dark region displayed a characteristic bright red colour staining whereas those of the light peripheral region, as well as the aldehyde-fuchsin-negative cells

showed no reaction at all (Plate13). The pseudo-isocyanin-positive cells responded to all the other differential staining procedures as well, in the same way as the beta cells of Tilapia mossambica did. These cells were, therefore assumed to correspond to the insulin producing beta cells of higher vertebrates. The granules of pseudo-isocyanin-negative cells of both the central dark region and the light peripheral region were shown to be specifically stained with phospho-tungstic acid-hematoxylin. They stained dark purple with Maldonado - Jose's staining sequence, red with Monroe-spector's method and dark red with chrome-hematoxylin. Their staining properties resemble very closely those of mammalian alpha cells. The pseudo-isocyanin positive cells were cylindrical in shape as a rule and arranged compactly in cord fashion (Plate 13). These cells were usually observed to surround the vascular tissue in a radiant manner. The size of these cells was found to be 4.0 x 10.0 μ but sometimes slightly longer (5.0 x 14.0 μ) cells were also observed. The cytoplasm of these cells showed a large number of small granules and an excentrally located nucleus, which was oval in shape. Nuclei of most of these cells were found to have the same dimensions (3.5 x 6.5 μ) but sometimes thinner nuclei (3.0 x 7.0 μ) were observed as well.

The pseudo-isocyanin-negative cells resembled very closely the pseudo-isocyanin -positive cells in their structure, shape and size. The cells were elongated and mostly uniform in size (4.5 x 10 μ), though many times their cytoplasmic processes have also been observed. Cell nuclei were oval in shape, having 3.5 x 7.5 μ as their nuclear dimensions(Plate 14).

Silver staining methods did not clearly establish the characteristic argyrophilia in any of the islet cells, but it appeared from close examination that all the pseudo-isocyanin-positive cells of the central dark region as well as the pseudo-isocyanin-negative cells of the light peripheral region failed to show any positive reaction. On the other hand, a very weak reaction was observed in the pseudo-isocyanin-

negative cells of the central dark region, though in fact it was very difficult to give any conclusive result regarding their argyrophilic nature.

The light peripheral region was observed to contain, in addition to pseudo-isocyanin-negative cells, abundant cells closely associated which were spherical to polygonal in shape with clear cytoplasm but without granules of any sort. Most of these cells were always spread out in the outer zone of the peripheral region. Their size varied from 6.0 x 10.0 μ to 8.0 x 12.0 μ . They contained almost round nuclei (size 3.0 x 3.0 - 3.5 μ) usually located in the centre.(Plate 14,15). Generally these cells did not show any reaction to any specific differential stain and took the faint background stain. Counter-staining with light green showed their cytoplasm to be homogenously translucent green in appearance.

All the special differential staining methods showed that the pseudo-isocyanin-positive cells, the pseudo-isocyanin-negative cells and the cells without any granules resembled in their tinctorial properties those of beta, alpha and agranular cells of higher vertebrates respectively.

iii. Electron Microscopy.

General findings: It has not yet become possible to stain selectively the different cell types of pancreatic islets for their identification at electron microscope level, which is usually possible for light microscopy. By the use of correlative thick and thin sectioning technique with light and electron microscopic studies respectively, and by secretory granule morphology, it was possible to identify different cellulose types at the ultrastructure level in the principal islets of Tilapia mossambica.

Exocrine pancreatic cell was easily recognised and differentiated from endocrine pancreatic cells (Plate 16) by its dense structures i.e. zymogen granules, in sections cut out from blocks trimmed at the capsule of the islet. Sometimes structures similar to zymogen

granules but having a matrix of lighter density were also observed. These structures have been regarded as their immature forms containing less zymogen material and have been called prozymogen (246). Exocrine pancreatic cells were observed to have large spherical nucleus with finely granular nucleoplasm, a number of ovoidal-elongated mitochondria and well defined Golgi zone. The cytoplasm of these cells contained a very large number of thin double membranes, parallel to each other which appeared to be close at both ends, called the endoplasmic reticulum. Ribosomal particles of various sizes attached to the endoplasmic reticulum were observed.

Electron microscopy clearly confirmed the light microscopic observations that islet tissue in Tilapia mossambica contains atleast two major types of granular cells. The non-granular cells were also identified in the electron micrographs. In addition to these, another type of cell, granular in nature but different from the major granular cell types, was observed, though less frequently. The general appearance of some of the islet cells and their inter-relationship in the islet tissue is illustrated in Plate 17. It could be seen that the two major granular cells (granular cell type 1 and granular cell type 2) tend to cluster around capillaries and were not difficult to distinguish from each other, at low magnification, even by the size of their granules. At low magnification, the third type of cell (cell type 3) was very difficult to identify but the nongranular (cell type 4) cells were easily identified by the absence of secretory granules (Plate 18).

Individual cells were seen to be surrounded by continuous plasma membranes, closely applied to each other. Sometimes these membranes appeared to be separated by distinct intercellular spaces. The nuclei of various cell types showed the usual double nuclear membranes. The cell membranes sometimes showed convolutions or invaginations. Islet cells were separated from the capillaries by two basement membranes. There appeared to be a connective tissue

space present between these two membranes which contained mostly a variable number of collagen fibres and occasionally fibrocytes.

a. Cell type 1:

The most characteristic feature of these cells is the presence of a number of specific secretory granules in their cytoplasm (Plate18). With high magnification these cells could easily be differentiated from other cell types by differences in appearance and size of their secretory granules. Generally these granules appeared rounded in contour but sometimes may be slightly irregular, or nearly (Plate 19a) hexagonal in shape. In some cases granules in the form of rectangular bodies perpendicular to the outer membranous sac have also been observed in the same cell containing spherical secretory granules (Plate 19b).

The matrix of the granules was observed to be homogenously dense, but there were gradations to a less dense matrix. Often they appeared paler and somewhat vesicular in texture which gave them a 'porous' appearance. The electron density of the granules varied fairly much in one and the same cell. Mostly these granules were less electron dense, less homogenous than the granules of cell type 2. The granules were individually bounded by a single layered smooth membrane. There is generally a lucid space or 'halo' of variable sizes separating the enclosing membrane from the material contained inside it - the proper granule (Plate 20a). Occasionally, these limiting membranes appeared as incomplete with a wavy outline and also the space between the membrane and dense vesicle content was not clearly marked, but generally these membranes were regular, compact and complete.

The size of the cell type 1 secretory granules varied from 160 mμ to 290 mμ in diameter but sometimes these were observed to vary from 120 mμ to 400 mμ in diameter in one and the same cell. It seemed that these granules are characterisied by a marked variation in size. It was also observed that more numerous 'porous' granules were

usually about twice the size of the fully condensed granules. The space between the central core of the granule and the limiting membrane was usually very small (varied from 15 mμ - 50 mμ) but sometimes the limiting membranes were observed to form a very large halo (up to 200 in width). This space in many granules, especially those that did not appear fully condensed, was seen to contain slightly dense fibrous material (Plate 20a).

Occasionally two or three central cores of the granules were observed within one limiting membrane. They were connected at their ends or were overlapped by one another at different angles. Frequently the smooth membrane-bound vacuolar spaces termed 'sacs' were seen by Munger (115) in the beta cells of rabbit islet tissue. It was (Plate 19b, 20a) observed that such sacs resembled very closely in their size, form and location, the limiting membranes of beta granules and there were a number of membranous sacs present which were empty. Some of these sacs were observed to contain particles of various sizes with an electron density similar to that of beta granules. It seemed possible that such sacs could be one of the transitional stages in the formation of beta granules, as was suggested by Munger (115). It could also be said that the less dense beta granules, 'porous' in appearance, probably represent an intermediate stage in their condensation.

These cells showed spherical nuclei, the nuclear chromatin of which usually was evenly distributed, though prominent accumulations of chromatin along the nuclear membrane were frequently seen. In many instances, the nuclear membrane showed a small shallow indentation (Plate 19a). Sometimes the nucleus was observed to have a patchy appearance due to the irregular distribution of nuclear substance composed of very fine filamentous material. Nuclear pores (226) were very rarely observed.

Though the 'macular zone' - the irregular zone of moderate electron density near the nucleus (227) was not regularly seen, often fibrillar material composed of extremely fine fibrils (approximately 30 mμ in diameter) was observed in the immediate outside vicinity of the nuclear membrane (Plate 19a). Occasionally such fibrillar material but less conspicuous was also observed throughout the cytoplasm, particularly near the cell membrane.

The cytoplasm of these cells was faintly electron dense and the endoplasmic reticulum mainly of rough lamellar type. The cisternae of the endoplasmic reticulum were observed not to follow a regular pattern in their distribution throughout the cytoplasm. These cisternae varied in number and usually appeared in small groups (Plate 19b) and in large parallel arrays (Plate 20b) but also occurred singly (Plate 19b). The parallel arrays of endoplasmic (Plate20a) reticulum cisternae (up to 14 in number) were usually observed near the cell membrane. The tubular or vesicular cisternae showed a varying degree of dilations and these were observed to contain moderate amounts of a fine granular or homogenous material of different electron densities. Ribosomes varying greatly in number were associated with the cisternal membranes. Free ribonucleoprotein particles, scattered throughout the cytoplasm could also be observed.

Though the continuity between the cisternal membranes of the endoplasmic reticulum and the limiting membranes of the secretory granules was not found to occur regularly, sometimes such a relationship was clearly observed. In such cases, the granules of varying size and electron densities were seen lying at the ends of cisternal membranes of endoplasmic membranes, mostly when the latter were arranged in parallel arrays. Their membranes were in continuation with those of cisternae (Plate 20b), and it appeared as if the cisternal membranes had formed a pocket-like structure to enclose the secretory granule.

Mitochondria were observed as ovoidal or thick short rod-shaped structures (Plate 21a). The matrix in the mitochondria was less dense and the mitochondrial cristae were not distinct. Transversely orientated cristae were difficult to observe. Moreover, typical intramitochondrial granules were not observed. It was difficult to assign any particular site for their location. They were found around the nucleus in the vicinity of the Golgi zone, close to the profiles of the endoplasmic reticulum and at the periphery of the cell.

The Golgi complex was found usually near the nucleus and consisted of irregular smooth membranes, vacuoles and vesicles (Plate 21a). The latter were usually observed to contain a homogenous material of low electron density. A number of vesicles of different sizes and shapes were also observed near the Golgi complex as well as throughout the cytoplasm. The interpretation of their fine organisation was found difficult. They appeared similar to those reported present in other tissues and in each case different terminology has been used to describe them. Usually they have been referred to as complex vesicles (228), coated vesicles (228, 229), dense rimmed vesicles (230) and alveolate vesicles (229). Whether these vesicles fall under any of the categories interpreted and described by Meyer and Bencosme (227) is not known.

A number of multivesiculated bodies were commonly observed in the cytoplasm (Plate 20b, 21a). The vesicles varied in size, measuring up to 850 mμ in diameter. They were mostly round or nearly round but oval shaped vesicles were observed as well. They were either electron transparent or moderately electron dense. Other cytoplasmic components, e.g. autophagic vacuoles, dense bodies were also observed in the cytoplasm of cell type 1. The dense bodies suggestive of lysosomes (82) were of varying size and internal structure.

Clusters of minute granules, observed by Munger and his co-workers (64, 65) as reminiscent of prosecretory granules were also seen in some of the electron micrographs (Plate 18). Sometimes one minute granule was observed in close relationship with the Golgi complex (Plate 21a).

The intercellular space between two adjacent cells was usually found to be approximately 30 mμ wide. It was difficult to observe clearly the occurrence of lacunae or microvilli at the cell surface. However, desmosomes as well as pits of varying depths as described by Roth and Porter (231) were occasionally observed (Plate 20a). It was very common to see a number of secretory granules and sacs containing variable amounts of electron dense particles attached to the cell membrane (Plate 21b). In such cases, the granules or sacs appeared very close to the cell membrane and pointing towards it. The cell membrane was seen to be thickened and slightly bulged outwardly at that point. The limiting membrane of such granules in one electron micrograph appeared to have fused with the cell membrane (Plate 21a). In one of the electron micrographs a pinocytic vesicle still attached to the cell membrane was also clearly observed. (Plate 20a)

Though some of the cells belonging to this category were observed to have less secretory granules and sometimes more ergastoplasm with a greater number of unattached ribosomes, it did not appear that two distinct phases - granular and agranular - of this cell type could be established.

b. Cell Type 2. : These cells, comparatively less in abundance, were easily differentiated from the cells of type 1, mainly by their characteristic secretory granules which were always more electron dense and comparatively smaller in size (Plate 22). The granules of these cells measured from 90 mμ to 150 mμ in diameter. On rare occasions these were found to be slightly bigger than usual measuring up to 190 mμ in diameter. They were mostly round but sometimes their outline was observed to be hexagonal or quadrangular. They were comparatively

less in number, diffusely distributed throughout the cytoplasm. It was apparent that these cells showed very little degree of variation in size, shape and density.

The central core of the secretory granules composed of homogenous material was separated from a smooth limiting membrane by an electron lucent space of variable width (Plate 23a). This space or halo was mostly observed to be comparatively big in comparison to that of cell type 1. Usually the width of this space varied from 75 mμ to 110 mμ. Sometimes the central core of one of these granules was very close to the limiting membrane on one side, showing a very large halo (up to 160 mμ wide). Also, the granules which were seen slightly bigger in size than usual, showed a very small halo measuring up to only 30 mμ in width. The limiting membrane was often complete but frequently irregular and wavy in appearance.

The nucleus of these cells was usually round and less electron dense as compared to that of cell type 1, but generally it did not show any significant difference. The nucleoli sometimes appeared conspicuous and distinct. The macular zone and the fibrillar material outside the nuclear membrane were very rarely observed.

The endoplasmic reticulum was largely of the rough surface type. The cisternae of the endoplasmic reticulum arranged in large numbers of parallel arrays were often observed (Plate 23b). Moderately dilated cisternae were also seen either in singles or in groups, scattered throughout the cytoplasm. The cisternae were mostly observed to contain variable amounts of fine granular material of moderate electron density. The secretory granules were also observed in close association with the endoplasmic reticulum. Free ribosomes were observed in the cytoplasm as commonly as in the cell type 1.

The Golgi complex, adjacent to the nucleus, was smaller and probably more well developed and consisted of irregular lamellar and vesicles (Plate 22). The mitochondria were similar in structure and distribution to those of cell type 1, but probably less in number.

Vesicles of various sizes throughout the cytoplasm particularly near the Golgi complex were also observed in these cells.

Other cytoplasmic structures including multivesicular, autophagic and dense bodies as well as microtubules were also seen in these cells. These structures did not show any significant difference in their appearance or relative localization from those observed in cell type 1.

As seen in the cell type 1, the secretory granules of these cells were also sometimes closely attached to the cell membrane (Plate 23b) and the latter appeared thickened in a similar fashion. The intercellular space between the two adjacent cells was measured up to 85 mμ.

c. Cell type 3

In addition to the two major granular cell types, another type of cell was observed in the islet tissue, though only infrequently. This cell type was identified and differentiated from the other two by the general overall electron opacity of its cytoplasm and by the fact that the nuclei were larger in relationship to the cytoplasm than in the other cell types (Plate 24). The nuclei of these cells appeared characteristically different from those of other cell types (Plate 24) in being not spherical but irregular in contour. (Plate). The chromatin material was evenly distributed and more resembled that of cell type 2. The cytoplasm was observed to be more of a rough lameller type and very sparse. The cisternal membranes of the endoplasmic reticulum were usually densely studded with ribosomes.

The striking feature of this cell type was the few characteristic secretory granules which always tended to be present more on one side of the nucleus and usually away from it (Plate 24). The secretory granules were regularly round in profile and the granular core was composed of fairly homogenous material of moderate electron opacity but always more dense than that of cell type 1. It was also observed that a mild degree of variability of electron opacity of the secretory

granules exists and some of the granules were seen as dense as those of cell type 2, but the majority of the granules were of lower electron opacity as compared to them.

As in cell type 1 and 2, the granules were enclosed by smooth limiting membranes which were very closely applied to the granule cores (Plate 24). The space between the granule core and the limiting membrane was sometimes negligible but usually it varied from 10 mμ to 25 mμ in width. The granule core was observed to be uniform in size and they were approximately 130 - 150mμ in diameter. Membranous sacs like those observed in cell type 1 could also be observed throughout the cytoplasm of this cell but very few in number.

Other cytoplasmic organelles in general were observed to be very similar to those observed in the other two cell types. No obvious difference was found between the ultrastructure, appearance and relative localization of such structures as mitochondria, multivesicular and dense bodies and vesicles and those seen in other granular cell types.

d. Cell type 4.

More frequently observed than cell type 3, this type of cell was characteristically identified by the fact that they were almost devoid of any secretory granules (Plate 25). The very few secretory granules whenever present were usually spherical in shape but the contour of these was not clearly seen. They were uniform in size, measuring approximately 140 mμ in diameter. The granule core was composed of an extremely homogenous type of material of very low electron opacity and usually surrounded by a limiting membrane. Sometimes the limiting membrane was either not complete or was lacking.

Another distinct feature of this type of cell was the presence of a very large number of membrane enclosed sacs, very close to each other and usually electron translucent. Sometimes they appeared to contain variable amounts of homogenous material of very low electron opacity. They varied from about 250 mμ to about 500 mμ in diameter.

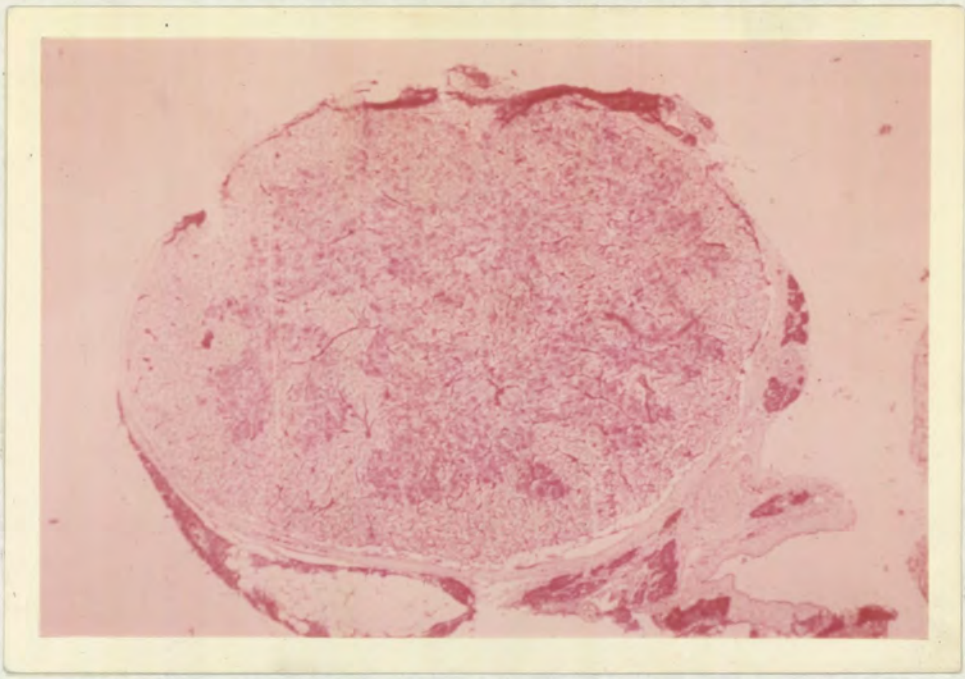


PLATE 5. Principal islet of Cottus scorpius. The islet tissue is separated from the thin layer of exocrine pancreas by a connective tissue capsule. Beta cells stand out bright red after application of psuedo-isocyanin stain.

(X 35)

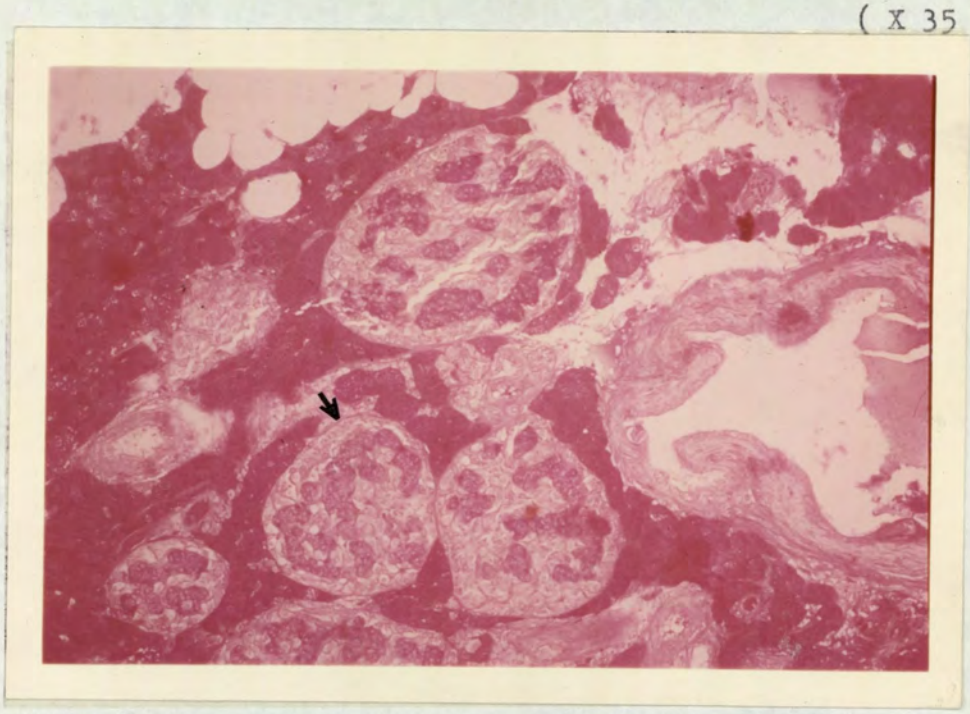


PLATE 6. Five principal islets of Tilapia mossambica, seen embedded in exocrine pancreatic tissue and surrounded by thin connective tissue capsule (arrow). Two islets have their capsules fused. Beta cells stained purple are scattered in the islet tissue.

(aldehyde-fuchsin stain; X35)

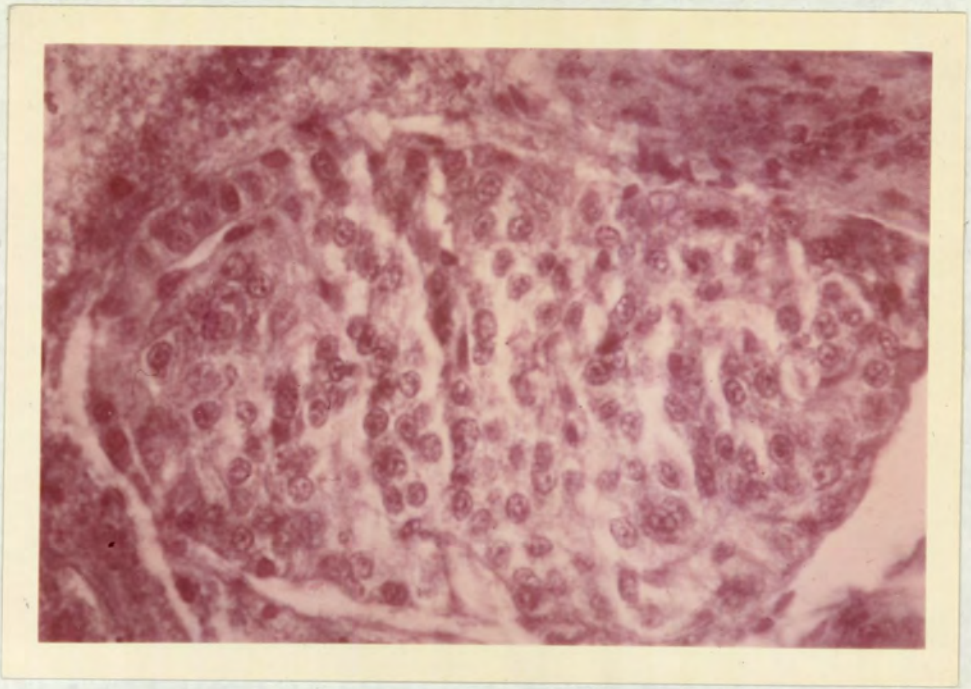


PLATE 7. A principal islet of Tilapia mossambica after staining with a non-specific stain. No differentiation of dark and light cell regions occured and all cells apparently looked alike.

(hematoxylin/eosine; X 820)

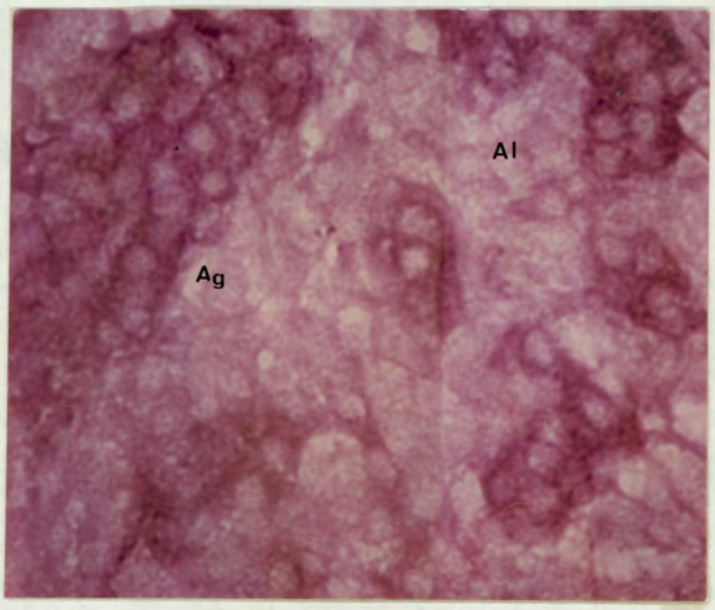


PLATE 8. Central part of a principal islet of Tilapia mossambica. Dark purple stained beta cells in association with alpha (Al) and agranular (Ag) cells.

(aldehyde-fuchsin/Ponceau de-xylidine stain; X640)

PLATE 9. High magnification of an area of principal islet of Tilapia mossambica. Oval to elongated shaped beta cells have psuedo-isocyanin-positive granules (bright red stained).

(Psuedo-isocyanin stain; X1600)

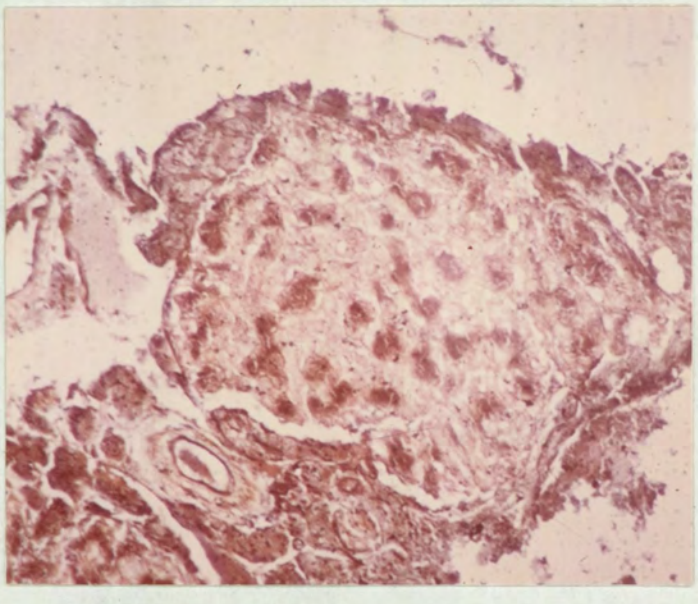


PLATE 10. A principal islet of Tilapia mossambica. Light to dark brown stained cells showing false argyrophilia.

(silver impregnation technique;
X 160)

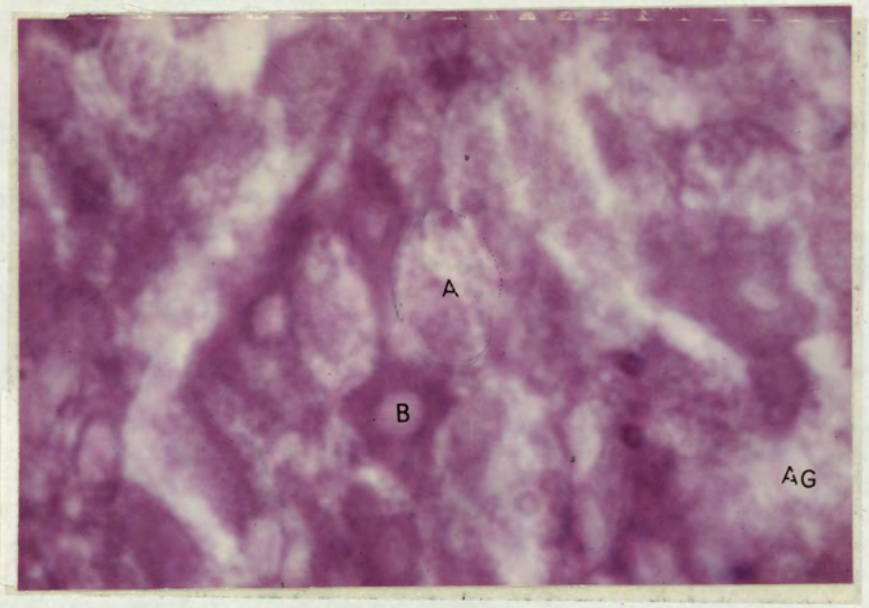


PLATE 11. High magnification of peripheral region of a principal islet of Tilapia mossambica, showing three types of islet cells(beta cells;B; alpha cells:A and agranular cells:Ag.).

(aldehyde-fuchsin/Phloxin ; X1600)

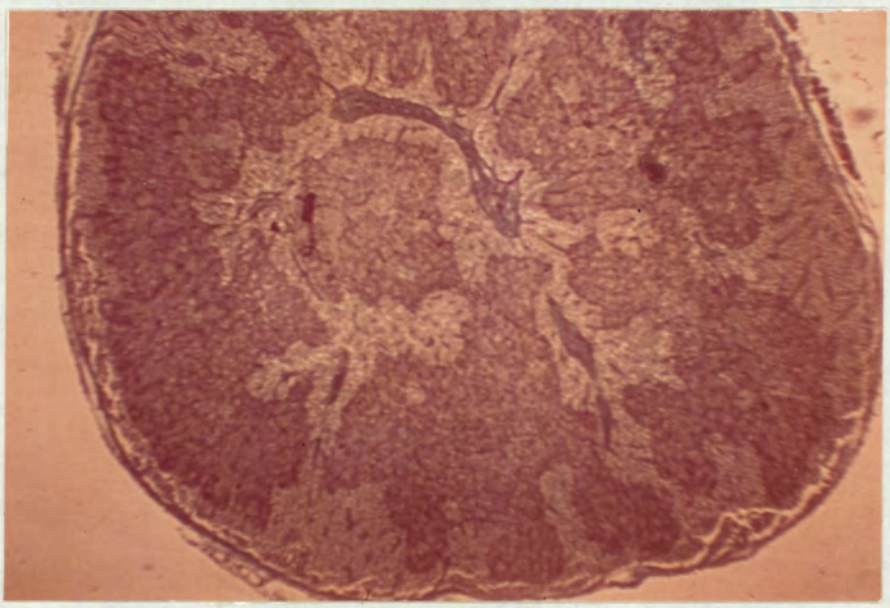


PLATE 12. Low power view of a principal islet of Cottus scorpius. Dark region consists mainly of beta cells whereas the lighter region mainly in the periphery contain alpha and the agranular cells.

(aldehyde-fuchsin/Ponceau de-xylidine; X 60)

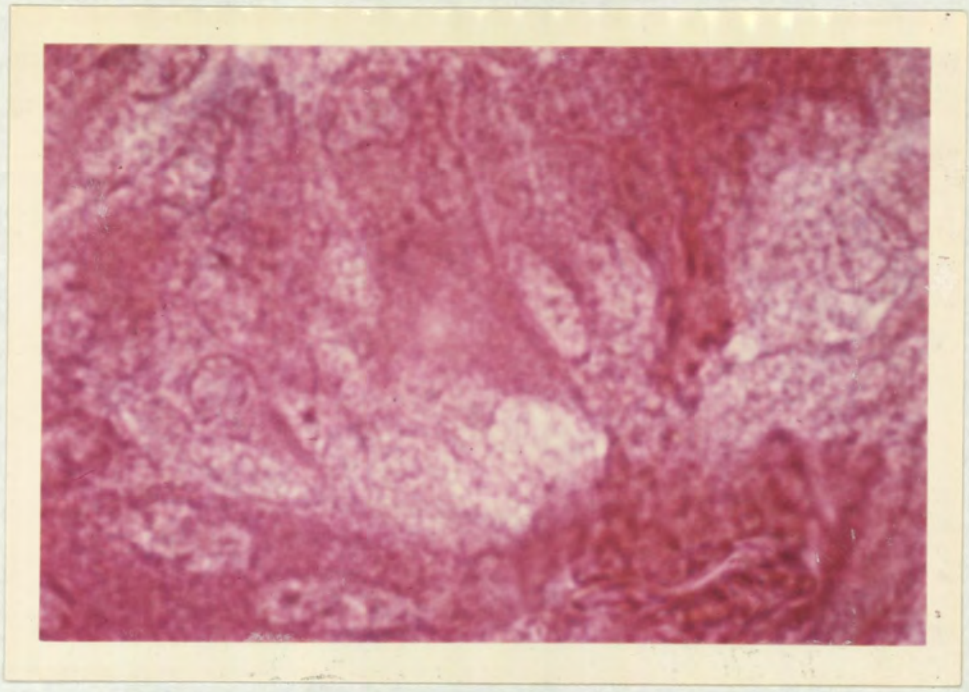


PLATE 13. High magnification of cylindrical beta cells grouped together in a principal islet of Cottus scorpius. Their cytoplasmic granules appear bright red.

(Psuedo-isocyanin stain; X1600)

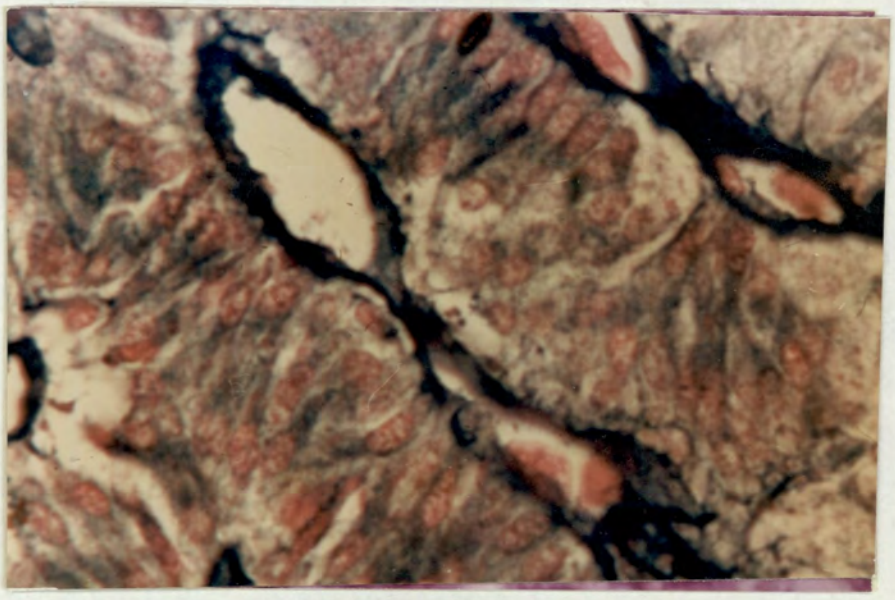


PLATE 14. Peripheral region of a principal islet of Cottus scorpius Group of cells consists of alpha and agranular types of islet cells.

(aldehydefuchsin/Ponceau de-xylidine X 1600)

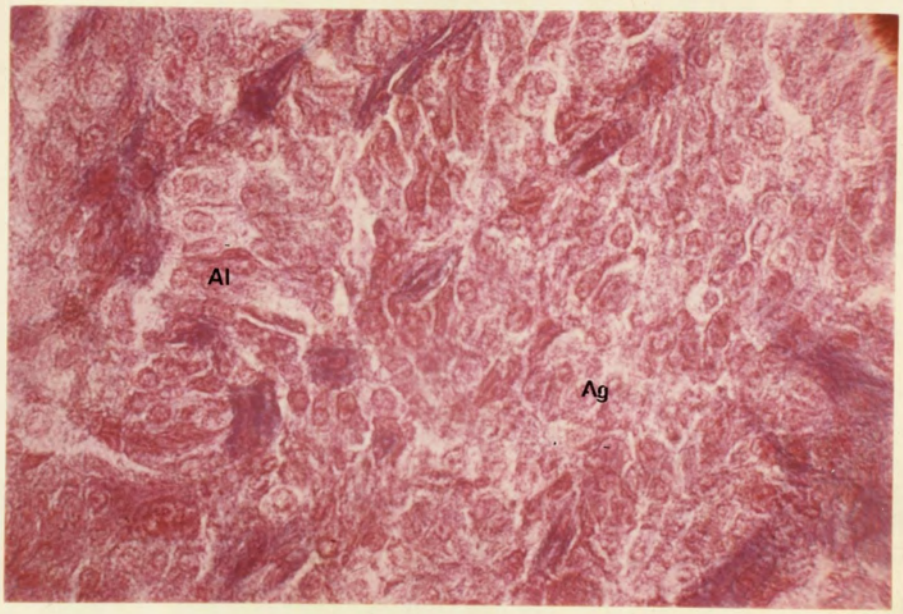


PLATE 15. Alpha and group of agranular cells seen in the peripheral region of a principal islet of Cottus scorpius. Spherical agranular cells (Ag,); elongated alpha cells (Al.).
(Masson's Trichrome stain; X 820)

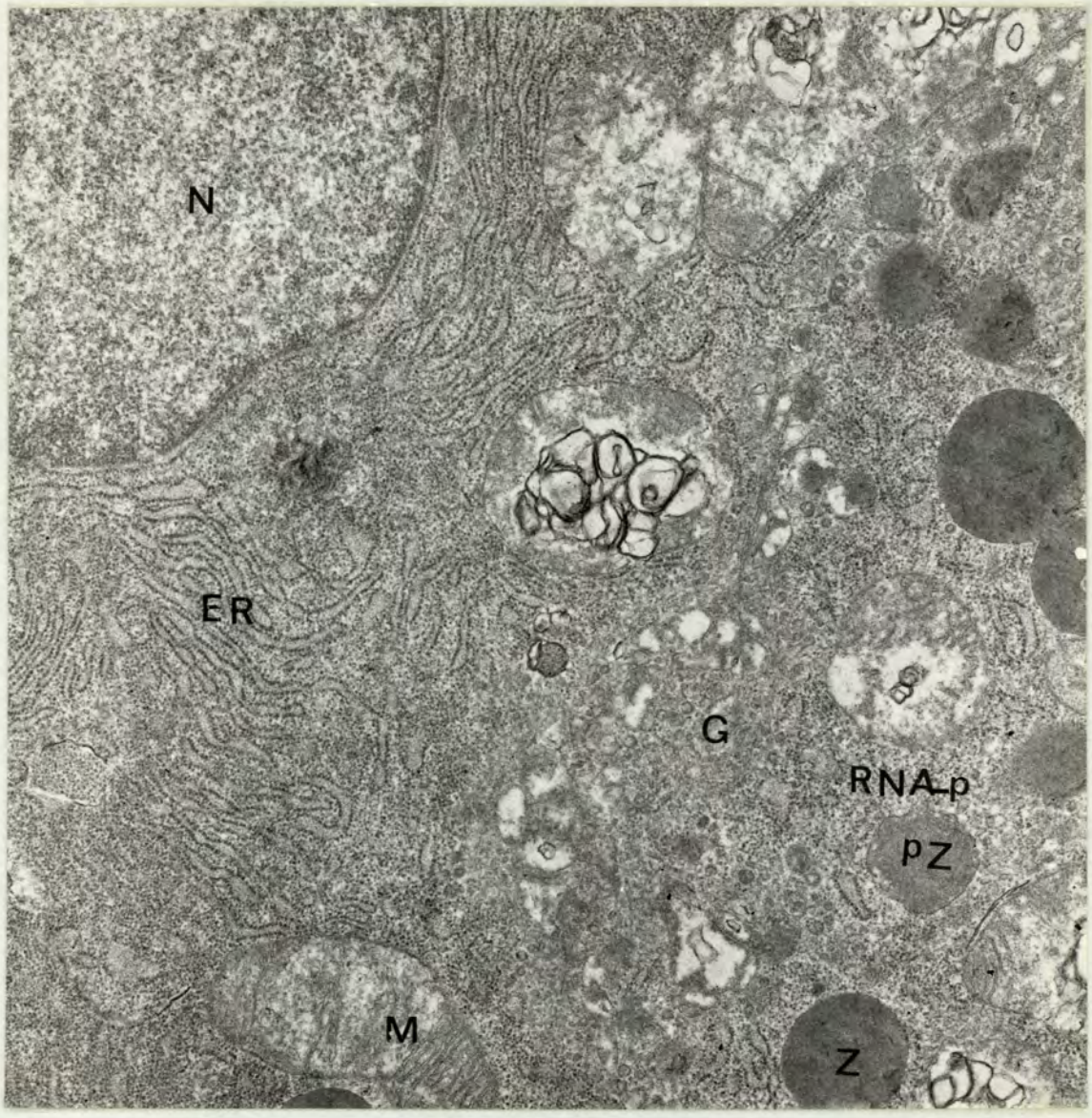


PLATE 16. Electron micrograph of a part of an exocrine pancreatic cell of Tilapia mossambica.

Key to labelling:

N, nucleus ; ER, ergastoplasm ;

M, mitochondria ; RNA-p, RNA particles ;

Z, zymogen granules ; PZ, prezymogen

G, golgi zone

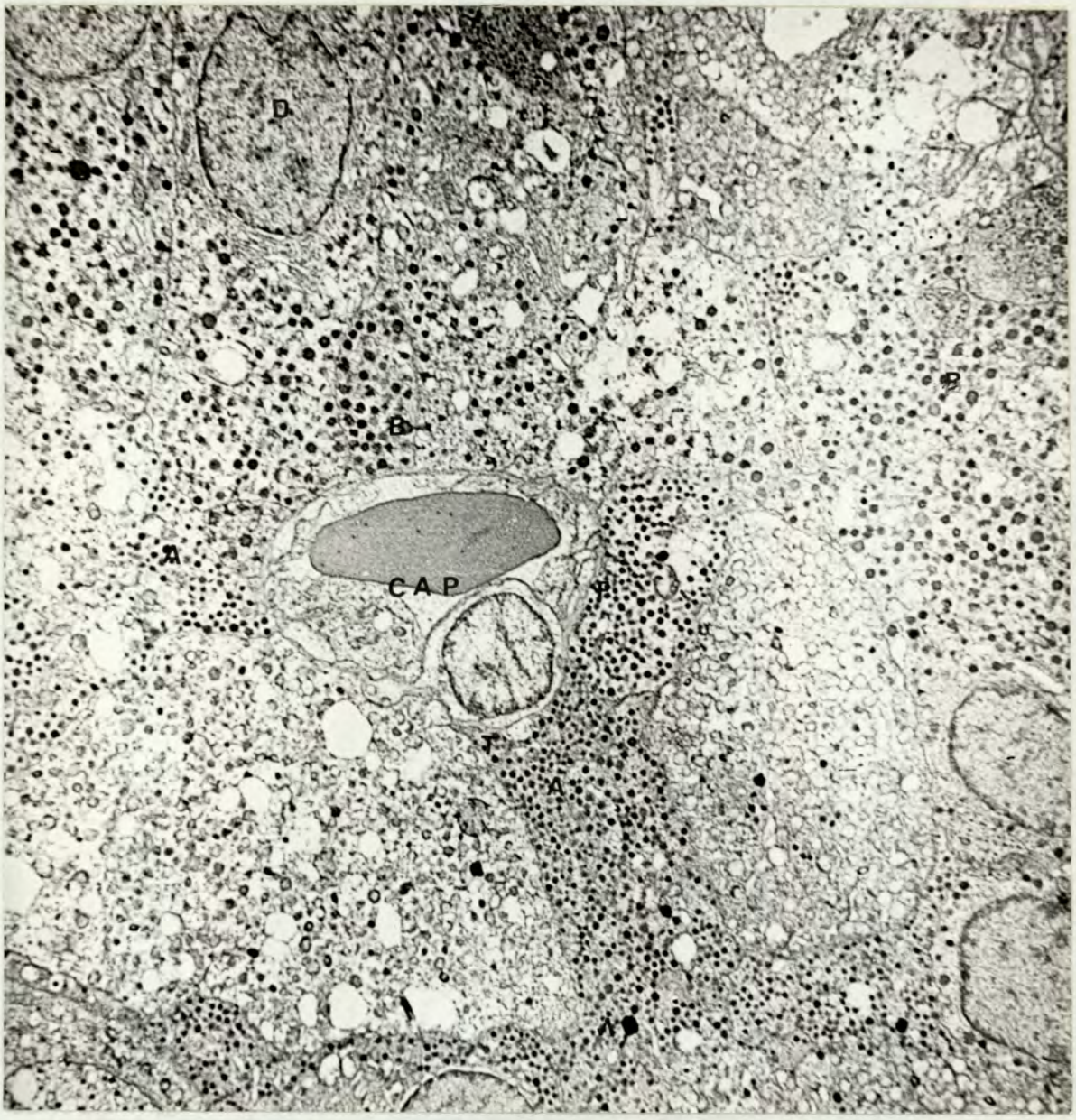


PLATE 17. Tilapia mossambica principal islet: Low magnification of its portion showing a general view of different types of cells present around the blood capillary (CAP); beta cells (B) are easily distinguished from the alpha cells (A) by their secretory granules of larger size and lesser electron density. The two cells on the left top corner are probably the D-cell (D) having relatively very few secretory granules present at the margin of the cell.

Magnification; 4,400 X

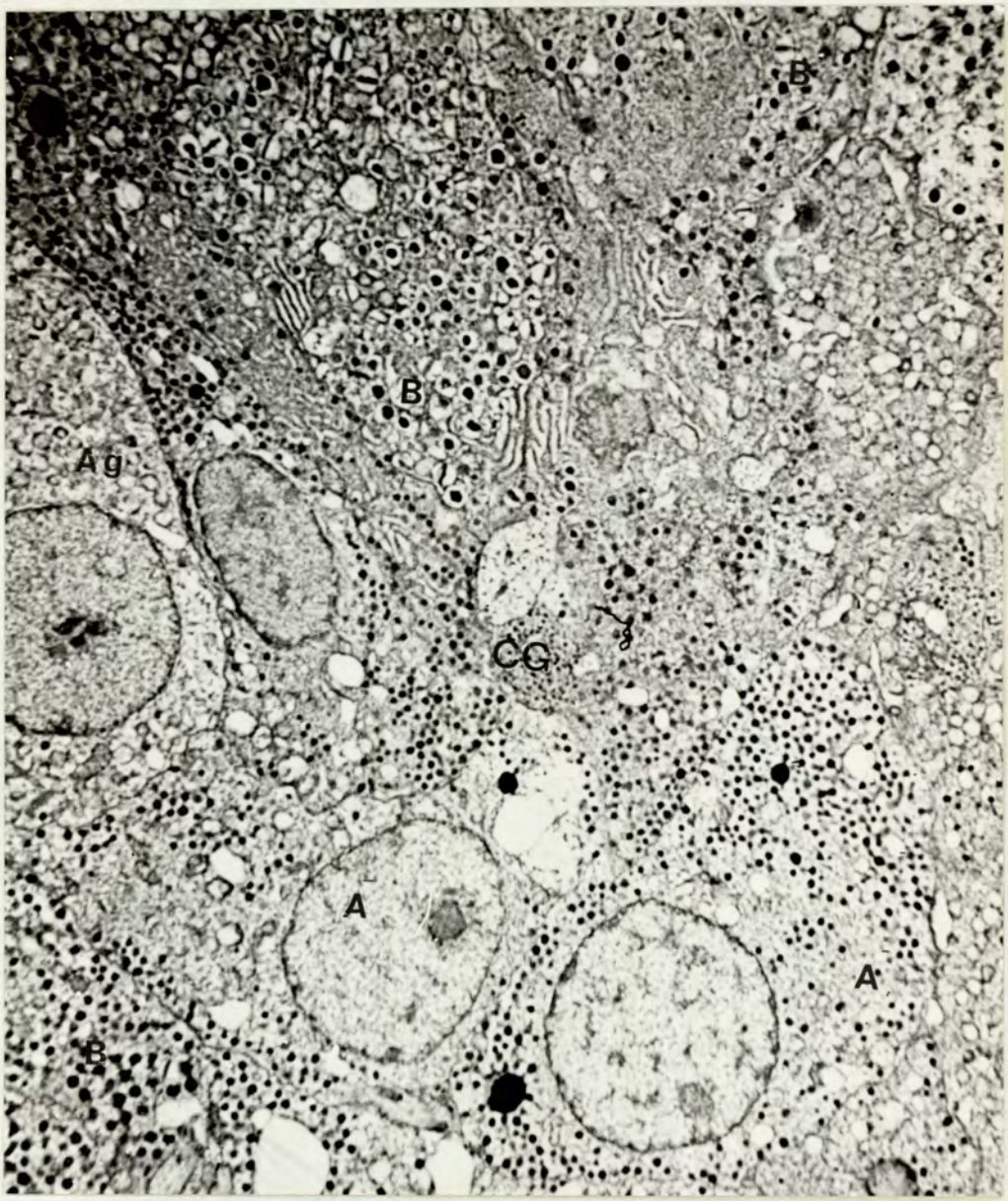


PLATE 18. Another general view of a portion of a principal islet Tilapia mossambica: Besides alpha (A) and beta (B) cells the agranular cells (AG) can also be seen, the latter being identified by their numerous ergastoplasmic sacs as well as by the fact that the secretory granules are almost absent. In one of the beta cells clusters of minute granules are also seen (CG).

Magnification: X 4,400

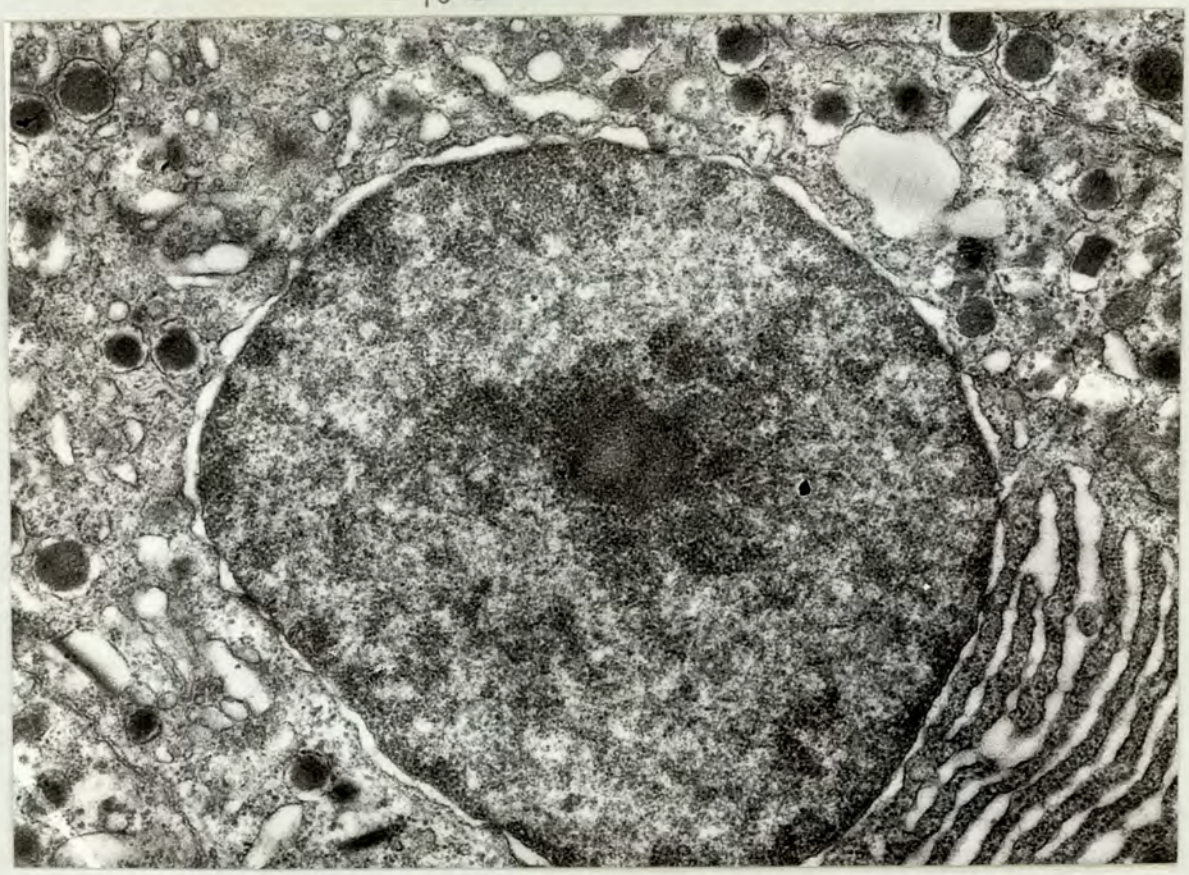


PLATE 19 (a). Portion of beta cell showing oval nucleus (N) with a double layered nuclear membrane, typical round secretory granules (SG), rough lameller ergastoplasm (ER) and Golgi zone (GZ) in the perinuclear region. Inconspicuous fibrillar material (FM) can also be seen. Magnification: X 24,000

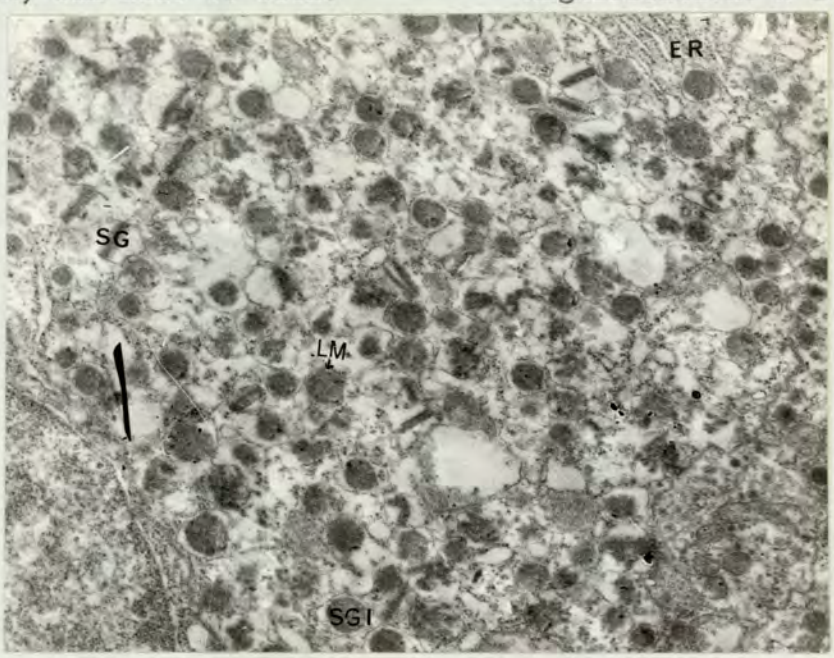


PLATE 19 (b). Another portion of beta cell showing secretory granules of varying sizes, shapes and electron densities (bar-shaped; SG; oval or round: SGI), and most of these have very narrow space between the core and the limiting membrane of irregular outline (LM). Granule-like sacs but without dense material are also seen. Granules in close association with GZ or ER can be seen as well. (Magnification : X 24,000)

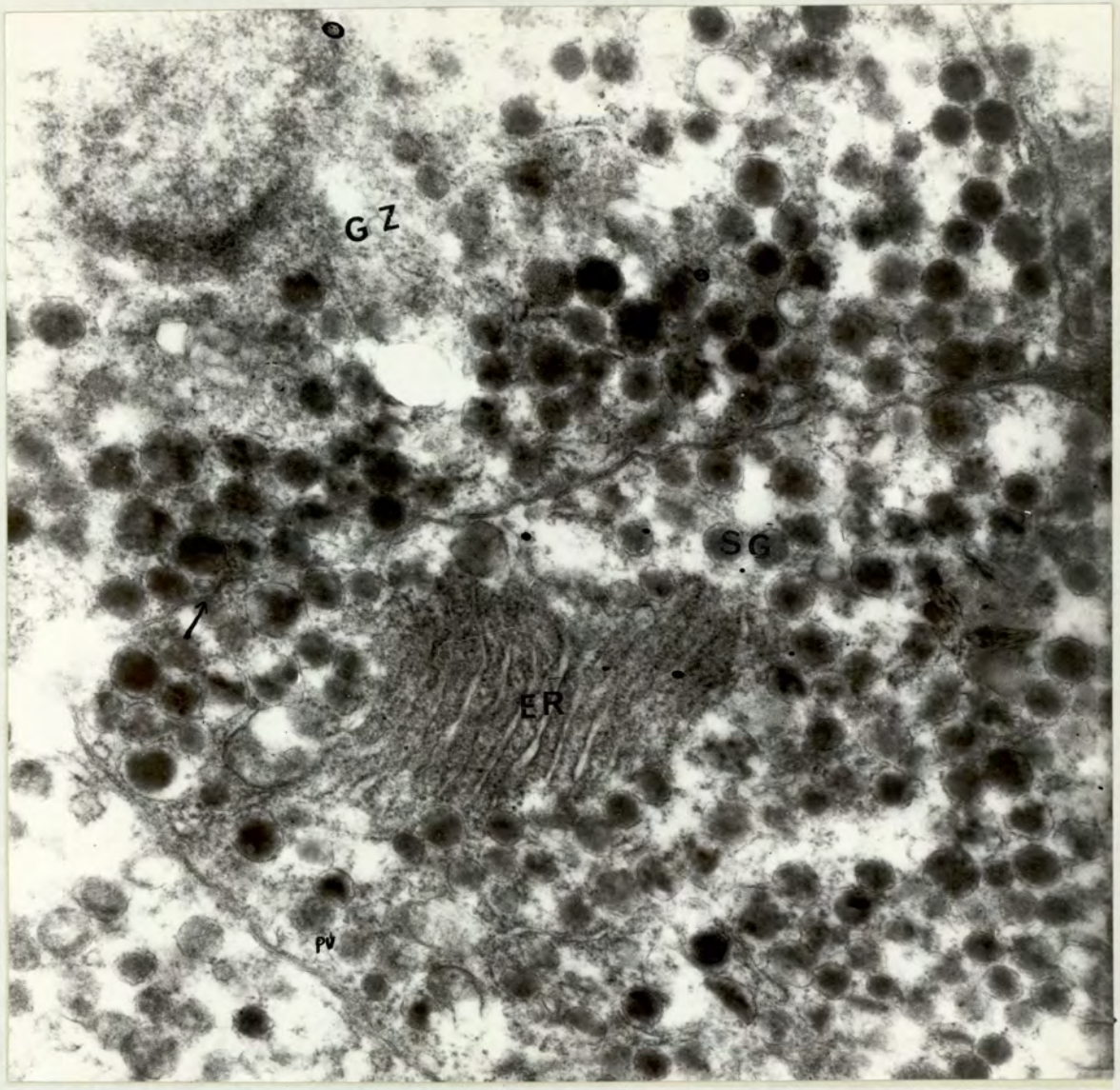


PLATE 20 (A). Principal islet of Tilapia mossambica: Portions of three beta cells with very small intracellular space, ergastoplasm (ER) in characteristic parallel arrays(one or two secretory granules in close association), typical secretory granules (SG) with narrow halo are seen. Some of the granules are in close contact with the cell membrane(one of them appear to have fused with it shown here by an arrow). In one of the cell Golgi zone (GZ) can be seen, as well as sac like structure near ER containing hazy material probably for three secretory granules. In one cell pinocytisis vesicle (PV) can also be identified.

Magnification: X 20,000

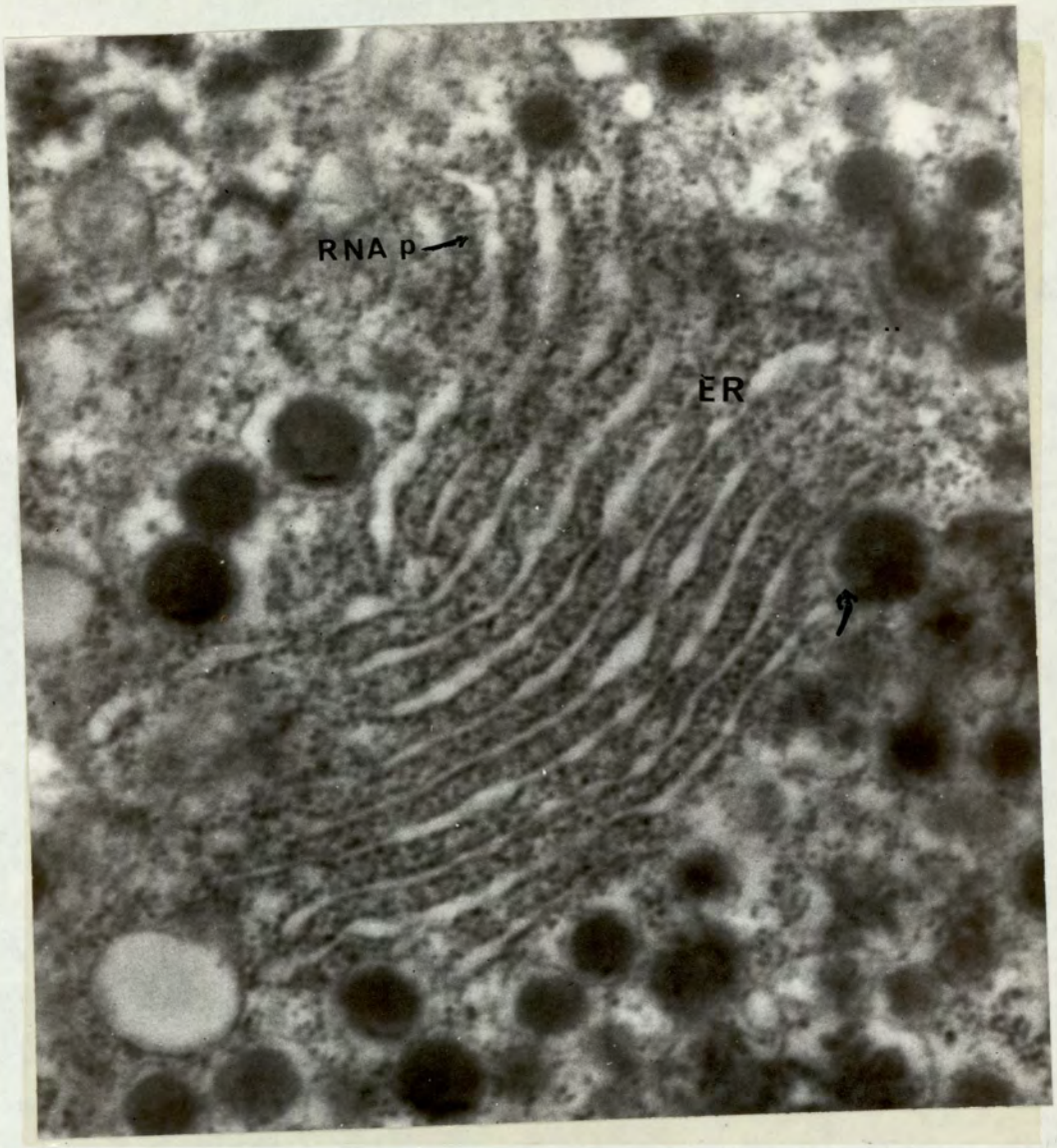


PLATE 20 (b). Principal islet of Tilapia mossambica: High magnification of a beta cell portion showing apparently the continuity of the ergastoplasmic cisternal membranes with the limiting membranes of the secretory granules (arrow). RNA particles (RNA-P) are seen attached to the ergastoplasm (ER) as well as scattered through the cytoplasm.

Magnification; X 42,000

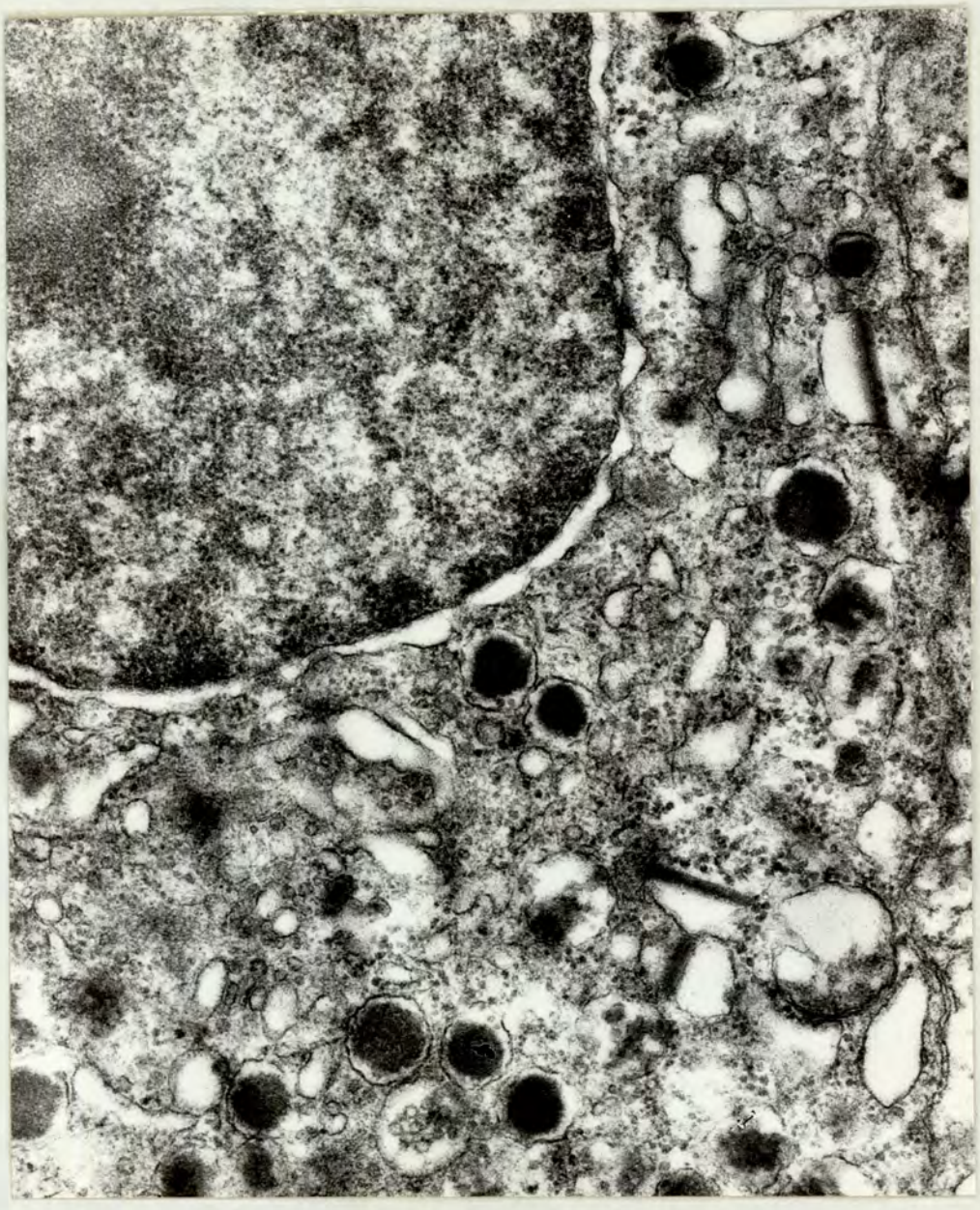


PLATE 21 (a). Principal islet of Tilapia mossambica; portion of a beta cell showing prominent Golgi zone (GZ) near the nucleus (N). One secretory granule (probably the prosecretory) appears to be in close association with GZ. Also seen in the electron micrograph are a few lysosome like bodies (L).

Magnification; X 48,000

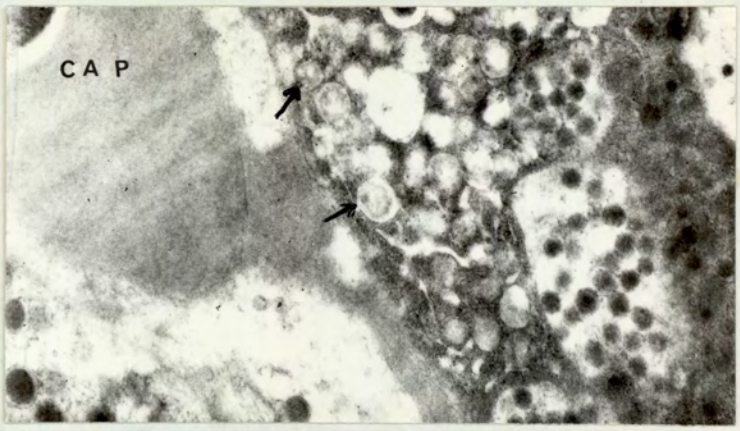


PLATE 21 (b). Principal islet of Tilapia mossambica; portion of a beta cell near the blood vessel (CAP) showing extreme margination of the secretory granules. Beta granules are seen in close contact with the cell membrane (arrow). Most of these granules are of very low electron densities. Their limiting membranes are not recognizable but in some granules a wide halo is also seen . The cell membrane at the site of contact with the limiting membrane of the granule appears to be slightly thickened.

Magnification: X 15,000

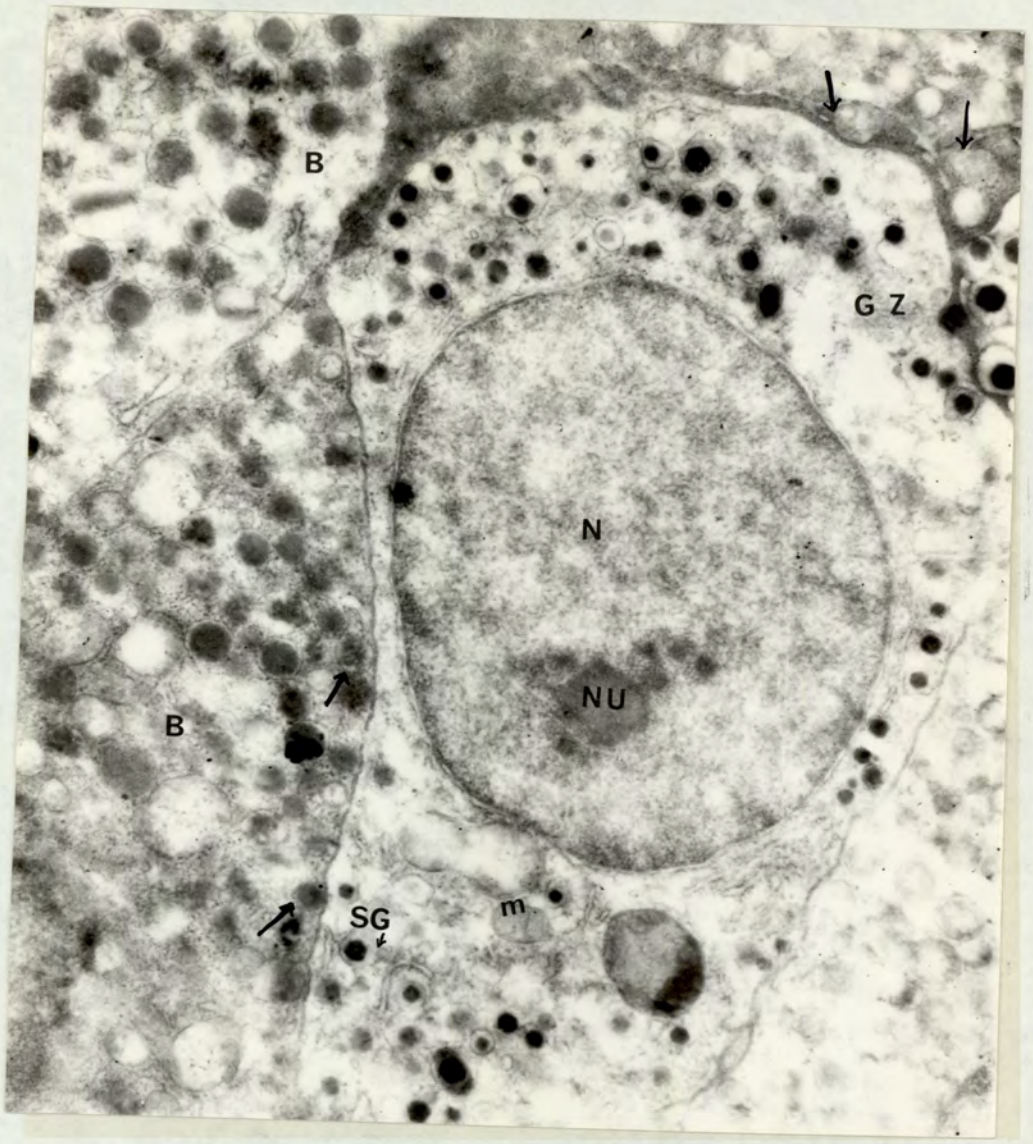


PLATE 22. Typical alpha cell (A) of the principal islet of Tilapia mossambica having more or less round nucleus (N) with a double layered nuclear membrane, a conspicuous nucleolus (NU) mitochondria (M), golgi zone (GZ) and specific secretory granules of greater electron density that those in the adjacent beta cells(SG). Also seen in this electron micrograph are portions of beta cells(B) with marginated granules along the cell membrane (arrow).

Magnification: X 19,500

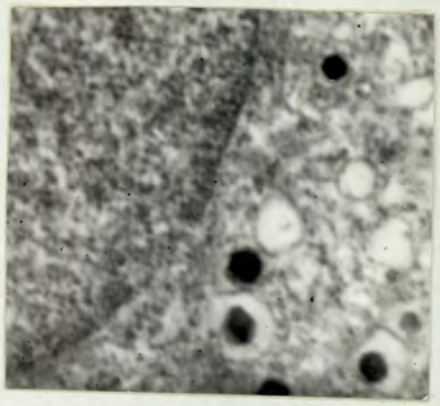


PLATE 23 (a)

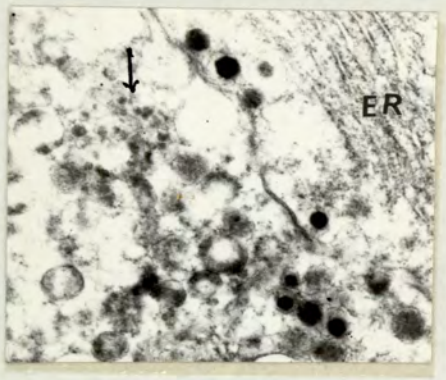


PLATE 23 (b)

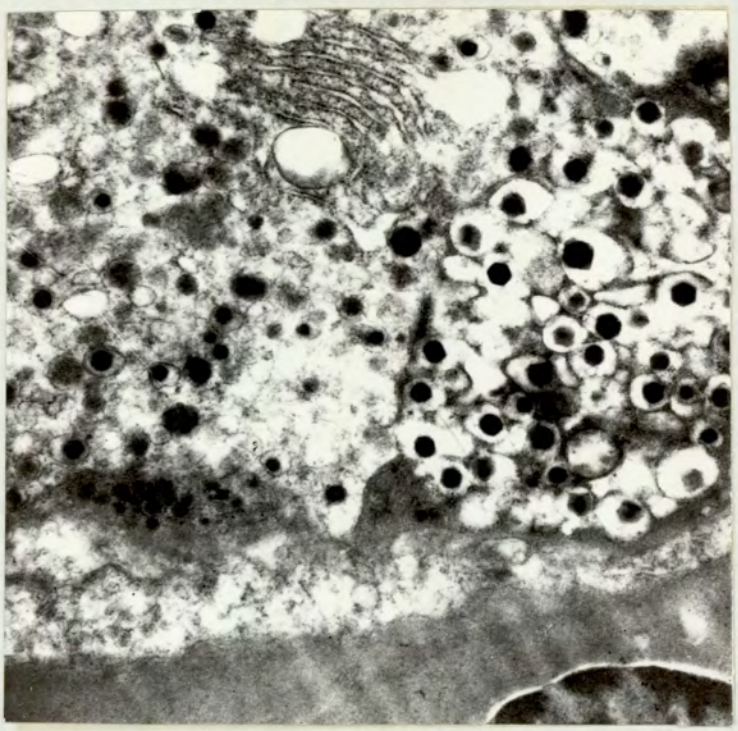


PLATE 23 (c)

PLATE 23 (a). Characteristic secretory granules of the alpha cells, round or hexagonal in shape, of uniform electron density and having a prominent large halo. Magnification: X 27,000

PLATE 23 (b). Portion of an alpha cell in a principal islet of Cottus scorpius showing parallel arrays of ergastoplasm (ER). Cluster of minute granules is also seen (arrow).
Magnification; X 15,000

PLATE 23 (c). 'Ballooning' of the limiting membranes of the secretory granules in the alpha cells some of which appear to be completely fused with the cell membrane thickened at the site of their contact. Spaces are seen both inside the cell and outside in the capillary lumen which probably were occupied by the granules. Magnification: X 15,000

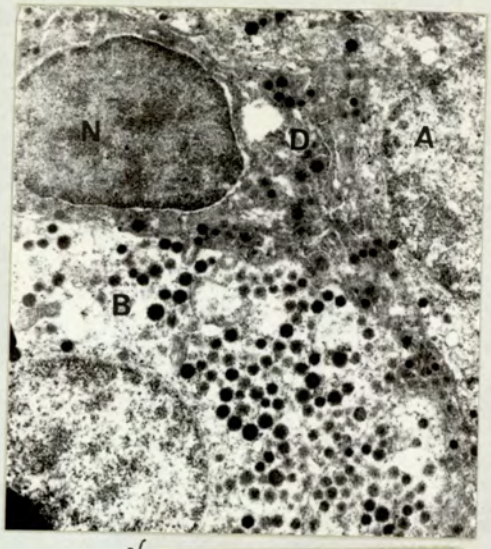


PLATE 24. Principal islet of Cottus scorpius: D-cell (D) seen adjacent to alpha (A) and beta (B) cells from which it is distinguished by its irregularly shaped nucleus (N), by overall greater electron density of both nucleus and cytoplasm, and by the presence of its specific secretory granules always towards one end of the cell as seen in this electron micrograph. Magnification: X 10,000

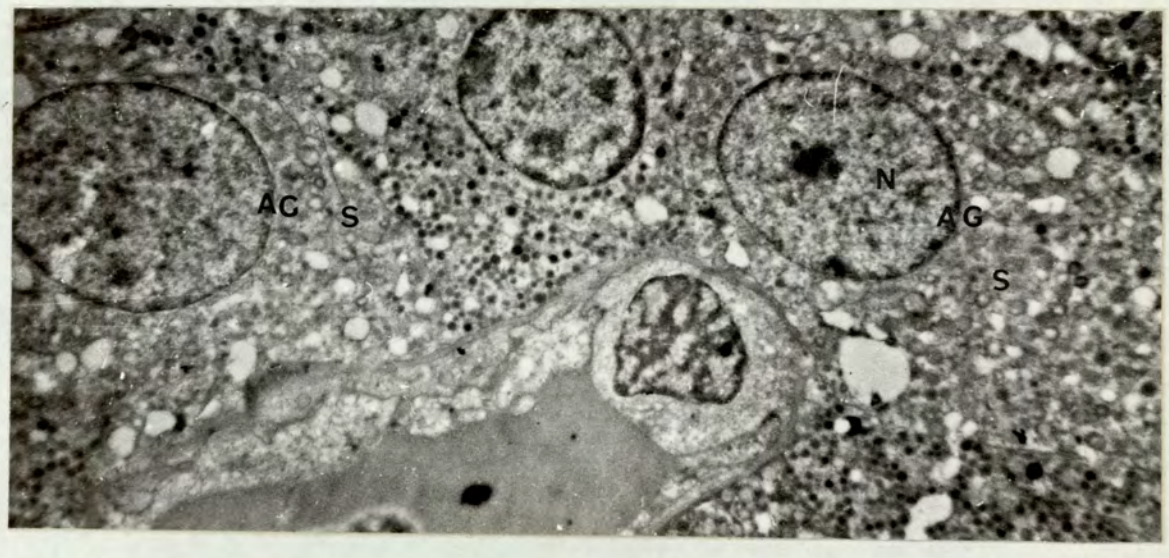


PLATE 25. Low power magnification of a portion of a principal islet of Tilapia mossambica showing two agranular cells (AG) with their prominent cytoplasmic sacs (S) and oval shaped nucleus (N).

Magnification: X 4,000

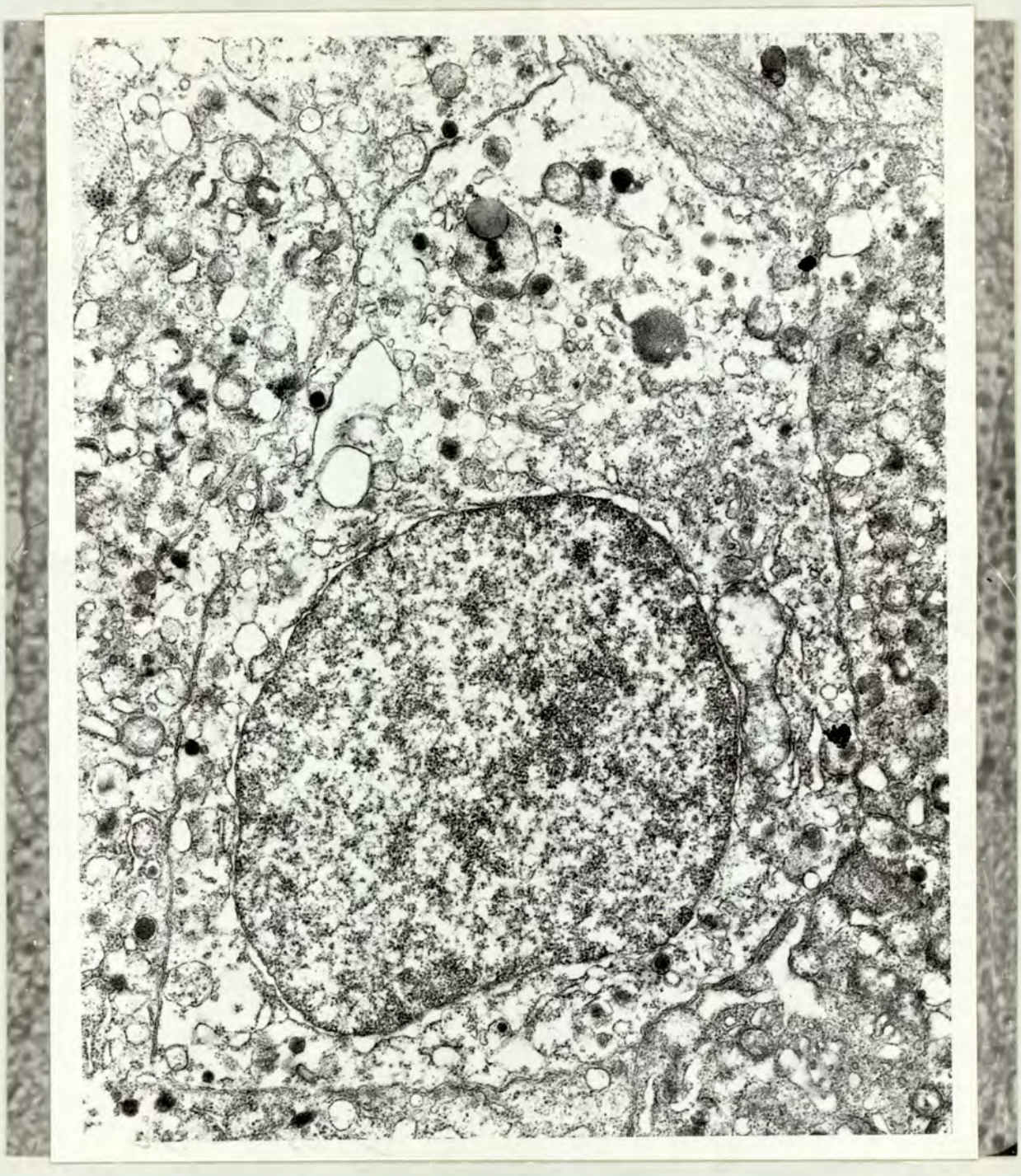


PLATE 26. High magnification of an agranular cell of the principal islet of Tilapia mossambica showing round nucleus (N) and numerous conspicuoues ergastoplasmic sacs (S). Secretory granules are almost completely absent: those present are moderate in electron density.

Magnification: X 9,600

These ergastoplasmic sacs were in such a large number and so prominent that the cytoplasm mostly appeared packed with them (Plate 26). The cells of this type, however, were observed to have most of the other cytoplasmic organelle seen in other cell types.

iv. In vitro experiments.

Initial experiments served to establish conditions suitable for incubation of principal islets. In these experiments morphological appearance was used as a convenient reference index for overall tissue viability. The frozen sections obtained from the islet tissue incubated in the three incubation media (V, Chapter 2, Section II) for about one, two and four hours were analytically examined and their cell structure was compared with that of normal fresh frozen section of the islet tissue. It was found out that though the islet cells, after one hour of incubation in Cottus and marine teleost Ringers were observed to have the same cytological appearance as that of the normal islet cells, they appeared to deteriorate on incubation for longer periods. On the other hand, cells of the islet tissue incubated in Kreb's Ringer bicarbonate buffer showed apparently no change in their cytological characteristics as well as in their affinity towards granule staining, and appeared almost intact and normal cells.

Observations to study the cytological changes in the islet cells after incubation of the islets with different hypo- or hyperglycemic agents were always made in comparison with those carried out on 'control' islets.

a. Incubation with Alloxan: Principal islets of Cottus scorpius were incubated, adding alloxan to the incubation media, for 5, 30 and 60 minutes and histological changes were observed. Light microscopy of the islets incubated for five minutes showed that no definite change occurred in their cell structure. The size of different cell nuclei was observed to be the same as in the islets incubated without alloxan. A slight but definite change could be observed in the

nucleus and cytoplasm of the beta cells after 30 minutes of incubation with alloxan. Some of the beta cells appeared slightly and occasionally moderately shrunken and the pericarpillary spaces more or less widened. In these cells, slight diminution of the beta granules in the cytoplasm could be detected (Plate 27). Mostly the cytoplasm of beta cells was seen packed with granules and it seemed difficult to say positively whether there was a definite increase or decrease of beta cell granules but it appeared sometimes that the granules probably reduced in number while the beta cells themselves remained distinct. Occasionally, in some of these cells slightly pycknotic nuclei with clumping of the chromatin could be observed. The size of the beta cell nuclei was found to vary from 2.5μ x 5.5μ to 3.0 x 6.0 μ in diameter, whereas that of alpha cell nuclei was approximately 3.5 x 7.0 μ in diameter. It could be said that the reaction of the beta cell nuclei was not always uniform with regard to the localization in the central or peripheral parts of the islet tissue, and that the affected beta cell nuclei showed a decrease in size.

After one hour of incubation the shrinkage of the beta cells was more commonly observed and mostly it appeared quite prominent (Plate 28), with cell boundaries becoming somewhat indistinct. Occasionally, the cytoplasm of these cells was also observed to be moderately vacuolated and somewhat granular. Nuclear pycknosis was frequently seen in these cells. The beta cells which were observed to be affected by alloxan, showed a decrease in size of their nuclei which measured approximately 2.5 x 5.5 - 6.0 μ in diameter. Again, the nuclei of the alpha cells did not show any decrease in their size. It was apparent that though mild degenerative morphological changes were observed in the beta cells after one hour's incubation, some of them were still showing the affinity for specific beta granule stains but the cytoplasm of these cells stained very faint and appeared foamy.

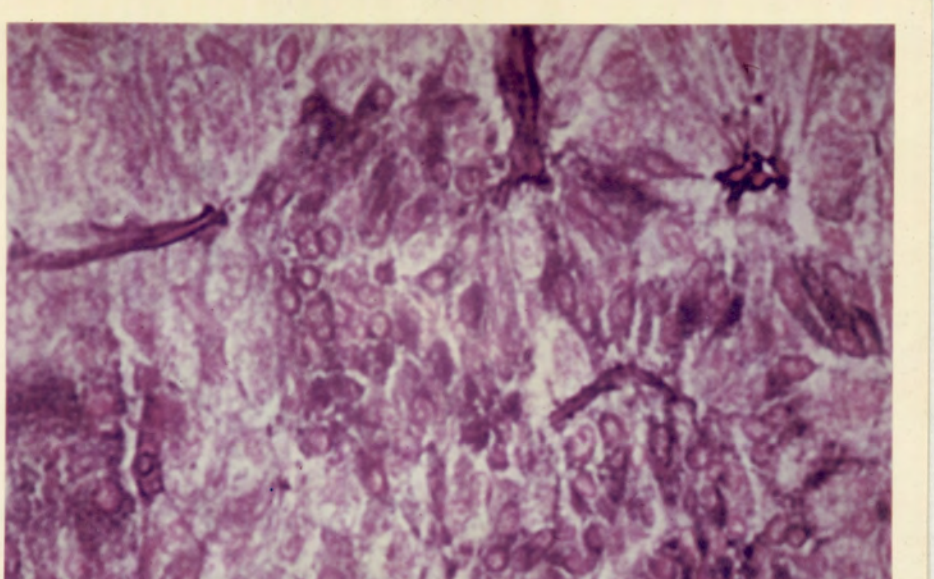


PLATE 27(a). Principal islet of Cottus scorpius showing early degenerating changes in the beta cells after incubation with alloxan for 30 minutes. Beta cells appear shrunke with diminution of their granules.

(aldehyde-fuchsin/Ponceau de-xylidine ; X 640)

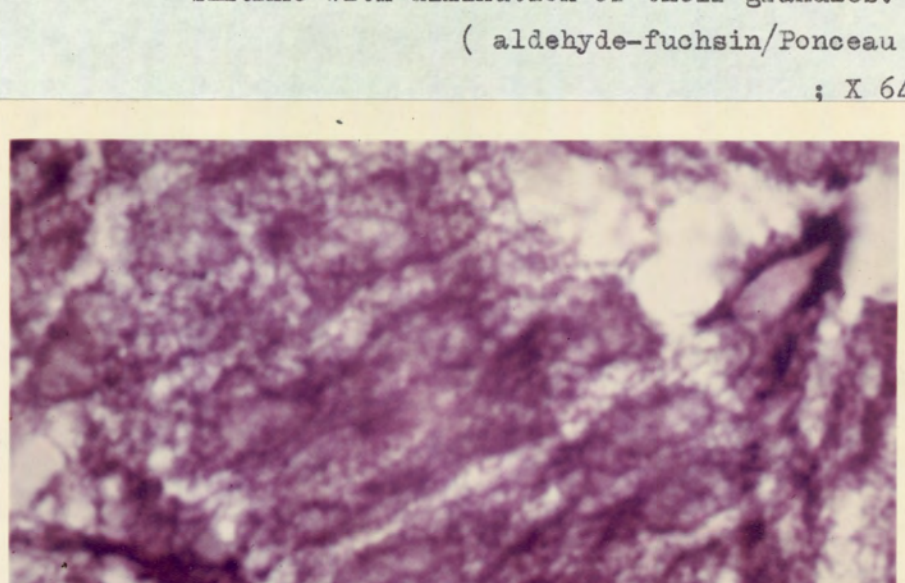


PLATE 27(b). High magnification of the damaged beta cells in a principal islet of Cottus scorpius after incubation with alloxan for 30 minutes. Indistint cell boundaries, wide pericarpillary spaces with loss of cohesion among the cells and loss of granules are apparent.

(aldehyde-fuchsin/Ponceau de-xylidine ; X 1600

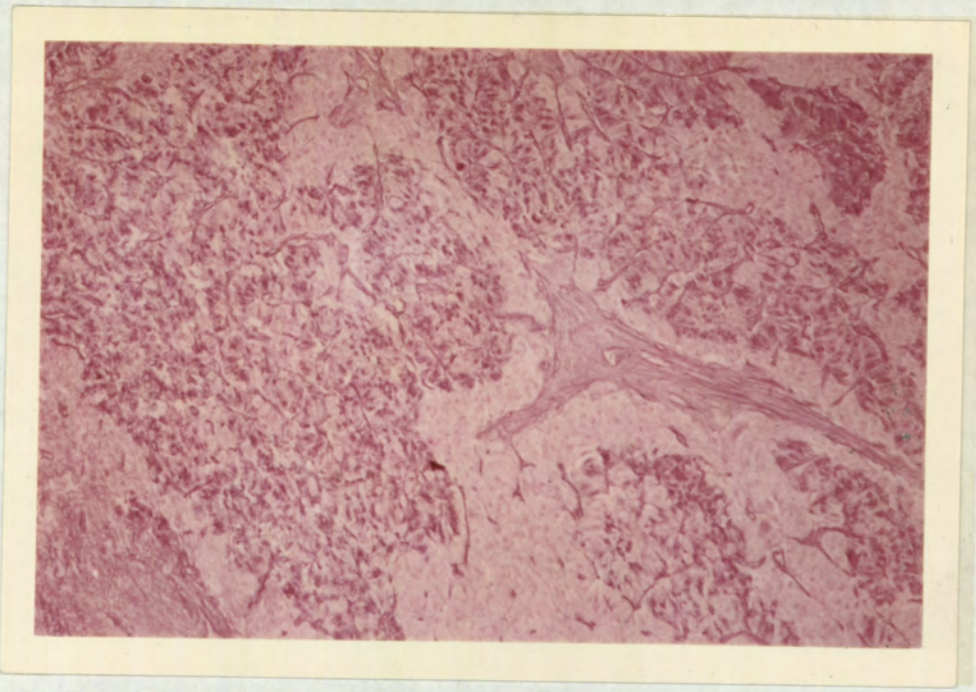


PLATE 28(a). Low power view of a principal islet of Cottus scorpius after incubation with alloxan for one hour. Nearly whole of the beta cell area shows far less affinity towards specific staining than in the normal islets.
(aldehyde-fuchsin/Phloxin; X 80)

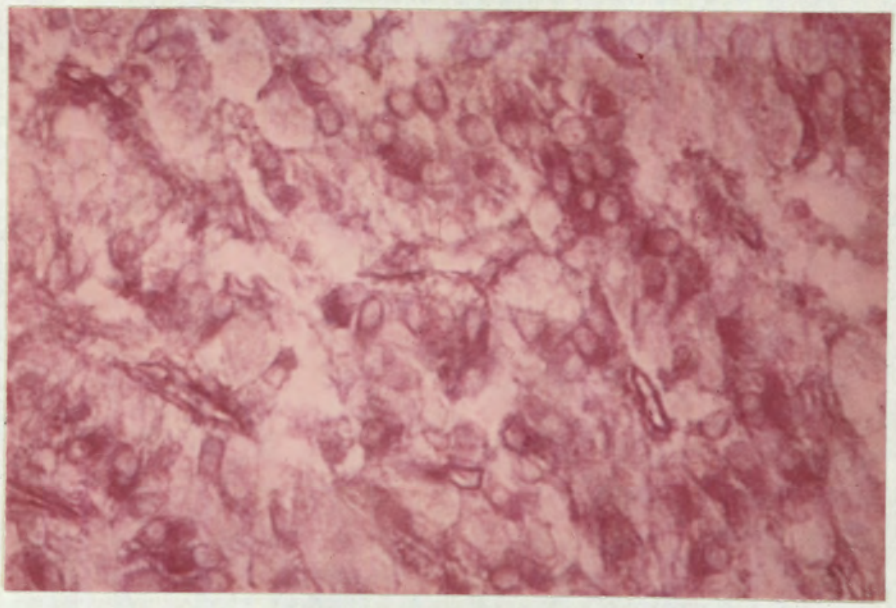


PLATE 28(b). Mild degenerative changes seen in the beta cells of a principal islet of Cottus scorpius after incubation with alloxan for one hour. In some cases nuclear pycknosis has occured.
(aldehyde-fuchsin/Phloxin; X 820)

b. Incubation with Growth hormone and Hydrocortisone: In the experiments carried out on the principal islets of Cottus scorpius incubated in vitro with growth hormone, no appreciable change was observed after first or second hours of incubation but it was possible to detect slight depletion of the beta cells four hours after the incubation. Most of the beta cells appeared normal and intact without any significant shrinkage of the cytoplasm (Plate 29). There was apparently no decrease in the nuclei of either alpha or beta cells; some beta cells, however, were approximately 3.5 x 6.5 μ in diameter, when measured, showing a probable slight increase in their size.

The principal islets of Cottus scorpius incubated in vitro with hydrocortisone, showed, as in the case of growth hormone, slight degranulation of the beta cells only after four hours of incubation. Nuclear pycknosis of any sort was never observed in any of the islet cells. As in the case of islets incubated with growth hormone, it was difficult to assess any significant change in the size of alpha or beta cell nuclei. The principal islets apparently were unaffected after one or two hour's incubation. (Plate 30)

c. Incubation with Tolbutamide: It was observed during this study that tolbutamide has an in vitro effect on the beta cells of the principal islets of Cottus scorpius. Though no significant change in the beta cell granulation was observed after one hour of incubation, nuclei of some of these cells appeared slightly enlarged. The nuclei of these beta cells varied from 3.5 x 7.0 μ to 4.0 x 7.0 μ in diameter in their size and it seemed that an increase in the size of the beta cell nuclei had occurred. Similar enlargement of some of the beta cell nuclei was also observed in the islets incubated with tolbutamide for two hours. The beta cells, in general, appeared moderately fat and somewhat healthier (Plate 30b). Though there

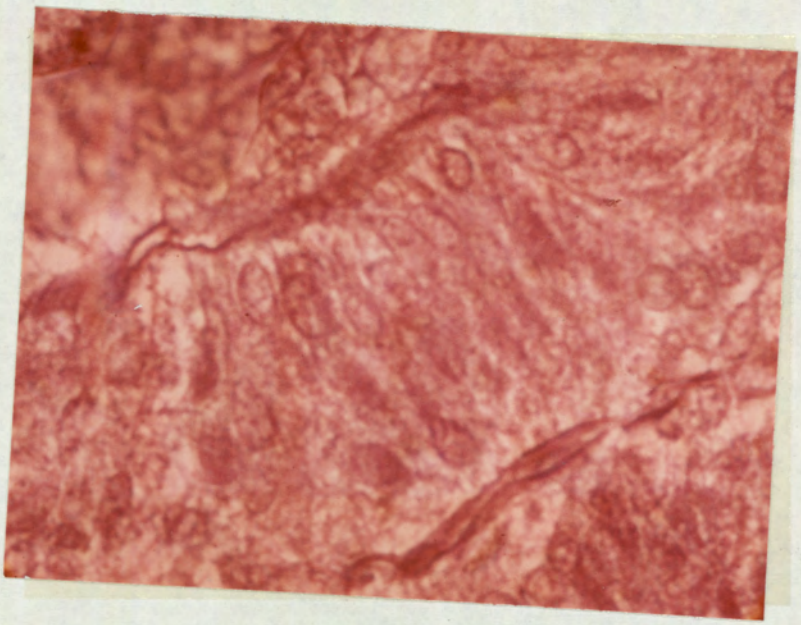


PLATE 29. Principal islet of Cottus scorpius after incubation with growth hormone for four hours. The beta cells appear intact and apparently showing no degenerative changes. (Masson's Trichrome stain), X 1170

were signs of partial degranulation in some of the beta cells, no distinct change in the internal structure of these cells was observed. The change in the beta cell granulation after four hours of incubation was also not uniform. Some groups of the beta cells apparently showed moderate to complete margination of the beta granules. Partial degranulation of the beta cells was also observed. Vacuolization of the cytoplasm, as observed in the beta cells after alloxan incubation was not infrequently seen in some of the beta cells. The nuclei of most of the beta cells appeared somewhat smaller than those observed two hours after the incubation. They measured approximately 3.0 μ x 5.5 - 6.0 μ in diameter. Occasionally the beta cells with slightly enlarged nuclei (3.5 μ x 6.0 μ in size) were still observed. Also, nuclear pyknosis in some of the beta cells of two- as well as four-hour incubated specimens was also seen. It seemed that nuclei of all the beta cells were not affected by tolbutamide in vitro at the same time.

Though no quantitative assessment was made to study the staining affinity of the beta cell secretory granules towards specific granular staining reactions, observations clearly indicated that most of the beta cells in islets incubated for one hour showed rather intense staining. The intensity of the staining did not increase appreciably after two hours of incubation but was slightly less intensified in the beta cells of four-hour incubated islets. It seemed that the secretory granules were not distinctly stained and the cytoplasm in these cells appeared faint and very foamy (31a).

Any significant effect of the hypo and hyperglycemic agents used in this study on the structure of cytoplasmic organelle observed with light microscope, was difficult to assess. A marked increase or decrease in the size of the nuclei of the beta cells probably correlates with their different functional states of insulin secretion due to the

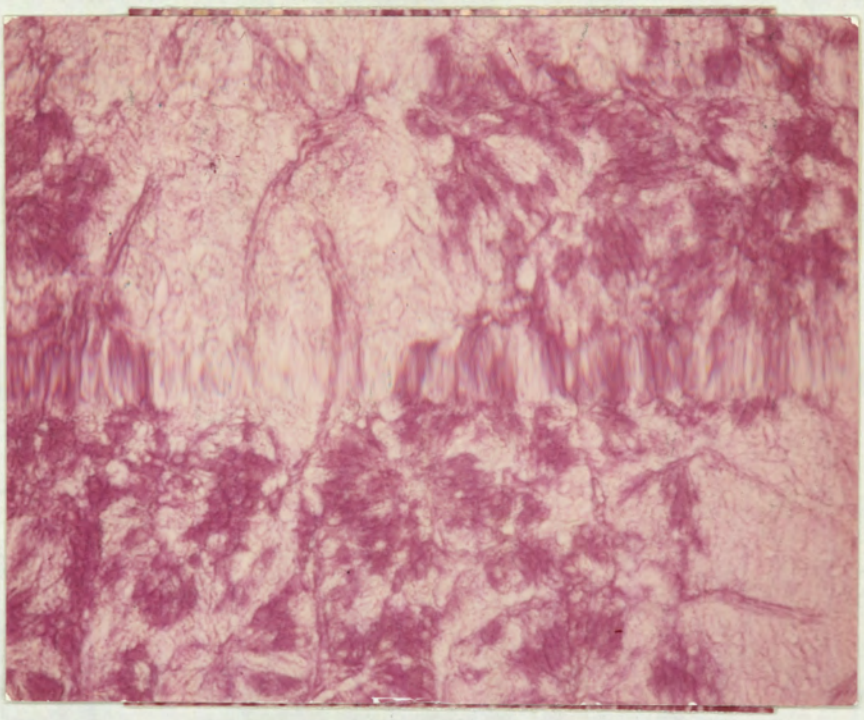


PLATE 30(a). Lower magnification of beta cell area in a principal islet of Cottus scorpius after incubation with tolbutamide for one hour. No degenerative changes as in case of alloxan incubated islets are present. Affinity towards specific staining appeared slightly increased.

(aldehyde-fuchsin; X 256)

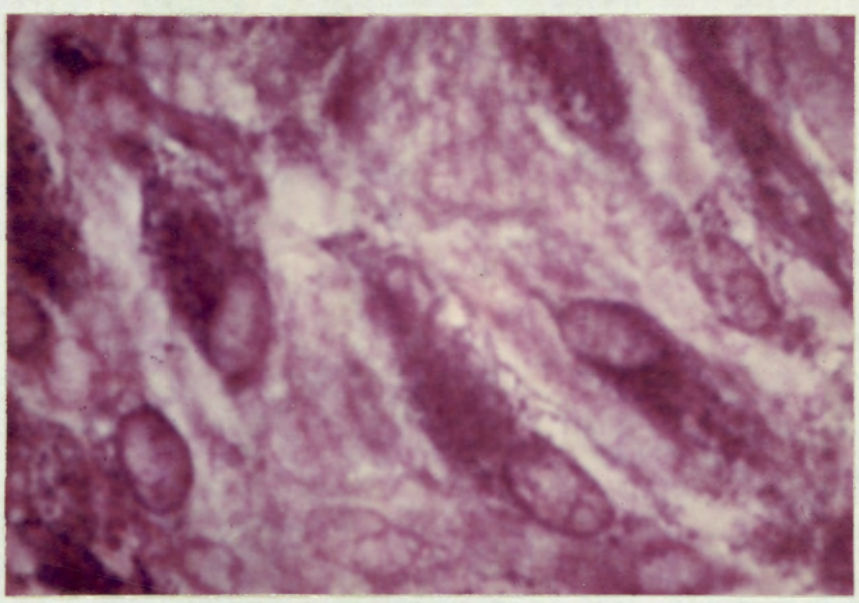


PLATE 30(b). High magnification of beta cells (as in 30a) showing slightly enlarged nuclei and cytoplasm packed with specifically stained granules.

(aldehyde-fuchsin; X 1600)

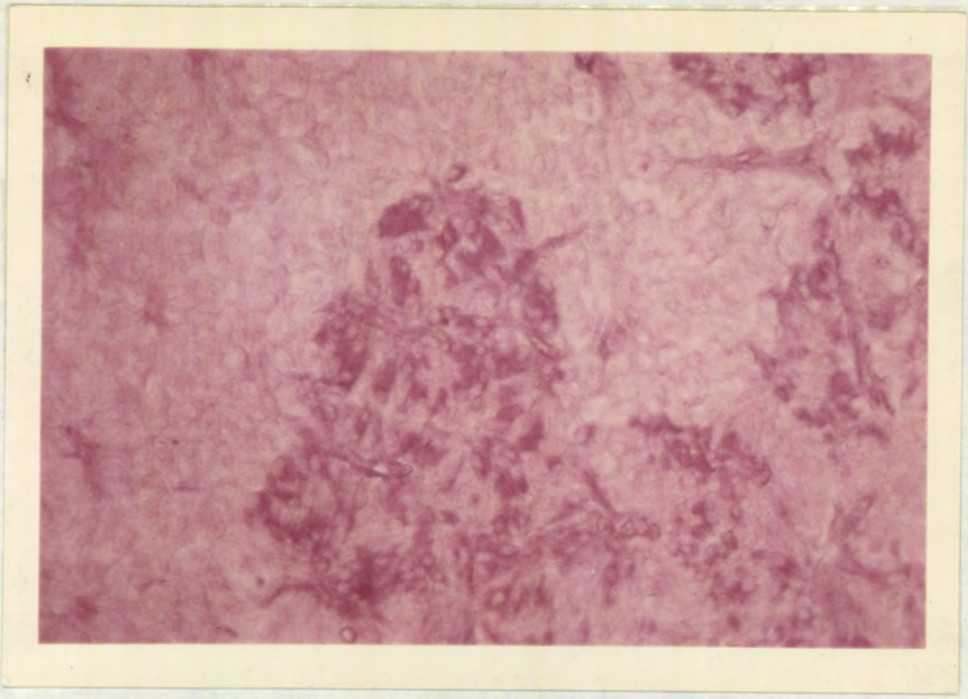


PLATE 31(a). Principal islet of Cottus scorpius, incubated with tolbutamide for two hours, showing faintly stained beta cells some of which appear slightly shrunken.
(aldehyde-fuchsin; X 410)

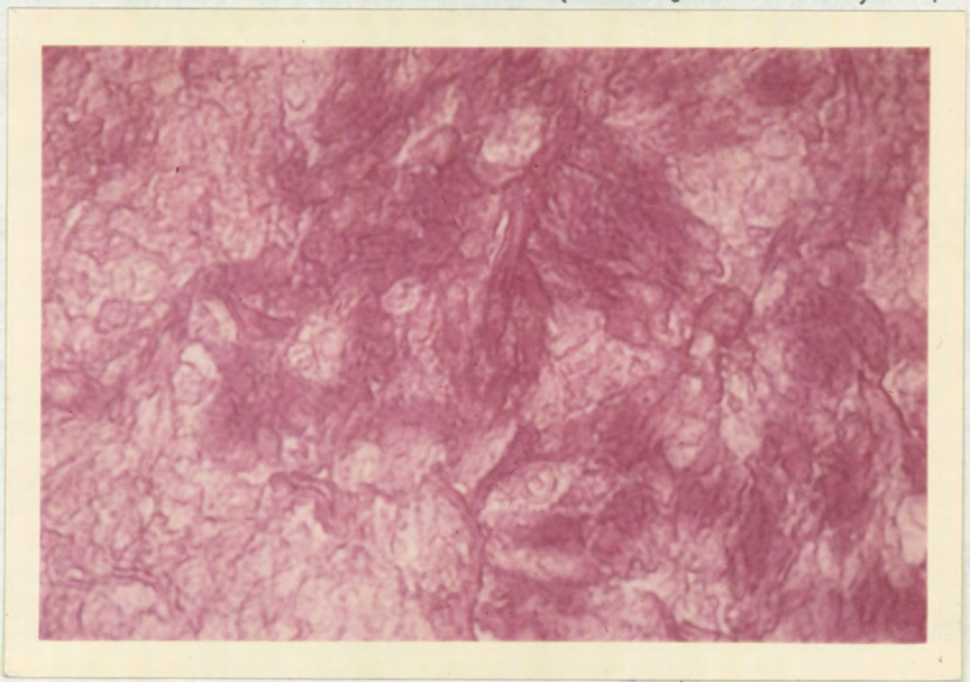


PLATE 31(b). Tolbutamide-incubated(4 hours) principal islet of Cottus scorpius showing partially degranulated and depleted beta cells some of which show nuclear pycknosis as well.
(aldehyde-fuchsin ; X 1024)

presence of these agents in the incubation media. The details of relative changes in the fine structure of cytoplasmic organelle of these beta cells have been best studied by electron microscopy. The sequence of increase or decrease in the nuclei of the beta cells during incubation with these agents for different periods of time probably showed the difference in the behaviour of the beta cells towards them.

v. Electron microscopy of islets incubated with tolbutamide:

The principal islets of Cottus scorpius incubated with tolbutamide for one hour were also examined with the electron microscope. The ultrastructure of the normal beta cells of this species in general is similar to the one described previously (71). The characteristic secretory granules, diffusely distributed throughout the cytoplasm were spherical in shape but with considerable individual variation in the electron density of the material contained within them as well as in their size. Each beta granule was enclosed by a membrane sac from which its central core was separated by an electron lucid halo of very short width. Contacts between the plasma membrane and the limiting membrane of beta granules were rarely observed.

The cytology of beta cells of incubated islets showed that despite reasonable preservation of its fine structure some cell damage had occurred and the extent of this varied from sample to sample. On the other hand, it did provide morphological evidence for in vitro survival of the beta cells, as most of the beta cells of the incubated islets were observed to be morphologically intact. These beta cells did not differ much cytologically from those of non-incubated islets. One of the major morphological changes seen in these beta cells was observed in their ergastoplasm. The endoplasmic reticulum in most of these cells appeared very prominent, the cisternae being very much dilated. Most commonly found were the parallel arrays of the cisternal lamellae, which apparently showed an increase in number (Plate 32a).

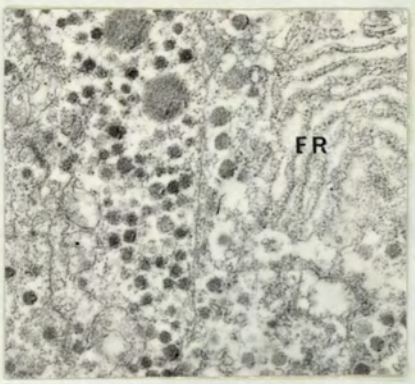
The particulate RNA granules attached to the lamellae appeared distinctly large and were also increased in number throughout the cytoplasm. In some cells the endoplasmic reticulum showed a semicircular arrangement (Plate 32a). In other beta cells, the ergastoplasm appeared vesicular with irregular contours (Plate 33b), with ribonucleo-protein granules attached to their outer surface. These vesicles were observed to contain a homogenous material of low electron density.

A striking change in the lameller ergastoplasm of some of these beta cells was its arrangement in concentric arrays so as to exhibit an appearance of 'finger-print-like' whorls. Such onion-like configurations (Plate 32b) were mostly observed in areas near the cell membrane.

Another significant change observed in the beta cells of incubated islets was in the appearance and localization of the secretory granules. There appeared to be no significant change in the population density of the beta granules. Most of the secretory granules showed a clear wide space between the dense portion of the granule and its limiting membrane, the latter being 'ballooned' out and appearing as a large sac-like structure (Plate 33a). The membrane of such granules appeared largely oval, round or irregularly shaped. The granular core of all the beta cells was of variable electron density as well as size. In many instances the granular sacs were empty (Plate 33a) with no electron dense material and appeared as membranous sacs of the beta cells probably showing their degranulation. The granules were mostly in close association with one another and in many instances the limiting membranes of more than one granule were observed either in very close relationship or apparently united with one another (Plate 33b).

It was apparent that in most of the beta cells a number of secretory granules showed migration towards the plasma membrane. The beta granules with their surrounding sacs were commonly observed adjacent to the surface

of the cell showing apparent margination of the beta granules (Plate 33b). A large number of beta granules were still present throughout the cytoplasm without any particular localization. Whether a significant increase in the number of beta granules which migrated towards the plasma membrane occurred due to tolbutamide stimulation of insulin secretion is not known. On many occasions, however, the limiting membrane of the beta granule was observed to have fused with the plasma membrane, the latter being slightly bulged outwardly at this place. Though neither intact beta granules nor a portion of them was ever observed outside the beta cells, outlines of empty spaces were seen just outside the beta cell membrane (Plate 33b). Probably the beta granule was occupying this space just before its release from the cell.



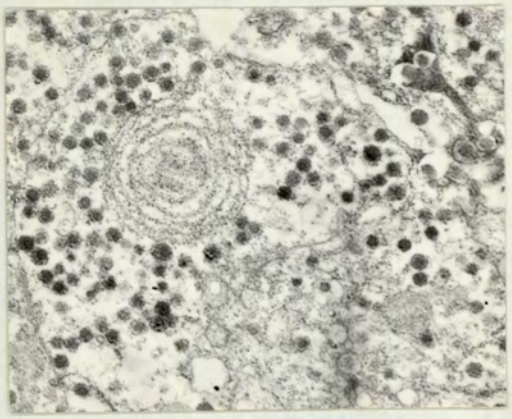


PLATE 32 (a) PLATE 32 (b)

PLATE 32. Tolbutamide-incubated principal islet of Tilapia mossambica

(a) . Beta cell portion showing semi-cicular arrangement of ergastoplasm (ER) with secretory granules having unrecognizable limiting membranes. Two zymogen type granules are also seen .
Magnification: X 10,000

(b) . A cicular configuration of ergastoplasm seen in a portion of a beta cell. A few granules are also seen having a wide halo. Some granule like structures without any content are also present.
Magnification: X 10,000

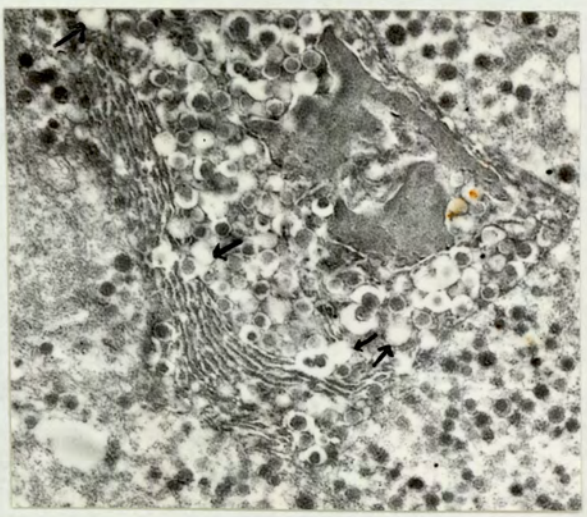


PLATE 33 (a). Portion of a beta cell in the principal islet incubated with tolbutamide, showing clearly distinct margination of the secretory granules. A number of granules are seen along the cell membrane in close contact. The space between the granular core and the limiting membrane is seen very much widened. Some spces are also seen which probably were occupied by the granules, (arrow). Magnification: X 10,000

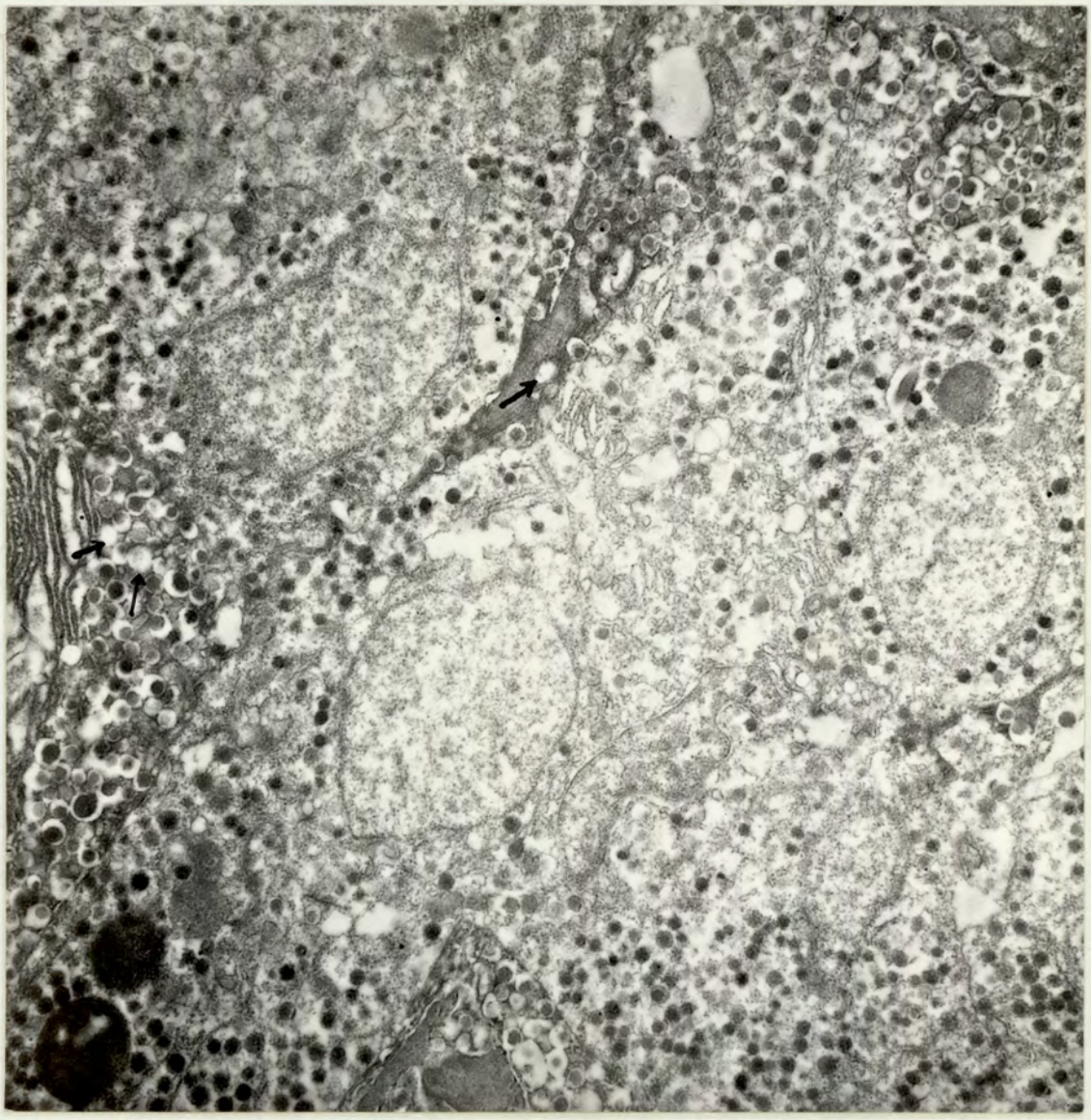


PLATE 33 (b). Portion of a tolbutamide-incubated principal islet of Tilapia mossambica showing beta cell survival as most of the celluar components e.g. cytoplasm, plasma membrane and secretory granules are intact and well preserved. The electron micrograph shows vesiculsr ergastoplasm (ER), increasing number of RNA particles 'ballooning' as well as margination of the secretory granules in the beta cells(B). Two zymogen like granules are also seen suggesting this cell probably to be a 'mixed' cell type. Spaces previously occupied by the granules are commonly seen in this electron micrograph.

Magnification; X 10,000

Chapter 4. DISCUSSION

A general survey through the literature revealed that in very few instances has the cellular composition of the principal islets of teleostean species, based on modern differential staining study been confirmed by electron microscopic observations. Though the special differential methods in general produced good results in the present investigation on the principal islets of Tilapia mossambica and Cottus scorpius, on certain occasions the limitations were also realised, even with these methods, and seemed to be the cause of controversial results regarding the identity of different cellular types of islet even within the same class of species. Realising these limitations, it was in this concept that light microscopic observations followed by electron microscopic study on the principal islets of Tilapia mossambica were found very useful for identification of different types of cells.

The present light microscopic observations on principal islets of Tilapia mossambica revealed as many as three types of cells - alpha, beta and agranular, but electronic microscopic observations clearly indicated the presence of a fourth cell type - D cell, as well. Previously the only light microscopic study on this species has shown the principal islet to be composed of one type of cell, i.e. beta cell (26). In other teleosts, on the other hand, in addition to the alpha and beta cells, a third cell type in the principal islet tissue has also been described and was termed as 'agranular' in the case of toad fish islet tissue and as 'clear' cell and 'δ' cell in the principal islet of bullhead fish (94) and carp (91) respectively. These observations were later confirmed by electron microscopic studies (72, 91, 95). The principal islet of Cottus scorpius seems to contain not less than three kinds of cells when examined light microscopically. On the other hand, earlier study on the principal islet of this species has put forward the evidence (17, 209) for the presence of four types of cells - beta, two types of alpha cells (a_1 and a_2) and agranular (208) but the results were not conclusive at the ultrastructural level (71).

Other studies on teleost principal islets were either brief reports (16) or with inconclusive results (93, 90, 180) but a most comprehensive review of the previous light microscopic investigations on the morphology of islet tissue of teleost fish - Lophius pictorius - did show (207) the presence of three independent cells, as were in Lebistes reticulatus (90), though in both cases the results have not been confirmed by electron microscopic observations as yet. In another teleost, tunny fish, only two types of cells, alpha and beta, have been reported (92) when studied light microscopically.

Though some of the cells of endocrine pancreas were rather difficult to classify, those of exocrine pancreas were very easily identified in the electron micrographs, by their characteristic features, e.g. large zymogen granules and prominent endoplasmic reticulum (232, 233). The ultrastructure appearance observed in the exocrine pancreas of Tilapia mossambica is in agreement with that in higher vertebrates (232, 233). In some cases, however, cells with zymogen granules of approximately the same size but having a matrix of lighter density were also observed and correspond to prezymogen granules as described in mouse (64).

Histological and histochemical studies in mammalian pancreatic islets have characterised essentially two types of granular cells - alpha and beta cells (56). Though there is disagreement over the identification of alpha and other types of islet cells, as discussed later, no controversy exists about the definition and function of beta cell in the pancreatic islet tissue (6 - 8, 53, 54). The cytoplasmic granules of these cells are said to be alcohol soluble (234) and are characteristically stainable with a variety of techniques, the most specific of which is the aldehyde fuchsin one of Gomori (235, 236) and they show strong affinity for chrome hematoxylin (235, 237) as well. The aldehyde fuchsin technique can be regarded as a histochemical method, and presumably demonstrates specific (238) SO_4 groups on the insulin

molecule or SO_4 groups on the matrix material in the secretory granules. The aldehyde fuchsin positivity is specific for β granules (239) and has been correlated with the insulin content of the pancreas (9). Moreover, the beta cells give metachromasia in the pseudoisocyanin procedure (221) and have been defined as insulin producing cells.

From my light microscopic observations it was evident that the affinity for the staining methods applied in this study was the same as in most of the other investigations on teleostean or mammalian pancreatic islet tissue, and there seems to be little doubt that pseudo-isocyanin-positive cells in the principal islet tissue are analogous to the beta cells in other vertebrates (6 - 8, 53, 54, 234 - 236). These cells appear similar to those described in the previous report on the principal islets of this species (26) and which were attributed to represent an active secretory stage, with cytoplasmic granules appearing finely and homogenously distributed with beta cell staining properties. There appeared no possibility of distinguishing two distinct stages in the activities of islet cells, based on the appearance and distribution of specific granules, as reported previously (26), despite the fact that slight variation in the distribution of cytoplasmic granules was observed within the group of beta cells.

Routine staining of principal islets of Tilapia mossambica did not result in any well defined division either in peripheral and central tissue, or of light and dark regions, as was found in Cottus scorpius, previously (17) as well as in the present study. Special differential staining methods, however, showed the principal islets as being composed of a ground substance of light cells with some centrally interspersed irregular islands of dark brown beta cells. In other teleosts the central dark region composed of beta cells has been observed (90, 207). On the other hand, some studies have shown the beta cells to be located in the light peripheral region (240).

Though planimetric determination was not carried out on the principal islet, it was apparent by visual assessment that no obvious difference in the proportion of beta cell population with respect to other types of cells could be observed in different principal islets in the same animal. Planimetric determination in Cottus scorpius has shown (17) that aldehyde fuchsin positive, i.e. beta cells, are roughly of the same proportion in both islets - pyloric and splenic. On the other hand, the two nodules of endocrine tissue in tunny fish (92) were seen to have different ratios of alpha and beta cells - one islet had about 44% increase in the beta cell population than the other islet in the same animal.

It appeared, again from the visual assessment, that in one principal islet the beta cells were more abundant than the alpha cells. This is in agreement to a certain extent with the observations made on several mammalian species (57). In the other teleost species, however, the number of two types of cells - alpha and beta - was found to be the same (72, 90, 92 - 94). Since the alpha cells have not been distinguished from the D cells, or rather the D cells have not been identified in the studies on these teleosts, as discussed later, and as D cells are now being considered to be a regular part of the islet components (62, 1 - 3, 113, 123, 222), D-cells might be present in them, contributing to the estimated population of the alpha cells.

Criteria for the identification of beta cells of pancreatic islet tissue at the ultrastructural level have been defined and confirmed in a number of mammalian (74, 76, 122, 241) and teleostean species (71,72,95). As a result of electron microscopy, it was possible to identify clearly the beta cells in the principal islet of Tilapia mossambica because of the characteristic features of the specific secretory granules, as described in the pancreatic islet tissue of mammals and teleosts. The present study shows that the granular cell type 1 is apparently the insulin producing beta cell. The cytoplasm of the beta cell was observed to contain beta granules enclosed in smooth membranous sacs,

smooth membrane vesicles of the Golgi body, mitochondria and prominent endoplasmic reticulum composed of double lamellae or membranes with attached ribonucleoprotein granules on their outer surface. The ultrastructure of the granular cell type 1 - its granules and cell organelle - corresponded well with that of beta cell in other teleostean species (71, 72, 95). The beta cell granules, as generally accepted, were always larger in size and less electron dense than the alpha cells, in the principal islets of Tilapia mossambica. On the other hand, in certain species, e.g. mouse (82), cat (74, 83), or man (76, 119), the beta granules have been observed to be of the same size as the alpha cells. In the carp, however, the maximum size of the beta granules has been described as being smaller than the maximum size of the alpha granules (91). The beta granules of principal islet of Tilapia mossambica, having a moderate electron density, varied considerably in size as well as in density, even within one cell, conforming well with the observations made in other species (71, 74, 76, 222).

The membrane surrounding the granule core is usually more closely adherent in the alpha cells than the beta cells and it has been considered a feature common to most of the beta granules in many vertebrates (74 - 76, 110, 115, 123, 227, 241 - 245) but during the present study it was found difficult to characterise beta cells on this feature. It was observed that mostly the electron optically empty space between the enclosing membrane and electron dense core was not wide, but quite narrow and sometimes unrecognisable. A similar minute space between the limiting membrane and the dense vesicle was observed in the principal islet of other teleosts (71, 72) and probably this feature in teleost species is constantly different from that in higher vertebrates. Beta granules with large halo, however, were observed as well.

Mostly the beta granules were spherical in shape, as in other teleosts (71, 72, 91) but sometimes irregular beta granules, as in bullhead fish (72) were also observed. The beta granules on rare

occasions were found to be bar-shaped with indistinct fibrillar material. Such granules have usually been seen in the pancreatic islets of chicken (74). In the carp (91), however, the beta granules have been reported to be spherical as well as elliptical in shape. It has been suggested previously that morphologic variations of beta cells granules in species of the same class or different classes, may reflect differences in the molecular structure of either insulin or its associated binding protein (74 - 77, 110, 115) and that the beta cell granule morphology seems to be characteristic for most species. Whether occurrence of two different shapes of beta cell granules in the principal islets of one species, i.e. Tilapia mossambica or even within one beta cell, is due to different molecules of insulin being produced in the cell, is not known. In a comparative electron microscopic study of the pancreatic islets in species ranging from Congo eel to dog (74), though some morphologic similarities were observed within the same class, it was found difficult to assess the pattern of variation relative to environmental activities or to any evolutionary (phyto-genetic) sequence in these animals. Different types of beta granules have been reported in the pancreatic islets of toad (247) and these have been classified as 'a', 'b' and 'c' type depending upon the size and density of the beta granule as well as on their number within one beta cell. Of these varieties, the 'c' type of beta granule has been considered to be similar to those seen in the exocrine cells and, therefore, were ascribed to be as zymogen granules (247). No such variation in the characteristics of beta granules was found in the principal islets of Tilapia mossambica, although in one electron micrograph, very large sized granules, along with characteristic beta granules, were observed in the same cell. Whether it is a natural or artificial state of the zymogen granule is not known. This cell may or may not be of the type described as 'mixed cell' in the pancreatic islet of toad (247).

Some authors have presented evidence (241) for an inverse relationship between the amount of ergastoplasm and ribosomes and the beta granules and for this reason they have postulated that beta cell goes through a cyclic transformation recognisable at the extremes by two major structural patterns - which were called 'granular' and 'agranular' - with gradations between these two forms. The beta cells, representing two distinct stages - 'granular' or agranular' were not observed in the principal islets of Tilapia mossambica.

The present histological and histochemical study on the principal islets of Cottus scorpius showed that the pseudoisocyanin positive cells were similar to those of mammalian beta cells in their tinctorial properties, as in the case of Tilapia mossambica, and were, therefore, identified as the beta cells. These results agree very closely with those obtained in the previous investigation (17) and therefore confirm the presence of beta cells in this teleost species.

It is now well established fact that glucagon is produced in the pancreatic islet tissue, as modern immunofluorescent techniques applied recently in the studies of pancreatic islet cells have confirmed (248, 249) the earlier findings (56, 250) that alpha cells of the pancreatic islet tissue are the source of this hormone. The principal islets of teleosts have also been clearly demonstrated to contain glucagon by the glycogenolytic effect (251) as well as by hyperglycemic action of this hormone (20) and it has also been indicated that this hormone produced by the alpha cells is identical to that of mammals (252). The present study has shown beyond doubt that the alpha cells exist in the principal islets of Tilapia mossambica, as there were good reasons to believe that the glucagon producing cells ought to be present in the islet tissue. The majority of the granular cells in the principal islet which were observed to be the pseudoisocyanin negative in character both showed tinctorial and other features like those of mammalian alpha cells, as described in numerous species (6 - 8, 53, 54, 56, 234, 237) and therefore, are the alpha cells. It was observed

during the present study that it is quite possible that a definite and clear identity of alpha cell may be confused with another type of cell - D cell. Though all the pseudoisocyanin negative cells demonstrated relative tissue acidophilia with counterstain (phloxine) after aldehyde fuchsin, slightly different cells can be identified as alpha cells. The previous study on this species (26) failed to observe any cell with granules having any affinity for phloxine. It has been pointed out that correct identification of alpha cell by light microscopic observations could be possible by using staining characteristics of the polychrome method (6, 53, 57). PTAH stain has been seen to be specific for the type of cell described originally in a number of species (6, 53, 57) as the alpha cell and which was later on found to be non-argyrophilic in nature (61, 61a, 62). Recently the alpha cells have been re-examined (75) in a number of mammalian species to characterise its identity. The present study indicated that all the pseudoisocyanin negative cells were PTAH positive and completely non-argyrophilic and were therefore identified as alpha cells. Other cells, similar in appearance and which although did not show argyrophilia, ~~as argyrophilic staining reaction did not prove successful in the present day~~, appeared to be PTAH negative. Despite careful staining it was rather difficult to identify clearly these cells and hence they were often confused with the alpha cells.

These alpha cells were definitely included in the beta cells during the previous study on this species (26). It may be mentioned that the use of orange G following aldehyde fuchsin, or phloxine following chrome alum hematoxylin used in the previous study, merely demonstrates relative tissue acidophilia and there is a possibility that quite a different cell could be taken for the beta cell. Or probably the alpha cells correspond to those cells observed (26), in some cases with reduced number of granules and were therefore considered to be of a less secretory stage. It may be mentioned that pancreatic islets of

Xenopus laevis, a toad, which were once reported to possess only the beta cells (102) were later on demonstrated to contain alpha cells as well (62). Similarly the demonstration (99) of only one type of cell - the beta cell and an absence of alpha cells in urodeles (newts and salamanders) was later on proved wrong by a recent study (74) with correlative thick-thin microscopy which revealed the presence of blue-stained and red stained granules which, with electron microscopy, appeared either oval to irregular (beta) or round, dense (alpha) respectively. In contrast, in a light microscopy study (253) of neotenous urodele the presence of unilayered tortuous bands of beta cells has been reported with the absence of the alpha cells. The negative findings of the alpha cells in the principal islets of Tilapia mossambica (26) by histological study could be a result of seasonal or nutritional stages. It may be due to staining properties and distribution of acidophil islet cells as well. It is quite probable that previous workers studying the principal islet tissue of Tilapia mossambica (26), urodeles (99) and Xenopus laevis (102) would obtain similar results as observed in the present study, if they were to use additional stains or modifications and follow by confirmation with electron microscopy.

From light microscopic observations on the principal islets of Cottus scorpius, it was apparent that pseudoisocyanin-negative cells were similar to those seen in the principal islets of Tilapia mossambica and were analogous to the mammalian alpha cells, as they resemble in their tinctorial properties those described for the alpha cells in the pancreatic islets of a number of mammalian species (6 - 8, 54, 56, 234, 237). On the other hand, in the previous study on Cottus scorpius evidence was presented to prove the existence of two types of alpha cells based on their response to silver staining techniques (17, 209). It was indicated that the alpha cells exhibiting argyrophilia (silver positive) in the dark central region appeared to be identical and

corresponding to the argyrophilic type of the alpha cell classified in rat pancreatic islet (61) as a_1, which since has been consistently reported by the Uppsala group in frogs (78), reptiles (79), birds (111) and a number of mammals (254 - 258) including man (254). Likewise, the alpha cells showing no positive reaction to silver staining procedure in the principal islets of Cottus scorpius were considered (209) analogous to a_2 type of alpha cell described in various species (61, 78, 79, 111, 254 - 258). No such distinction between alpha cells to be classified as sub type or independent, according to the Uppsala group were found in the principal islets of Tilapia mossambica. My observations on this species regarding the alpha cell are in general agreement with those described in other teleostean speices (23, 90, 91, 94, 207, 259, 260) including toadfish (23), bullhead fish (94) and carp (91). This difference in the present results on Cottus scorpius from those of previous study (17, 209) can easily be explained by the fact that D cells were not identified in the latter studies and a_1 cells reported by them probably correspond to the D cells (observed in the principal islets of Tilapia mossambica by electron microscopic observations) as many other workers have demonstrated in a number of mammalian as well as teleost species that in fact these argyrophilic cells (referred to as a_1 cell) are the D-cells of the islet tissue (261 - 267), observed by light microscope as well as by electron microscope. It is interesting to note that the distinction between a_1 and a_2 cells in the principal islet of Cottus scorpius as observed light microscopically was not conclusively characterised. Though typical alpha cells were identified as the a_2 cell , a_1 cells were not shown (71) at the ultrastructural level. In the principal islet of tunny fish (92) two types of alpha cells were distinguished, mainly on the basis of the size of the nucleus and the variation in the intensity of cytoplasmic coloration. These, however, were considered as an independent type of cells but were regarded basically as the two functional entities, as a reaction of the adaption to the rising of

metabolism and were attributed to active and resting cells. The functional variation in the endocrine cells in the principal islets in marine teleost, Scorpaena scorpa has also been assumed (259, 260).

It has been possible to identify the alpha cell in the principal islet of Tilapia mossambica at the ultrastructural level, thus confirming the light microscopic observations. It was clear that the granular cell type 2 possessed all the characteristic features particularly the secretory granules, of the alpha cells as characterised originally in mammals (76, 77, 83, 110, 243 - 246) and later on confirmed in the pancreatic islet of a variety of species (71, 72, 74, 75, 95, 241, 242). The alpha secretory granules in the principal islet of Tilapia mossambica were always spherical in shape as in toadfish (95), bullhead fish (72) and Cottus scorpius (71). On the other hand, these granules have been described as being polymorphic, spherical, horse-shoe shaped or bars, in the principal islets of carp (91). The observation that the alpha secretory granules were always greater in electron density, smaller in size than the beta cell granules and that there occurred little degree of variation, if at all in size and electron density, conformed well with those seen in the principal islets of other species (74 - 76, 83). Again in carp (91), the alpha cell granules have been shown to be of bigger size than the beta granules. It has been considered a common feature of the alpha granules that the limiting membranes are relatively more closely applied to the granule core (74 - 77, 83, 241 - 246), but the present observations indicated that most of the alpha granules, as compared to the beta granules, had much wider space between the limiting membrane and the granule core. Similar observations have been made in the bullhead fish (72). It appears that this cell is similar to the alpha cell (designated as P_3) in the Cottus scorpius (71). It is quite possible that the alpha and beta cells identified in Tilapia mossambica and other teleost species (71, 72, 95) might have been wrongly identified as beta and alpha cells

respectively in carp (91), on the basis of characteristic features of the cells described in mammalian species.

In the present study alpha cells were not found to exist in two types as strongly suggested even by ultrastructural studies (81, 82, 120). These authors have characterised a_2 cells to contain secretory granules, enclosed in concentric membranous sacs. The granules were dense but revealed a reticular or granular internal structure (81). In mouse pancreatic islets the type which appeared to be arranged in syncitium was thought to represent a_2 cells whereas another type having a complete membrane was identified as a_1 cell (82). No such differentiation among the cell types was observed in the principal islets of Tilapia mossambica. However, it is probable that the alpha cells identified in the present study correspond in general to the a_2 cell as described in various species by the Uppsala group (81, 82, 120). Throughout the present study, 'mixed cell' of alpha type, as described in the pancreatic islet of toad (247) with a different type of secretory granules 'f' type considered similar to zymogen granule of exocrine cell) was never found in the principal islet of Tilapia mossambica. Recently, three sub-types of alpha cells denoted as A_a, A_b, A_c have been described when pancreatic islet of some mammalian species were re-examined electron microscopically (123). This distinction was made predominantly on the size of the secretory granule and their relationship to the limiting membrane surrounding them (123). No such sub-types of alpha cells were possible to be classified during the present study. It was not clarified whether such sub-types of alpha cells represent distinct cell types secreting different hormones or are simply stages in the life cycle of the alpha cell. It seems that if at all there are three types of alpha cells, the granules of which are slightly different from each other, they represent simply the different functional stages of the alpha cell. In another study also, two functional stages of the alpha cell, - secretory and non-secretory stages - have been postulated (241).

It was not possible to get any support for even this suggestion in the present electron microscopic observations.

In addition to alpha and beta cells, principal islets of both Tilapia mossambica and Cottus scorpius were observed to consist of another type of cell, when examined light microscopically. These cells in their characteristic features, i.e. absence of any secretory granules, appeared similar to those defined as 'agranular' or sometimes 'clear' cells in the pancreatic islets of a number of species both mammalian and teleosts (17, 23, 91, 94, 208, 259). They appeared to have many points in common with these cells of pancreatic islet tissue of other bony fish (51, 90). When examined with the electron microscope, the principal islets of Tilapia mossambica showed certain cells (cell type 4) which were similar to those observed in Cottus scorpius (71) where they have been called 'agranular' and to those seen in bullhead fish (72) termed 'clear' cells. It was apparent from the present study that these cells are the agranular observed by light microscopy. Early description (6) of a clear('C')cell in the guinea pig, which on the basis of solubility and staining characteristics could not be considered as alpha or beta cell, was later on confirmed by reports in man (53) (third cell type) and guinea pig (54) but not in any other species, and these cells were characterised as lacking any secretory granules. However, studies in man (53) described granulated and non-granulated delta cells. Since then a number of light and electron microscopic studies have shown cells with absence of secretory granules. It has been pointed out recently (75, 113, 123) that a degranulated cell if present should be identified as correctly as possible, reserving the term clear ('C') cell for the cell lacking any sign of secretory granules. Hence, strictly following the original definition (6, 53, 54) as having an empty cytoplasm, these authors stressed that the term 'C' cell should be used for only cells with complete absence of secretory granules (75, 113, 123). Following this definition they were able to identify 'C' cell only in the pancreatic islet of guinea pig (75 - 77, 113, 115, 123).

It was quite clear that no such 'C' cell was present in the principal islet of Tilapia mossambica. Most of the cells (cell type 4) did have a few secretory granules. On this definition, the clear cells as described in bullhead fish are, in fact, agranular cells. There has been a controversy regarding the identity of agranular cells in many species, as a clear cell whose identity could not be established was noted in the rabbit (201) and it was suggested that they might represent the delta cells. In another study (268), in the same species, no granules were present in the delta cell by light microscopy. The agranular cells of Cottus scorpius were found similar to the δ cells of the cat (83) and the γ cells of guinea pig (76, 77). The present study does show resemblance of agranular cells with δ of cat (83) in having vacuolated ground cytoplasm. Very recently sparse occurrence of such cells has also been observed in the principal islets of Chinese hamster, and were described as 'clear' cell by one worker (267), and as agranular by another (269), the latter showed it to have features common with the 'C' cell in guinea pig (76, 77). Such agranular cells have been observed even in the pancreatic islets of primitive vertebrates (lamprey) where they were localised around large intralobular cavities (70, 87). Similar agranular cells have also been reported in the regenerating islet tissue (270) as well as in islet tumor of man (271). It was suggested as a result of light microscopical investigations on some cartilagenous fish (272) that, like alpha cells, agranular cells could be divided into two sub-groups depending on the presence and absence of argyrophilia. However, silver positive cells were then found to be the a_1 cell of higher species suggesting that a_1 cell may have an agranular cytoplasm inhibiting affinity for light green (273). No such differentiation among the agranular cells in the principal islets of either Tilapia mossambica or Cottus scorpius was obvious. In fact, these cells completely lacked argyrophilic properties which is in agreement with previous findings (17, 208, 209) on the principal islet of teleost species.

The nature of these agranular cells is completely unsettled and remains to be determined (123), the main reason for this may be their very poor response to any specific histological, histochemical and experimental characteristics. It was suggested that agranular cells of Cottus scorpius may correspond to the young immature forms of the granular cells of the islet tissue (17, 71, 208). The light microscopical and ultrastructural demonstration of such cells in the islet tissue of human foetus is in line with this view (273). Very recently the observations made on these cells in the principal islets of normal, subtotal pancreatectomised and alloxan treated Chinese hamster (269) rather strengthened the previous view (208), and these cells were thought to represent immature precursors to the beta cells, and in the diabetic animals may be interpreted as a sign of regeneration of the islet parenchyma. They may represent undifferentiated reserve cells capable of developing into any other cell type. Their abundancy in mitochondria as observed in the lamprey islet tissue (87), however, seems to indicate a more energy demanding activity. It may be a possibility that during their differentiation they correspond to the amphiphil cells described in some species (103, 264).

On the other hand, studies in the Chinese hamster by another worker (267) indicated the abundantly occurring agranular cells in the diabetic state to represent degranulated beta cells. Similarly, these cells in the principal islet of toadfish (95) have previously been considered to represent a secretory stage of the beta cells, as they observed numerous empty sac-like golgi body and abundant endoplasmic reticulum. Likewise these cells in another teleost - Scorpaena scorpa - were regarded in most cases to be only alpha cells in the degranulation stage (259, 260). The suggestion (115) that 'C' cells in rabbit islet are degranulated alpha cells was re-evaluated in a re-examination of the pancreatic islets by electron microscopy recently and 'C' cell was not used for these cells afterwards(123).

Electron microscopic observations on the principal islets of bullhead fish (72) could not reveal any transitional form between clear cells and alpha or beta cells and, therefore, it was stressed that these cells do not represent a functional stage of alpha or beta cells but were interpreted as a distinct islet cell.

Electron microscopic observations on the principal islet of Tilapia mossambica demonstrated that granular cell type 3 apparently shows most of the characteristic features described for the D-cell in a number of mammalian species (85, 113, 123). This cell type, though with few secretory granules, was quite different from the cell type 4 (agranular cell) in not having abundant ergastoplasmic sacs. It was easily distinguished from the alpha cell (granular cell type 1) on the basis that the secretory granules were larger in size and of low but uniform electron opacity. Beta cells, on the other hand, have been seen to show a degree of variation in size as well as in electron density in their secretory granules. Its overall electron density was distinctly greater than the beta cells. Furthermore, the secretory granules were often found close to the plasma membrane, mostly towards one end of the cell, as observed in typically defined D cells (75, 113, 123). It was, therefore, concluded that D cells analogous to those of mammalian (113,123) pancreatic islets are present in the principal islets of Tilapia mossambica. Surprisingly, it was not possible to identify distinct D cell in this species when studied light microscopically. It may be due to the technical fault during the staining procedure. These cells are probably of the same size as the alpha cells and on failing to respond specifically to the stain they probably appeared as alpha cells and not distinct cellular types. D cells have been found difficult to stain in the pancreatic islets of Amphiuma (62). It is also reasonable to assume that as they showed few secretory granules (observed by electron microscopy) these cells might have been mistaken for agranular

cells. The former possibility seems more plausible since it has been now consistently pointed out that D cells in the pancreatic islets are similar to the a_1 cell type (according to the nomenclature of the Uppsala group) which have been differentiated from typical alpha cells (a_2) by them mainly on their argyrophilic properties (61, 209, 254 - 258).

The existence of D cells in the pancreatic islets described originally in man (53) has been the subject of considerable controversy (56) particularly with regard to its correlation with the a_1 cell type of the Uppsala Group on a common property of argyrophilia. Light microscopic investigation later on confirmed its presence in the pancreatic islets of numerous mammalian species (54) and were thus considered a constant cell type of vertebrate islet tissue. They were overlooked and confusion arose when such classification of alpha cells - a_1 (silver positive), a_2 (silver negative) was suggested in a variety of species (61, 79, 209, 111, 254 - 258), as the D cells have also been shown to exhibit positive argyrophilic properties (62, 89, 261, 263, 265) and it was demonstrated (89) in a teleost fish as well as in dog and rabbit that argyrophil cells (a_1) correspond to the D cell. Argyrophilic tincture of a cell cannot be regarded as basis of classification as some studies showed that argyrophilia in the beta cell as well as alpha cell (see 274) while other studies indicated this character in all the alpha cells (56, 58). On the other hand, the Uppsala group (79) found a special D cell lacking argyrophil nature which instead was seen confined to a cell type with different granule staining characteristics (a_1). They also put forward the evidence (273) that some islet cells in human foetus completely lacked argyrophil reaction and after silver impregnation were found to be like D cells when their granules were stained and therefore they hesitated to accept the concept that D cell is identical with a_1 cell. They, however, agreed (79) in finding a type of cell in snake islets which from its granule staining characteristics was described as a D cell (275), and which at ultrastructural

level was seen to contain sparse cytoplasmic granules of low electron density (276). It was pointed out, however, (263) that although Fujita's method of bleaching and restaining the silver specimen (89) coincides in principle with that of the Uppsala group, an important difference existed. The latter workers did not intend to examine cyanophilic (affinity to aniline blue or light green) of the cells in question, which is the only known criterion of D-cells. The fact that argyrophilic a_1 cell in some animals (255) showed a lower affinity to phloxine (in guinea pigs a_1 cells contained no fuchsinophil granules and were interpreted as 'C' cells (6) of Benseley, strengthens the suggestion that islet D cell and argyrophil a_1 cells are identical (61, 89, 261 - 266). It was, however, indicated that not only the a_1 cells but also the agranular cells display the cytoplasmic staining properties (affinity for light green) and therefore, complete identity of a_1 and D cells was not accepted by some authors (269). Light microscopic observations on Chinese hamster revealed a PAS trichrome stained cell having different localization from that of the a_1 cell (269) and it seemed that these green cells could present both a_1 and agranular types. The observations (111) that after adequate impregnation, no obvious transitional forms between a_1 and a_2 were found, were thought to represent the presence of two entirely independent categories of cells. It was admitted, however, that the biological significance of the fact that it is possible on the basis of argyrophilic reaction to divide the cell content into two fractions, is not clear (111).

Furthermore, estimation of D-cells (5% of all the islet cells) in dog pancreas (54) coincides with the observations and calculation of the number of D-cells in silver impregnated and granule-stained specimen (263), but it has also been pointed out (79, 272, 273) that descrepancy between the number of recognisable D cells and a_1 cells in some species probably exists. The Uppsala group, however,

believed that since the D cell is considered to contain cytoplasmic granules, which are difficult to demonstrate in the light microscope, further studies at electron microscope level might clarify this question (272).

D cells have now also been demonstrated at ultrastructural level in the pancreatic islets of a number of species, including rat (277, 113), rabbit (75, 227, 275), dog (75), cat (83, 279) and guinea pig (123). D-cells have been readily distinguished from alpha cells in Chinese hamsters (267). In the same species, however, concurrent studies (268) by the Uppsala group described the a_1 cell and the a_2 cells. The former cells were observed to show characteristics of secretory granules comparable to those of alpha and D-cells, while the latter were considered to correspond to the alpha cells observed by the previous authors (267). The present observation in the principal islets of Tilapia mossambica showed cells with D-cell characteristics, distinct from those of typical alpha cell (a_2). It seems that D cells identified in this study are probably the a_1 cells described in other investigations.

Very recently application of argyrophilic reaction on the osmium fixed plastic embedded sections (visualised both by light and electron microscopy) was demonstrated (281) but no attempt was made to distinguish between alpha and D cells. Argyrophilic and non-argyrophilic granular 'clear' cells, in addition to the beta cells, were distinguished.

No report has appeared about the occurrence of D-cell in the teleost except the one in Carp (91) where certain cells have been identified as non-granular D-cells. It is quite probable that those cells which were distinguished in the light microscopic study on the principal islets of Tilapia mossambica (26) showing very few stainable granules and considerably reduced cytoplasm, are in fact the D-cells identified in the present ultrastructural study.

These cells were considered however as belonging to the post secretory stage of the beta cells (26) and not as distinct D-cells. Similarly, the type of cells rarely observed in the principal islets of Cottus scorpius when examined electron microscopically (71) are probably the cells corresponding to the D-cells observed in the present study. These cells, however, were thought (71) to be in some way related to, or of the same nature as, the granulated delta cells of cat pancreatic islets (83) but were not considered to be a regular constituent of the principal islet tissue (71). It appears that further electron microscopy of the principal islet tissue of Cottus scorpius would probably indicate the existence of D-cells. It is interesting to note, however, that in a recent electron microscopic study on the pancreatic islets of rabbit, dog, guinea pig and opposum (75), as many as eight types of cellular types in all have been suggested to be present and were termed alpha, beta, clear 'C', D, E, F, A_b and A_c cells.

Recently, observations in human pancreas have suggested (119) that Δ (D) cells are modified alpha cells and that these are intermediate cells representing a transition from alpha cells to D-cells. I could not find any obvious intermediate cells in the principal islet of Tilapia mossambica. No support for this opinion (119), therefore, was obtained. Some authors believed that D cells are the precursors of alpha cells (235) or that these cells constitute either damaged or dying alpha cells (280). On the other hand, it was assumed that D-cells are immature beta cells (104). Similarly investigations on lamprey (70) showed vesicular cells with numerous electron optically empty membranous vesicles which were thought to be the reminiscent of the delta cells (non-granulated) as described in the cat (83), monkey (121) and man (118) and these cells were considered as precursors of the beta cells (87) but as, in these cells, richly developing ergastoplasm seems to indicate an active protein synthesis, the possibility

cannot be ruled out that only granulated forms should be considered as D-cells, since these cells originally were delineated by light microscopy (53, 54) as granulated cell with specific staining characteristics. In a comparative ultrastructural study on a variety of species (74) however, cells have been identified as 'delta' cells but their identity was confused by associating them with clear ('C') or agranular cells in different species.

The suggestion that D-cells are argyrophil cells and actually identical to argyrophil a_1 cells should be considered convincingly confirmed now both by light and electron microscopic studies in the pancreatic islets of a variety of animals including fish (61a, 89), urodeles (62, 264), birds (261) and mammals (61a, 89, 262, 263, 265, 266). The conclusion arrived at was strengthened by the present observations that the identity of D-cell and a_1 cell is a common phenomenon and therefore D-cell should replace the designation of a_1 cell, and a_2 cell should be called alpha cell.

Emphasis has been laid by most of these workers that D-cell has an independent status and also that they may present the source of the pancreatic hormone (62, 103). The distribution of D cells along blood capillaries as well as their conspicuous polarity towards these capillaries (especially in rabbits) suggests a possibility that these cells are the source of an unknown hormone (263, 266). It may be mentioned that even the Uppsala group found (111) a close relationship of the silver impregnation cell with the capillary and it was assumed that they (a_1) are functionally more active. They may either be a separate type of cell or only a more active stage of alpha cell. It was also shown that the argyrophil cell corresponds to more degranulated alpha cells when blackening is removed from silver stained sections and this supports the latter assumption. Although glucagon production has been associated with a_2 cells, these workers were unable to get any information on the biological significance of so-called a_1 cells (258).

Amphiphil cells of pseudoisocyanin-fluorescent type as demonstrated (103, 264) in the pancreatic islets of shark, urodeles (Xenopus laevis), were not observed in the principal islet of Tilapia mossambica. These amphiphil cells have also been reported as being absent in mammal and birds (62, 264). In toad, bufo bufo however, two types of amphiphil cells were observed (103). The possibility was indicated that amphiphil cells might represent an intermediate stage between beta and D-cell (103) which does not contradict the earlier postulated functional autonomy of D-cells (62).

Although degenerative changes in the nuclei of D-cells which have been pointed out were not recognised in either dog or rabbit (262) yet increasing evidence strengthened by the present observations, suggests the maintenance of D-cell, as acknowledged in earlier studies (54) to be a normal component of pancreatic islets.

In short, it seems reasonable to put forward the view that in the principal islet tissue of Tilapia mossambica as many as four types of cells could be distinguished, corresponding to the alpha, beta, D and agranular cells of mammalian species (74, 75, 83, 113, 123) and support the independent status of alpha, beta and delta cells (83, 113, 123), all of them producing their respective hormones (glucagon, insulin, hormone of unknown nature), whereas the nature of the fourth type - agranular, remains obscure, probably they represent degranulated stage of D-cells (83). It is not known whether there is any reaction of any of these cells (D- or agranular) to some of the non-functioning islet cell tumors associated with Zollinger-Ellison syndrome. However, cytogenetic relationship between Zollinger-Ellison islet tumors and D cells has been suggested (282, 283).

'Mixed cells', containing both types of granules - exocrine and endocrine, as observed in both categories of alpha and beta cells in a previous electron microscopic study on the pancreatic islets of toadfish (247) have not been observed in the principal islets of

Tilapia mossambica even at ultrastructural level. In one instance, however, a few beta cells were observed to have, in addition to typical beta granules, a few large-sizes granules which appeared similar to those observed in the exocrine cell. Mixed cells, on the other hand, have been observed in normal rat as well as in ethionine treated, during the early stages of which such cells occurred more frequently (284), and also in human pancreas (285), suggesting the evidence of acinar insular transformation. In the pancreatic islets of duck, but not in those of mammals such transitional cell forms with ultrastructure characteristics of both the a_2 cells and the exocrine cell were observed (81) which indicated a possibility of relationship between exocrine tissue and a_2 cells of pancreatic islets. Among teleost species only the bullhead fish (72) has been reported as showing a region characterised by the presence of cells with ill-defined cell borders, between clearly recognisable exocrine and pancreatic islet cells. The cytoplasm of cells in this region were seen to contain elements of zymogen granule, alpha and beta granules similar to those in both types of cell (72). Such cells were never observed in the principal islets of Tilapia mossambica. From the literature and the present work, no conclusion can be drawn concerning the nature of such mixed cell though evidence suggests the acinar origin, in the adult organism, of the islet cell type in different species. It may be mentioned, on the other hand, that the evidence in support of the hypothesis of an acinar insular transformation has been criticised and referred to as artefacts, associated with misinterpretation of light microscope observations (268, 287).

In Vitro morphological study

The pancreatic islet of different species has been studied in different phases of its normal functional activity by light microscopy. High magnification and resolution obtainable with the electron microscope has been realised to be of great value in this aspect and essential information regarding the synthesis, storage and release of secretory granules in the islet cells has been visualised. In this connection, study of structural features of islet cells, both by light and electron microscopy, following injections of various chemical, hypo- and hyperglycemic agents has been found very useful. The present study offers a different approach for studying the functional activities of islet cells by establishing a system in vitro, in which cytological changes occurring in the islet cells in response to various hormones or stimulatory agents could be investigated even at ultrastructure level.

The present study shows that it has become possible to establish such an in vitro model for such morphological study on the principal islets of Cottus scorpius. Though electron microscopy of the principal islets incubated for 2, 4 hours was not performed, the light microscopic observations indicated that islet cell survival was achieved in the present study. These findings are consistent with the conclusive biochemical evidence for in vitro beta cell survival (148) where the response of the islet beta cells to the various factors on the release of insulin was adequately proved. The influence of the stimulatory agents on pancreatic pieces in vitro was found to be qualitatively similar to those which were known to occur in vivo (148,149,163). Whether the rate of release of insulin or any other hormone in response to these stimulatory agents can be compared with that determined in vivo, is not known as no parellel

quantitative study on the islet tissue was attempted during the present investigation. It would be of great interest to perform such a quantitative study parallel to the cytological observations on the isolated principal islets, e.g. relationship between pancreatic insulin release and beta cell structural changes in response to various stimuli.

A brief report (286) suggesting the evidence for islet cells survival during the short term maintenance of rabbit pancreas *in vitro* appeared during the course of the present investigation. The authors also reported cytological changes within the beta cells stimulated *in vitro* with addition of glucose or tolbutamide (287). In both these studies preparation of *in vitro* involved incubation of pieces of pancreas and it is possible that the islet specimen selected for study may not reflect the conditions of all the islets, as the islets are embedded in the exocrine pancreas and have to be located in an incubated piece of pancreas. The assessment of cell cytomorphology is therefore subject to sampling errors in these morphological studies. The fact that islets are embedded in the exocrine tissue suggests that the behaviour of structural elements in the islet cells, particularly when observed at ultra-structural level may not represent the true response towards the stimulatory agents. Moreover, some cell damage may be expected as a result of handling the tissue specimen prior to incubation. On the other hand, advantage has been taken in the present investigation of the teleost fish, in which the principal islets are found as isolated structures which are very easy to dissect out and do not need division into pieces. Moreover, the exocrine tissue surrounding the islet in the rabbit pancreas can cause certain changes which in some way or another may affect the cytomorphology of the beta cells *in vitro*. In another *in vitro* study on rat islets (158), the premature degenerative changes found in the beta cells of the

incubated pieces of pancreas with tolbutamide were thought to suggest that the process studied is not the function of living islet tissue but only some of its enzymatic reactions responsible for the release of insulin already in the beta cells.

The structural changes, as observed light microscopically, in the principal islets of Cottus scorpius incubated in vitro, in response to alloxan, growth, hormone, hydrocortisone and tolbutamide for different periods of time have been studied in the present investigation. Although such incubated principal islets were processed for electron microscopy as well, it has not been possible to complete their study at the ultrastructural level. However, the cytological changes in the beta cells of principal islets occurring in response to tolbutamide when incubated for one hour have been observed with the electron microscope. (Up to now no in vitro study has been performed to see the influence of alloxan, hydrocortisone and growth hormone on the cytology of the islet tissue).

No definite change in the beta cell structure was found after incubation with alloxan for five minutes, though changes in the nuclei and cytoplasm of beta cells with diminution of their specific granules five minutes after injection of a diabetogenic dose of alloxan have been observed (197). Electron microscopy of islets incubated with alloxan for five minutes would reveal initial cytological changes in the beta cells, as it has been found by biochemical studies on toad-fish islets that in vitro action of alloxan takes place very rapidly (23). These findings supposed that when islet tissue slices are treated with alloxan in vitro, the membranes of the beta cells are damaged (23). It seems reasonable to assume that initial cytological changes in the beta cells may give useful information regarding the mechanism of action of alloxan. The ultrastructure of islet tissue has, however, been studied in alloxan-treated rabbits (124, 125), rats (246, 288) and lizard (108), describing the degenerative changes in the beta cells. Mild degenerative changes, e.g. nuclear

pyknosis were apparently evident in the principal islets incubated for 30 minutes or 60 minutes. Similarly progressive shrinkage of beta cells was also observed in both cases. It seems that response of these cells to alloxan in vitro is very quick.

Progressive degenerated morphological changes in rabbit pancreatic islets have been described as seen light microscopically (197). The present study shows structural changes in beta cells to be of very mild stages but on the same degenerative pattern as in rabbit pancreas. Aldehyde fuchsin staining of the beta cells never ceased to occur in any of the incubates specimens. Whether this granule staining could persist even after marked changes have occurred in the beta cells is not known. In electron microscopic study of alloxan treated rabbits, the membrane surrounding the granule core was seen even after the loss of granules (124) and it was suggested (197) that membranous sacs are (124) aldehyde fuchsin positive. Nothing can be said to support this view as the affinity for aldehyde fuchsin in some beta cells of alloxan incubated islets did decrease despite the fact that there occurred a definite decrease in their affinity for pseudoisocyanin stain showing loss of insulin granules. On the other hand, electron microscopic studies showed loss of electron dense granules and mambrane sacs after cortisone administration when light microscopically loss of aldehyde fuchsin positive granules from beta cells was observed (203). A marked increase or decrease in the size of the nuclei of the beta cells probably could be correlated with their different functional states of insulin secretion due to the presence of the stimulatory agents in the incubation media. As with alloxan, the beta cell nuclei were observed to be slightly decreased in size one hour after the incubation, indicating probably the degenerative changes occurring in the beta cells. Similar observations of decreased nuclear size

in alloxan-treated rats have also been observed (289).

Principal islets of Cottus scorpius incubated in vitro with growth hormone or hydrocortisone appear to show no response to these hormones even after four hours incubation, although depletion of beta cells could be detected in some cases under the influence of growth hormone. Both these hormones, however, have been shown to have an effect on insulin secretion (152) as well as on pancreatic morphology, observed light microscopically as well as electron microscopically after prolonged treatment of the respective hormone in a variety of mammalian species (116, 127, 128, 133, 290 - 293). In the present study, no loss of aldehyde fuchsin positive granules could be assessed visually and the beta cells as well remained intact in the growth hormone-incubated islets, and as electron microscopic observations on dog pancreatic islets showed loss of membranous sacs as well as of secretory granules after growth hormone administration (116) it can only be presumed that growth hormone did not have a direct effect on the beta cell functional activity. It must be pointed out that difficulty arises when in vitro effect, such as in this case, of an agent is compared with that in vivo, particularly when it is known to be effective after rather prolonged treatment. It is also possible that teleost principal islets have different response to growth hormone or hydrocortisone from that in mammals, although it has been shown that growth hormone has a diabetogenic effect on blood sugar level of Cottus scorpius (294). Light microscopic observations (292) on rabbit pancreas presented evidence for beta cell destruction as a result of in vivo effect of growth hormone treatment. The present in vitro study does not indicate any direct injurious effect on the beta cell structure, as suggested in the case of alloxan incubated principal islets. It is probable that the structural changes observed in mammalian pancreatic islets after growth hormone administration are secondary to the hyperglycemia produced in the body.

The principal islets incubated *in vitro* with hydrocortisone did not show any cytological changes in the characteristic features of beta cell which could indicate its stimulatory or degenerative stage. It may be mentioned that *in vivo*, however, cortisone or hydrocortisone produces hypertrophy of the pancreatic islets, and beta cell degranulation, as well as vacuolization of beta cells (290, 291). Slight degranulation observed in the beta cells in the present study is in no way indicative of a direct cytopathogenic effect of hydrocortisone on the principal islets of *Cottus scorpius*. Cytological changes, however, have been observed at the ultrastructural level in the pancreatic islet of rabbit after cortisone treatment (128, 293).

The present *in vitro* morphological study clearly showed a direct effect of tolbutamide on the principal islets of *Cottus scorpius*. Previously its hypoglycemic effect *in vivo* on pancreatic morphology has been established both by light (130, 294) and electron microscopic studies (129, 201) performed in mammals. The enlargement of the beta cell nuclei was observed in most of the tolbutamide incubated specimens during this study which confirmed the supposition that the established correlation between nuclear size and functional state concerns the beta cells as well. Similarly significant increased nuclear size of the beta cells after the administration of glucose and in other conditions resulting in an increased insulin was observed (295). On the other hand, it has been shown that tolbutamide *in vivo* has a selective effect on alpha cells, the nuclei of which are significantly reduced, while those of beta cell remain unchanged (296), which is untenable since tolbutamide has been demonstrated to stimulate insulin secretion from the beta cells and any change in the functional activity is possibly reflected in nuclear size variations. The observations that significant change did not occur

in the beta cell granulation in the principal islets incubated for two hours was rather slightly different from the observations on rabbit pancreas pieces (286) incubated in medium containing glucose, and the beta cells of the preparation showed intense staining. The aldehyde fuchsin positive staining depends on the beta granules, the concentration of which would depend on the rate of synthesis and/or release of insulin from the beta cell. As the rate of release of insulin response to glucose and tolbutamide has been shown to be different (148), it is reasonable to assume that after a certain period of incubation with these two factors the granular concentration in the beta cells would be different. In another preparation of rat pancreas pieces incubated in vitro with glucose or tolbutamide, specific granulation of the beta cells was found not to decrease up to 90 minutes after incubation (158) and the present observations are in agreement with this study. Also, in this preparation, a large number of beta cells were seen to show degenerative changes in the form of nuclear pycknosis (158). On the other hand, nuclear pycknosis and vacuolization was observed only in some of the beta cells of tolbutamide incubated principal islets in the present study. Similar observations have been reported in rabbit pancreas pieces in vitro (286). Beta cell necrosis when incubating rat pancreas with tolbutamide in very high concentrations (1000 mg/100 ml) has been reported (297). Using the same concentration of tolbutamide, deganulation has been observed (298) in beta cells in frozen sections of rat pancreas by dark field microscopy but both these studies have been considered to be based on unphysiologic phenomenon.

The beta cells of tolbutamide incubated principal islets when examined with electron microscopy were found to be morphologically intact. Their secretory granules as well as the rest of the cell structure was well preserved. Despite the fact that some damaged

beta cells were observed, electron microscopic study of the incubated islets on the whole confirmed the light microscopic observations that survival of beta cells can be obtained in the present in vitro experiments and it also indicated that the study of cytological alterations in the islet cells in response to different stimulatory agents in vitro is possible even at ultra-structural level. No study up till now has been performed to see the effect of tolbutamide on the cytology of teleost islets. However, the effect of tolbutamide in vivo has been described in the pancreatic islets of rabbits (129, 201). The most obvious changes observed in the beta cells during the present in vitro study are in their ergastoplasm and secretory granules. The endoplasmic reticulum in most of these cells appeared very prominent and some cells showed a semi-circular arrangement in their distribution. Vesicularisation of the ergastoplasm was also often observed in some of the beta cells. The secretory granules in the beta cells mostly showed a very wide space between the limiting membrane and the internal granule core and also their migration towards the plasma membrane was apparent. It appeared from a very close association of the granule with the plasma membrane that presumably fusion between the limiting membrane and the plasma membrane had taken place.

It can be concluded that tolbutamide has an in vitro effect on the cytology of the principal islets and this consists of degranulation and vesiculation of the ergastoplasm. These observations are in agreement with the ultrastructural changes observed in the rabbit beta cells, following the administration of tolbutamide (129, 201). Whether there occurs any regranulation of the secretory material in the incubated principal islets, as described in rabbit beta cells (129) is not known. It was not possible to characterise distinctly the secretory granules on the amount and density of their secretory material. The present study suggests also that an approach like this should prove very useful

in understanding the different functions of islet cells as well as the mechanism of action of certain diabetogenic agents.

During the course of the present investigation a report of cytological changes occurring in the beta cells of rabbit pancreas pieces *in vitro* as well as of measurement of insulin content of the incubation medium ((299). By maintaining the pieces of pancreas in a shaking incubator for different periods of time (maximum one hour), the response of islet beta cell under these conditions was observed to suggest that a study of the mechanism of secretion as compared with the ones described in the animal *in vivo* is possible (299). The results in general were corresponding to the observations obtained during the present study. Very recently the same group of authors have studied the mechanism of secretion of the alpha cell using the same *in vitro* model for morphological study (278). It is interesting to note that Schwarz et al., in 1968, reported ((300) the ability of a new sulfonylurea derivative to stimulate insulin secretion *in vitro* (300), which is in agreement with the present morphological findings of the *in vitro* stimulatory activity of a sulfonylurea derivative (tolbutamide) as indicated by light and electron microscopic studies on the principal islets of *Cottus scorpius*.

Observations made on the islet cells of principal islets of normal *Tilapia mossambica* and tolbutamide-incubated islets of *Cottus scorpius* during the present investigation provide some useful information in connection with the synthesis and release of secretory granules in the islet cells. The significant change observed in the ergastoplasm as a result of stimulation with tolbutamide indicates that formation of beta granule is closely linked with this component of the cell. Similar observations obtained by other workers lead to the same conclusion (129). Even in normal beta cells occasional relation of the cisternal membrane of the endoplasmic reticulum to the limiting membrane of the beta granule was observed which indicated an evidence towards the role of ergastoplasm in the

formation of secretory granules, as postulated for the synthesis of secretory products in the beta cells of a number of mammalian pancreatic islet tissues (76, 110, 128, 129). Similar observations have also been made in bullhead fish principal islets (72), the beta cells of which were also observed to possess a system of vesicles and other structures possibly involved in the synthesis, storage and release of secretory products (301). Formation of beta granules from ergastoplasm has also been visualised in the islet tissue of lamprey (70). The possibility of fibrillar material of the macular zone being closely related to the secretory function of beta cells has also been suggested (128); the fibrillar material was believed to represent an inactive form of insulin. During the present investigation no such relationship could be assumed as the fibrillar material in the beta cells is of very inconspicuous appearance. Even in the principal islet of bullhead fish such a possibility was ruled out despite the fact that the fibrillar material was prominent (72). On the other hand, it has also been postulated that the beta granules appear to be sequestrated in membranes derived from the golgi system (65), as observed in the beta cells of fœtal and new born rats (65) as well as in adult rats (244). This view has also been endorsed by the observations on the beta cells in the islet tissue of lizard (108) and those on rabbit (227) though it was possible to see very rarely a small granule in close association with the golgi system but no support for the view that golgi system plays a definite role in the formation of secretory granule was obtained during the present observations. It may be mentioned that round bodies in the vicinity of the golgi lamellae of the beta cell of the rabbit were found, and though these were similar to the one observed in newborn rats (65), they were considered to be lysosomal in character and it was postulated that they provided the storage

granules, which were formed within the ergastoplasmic reticulum, with proteolytic enzymes (302). Of great interest is the suggestion (303) that the beta granules are formed by progressive polymerisation of insulin which has been sequestrated within the membranous sacs. The present study therefore, favours the origin of beta cell granules from the ergastoplasmic lamellae, since these structures were frequently seen to contain a hazy electron dense material which was similar to that in some of the smooth-surfaced membranous sacs.

Clusters of small granules as observed in mouse pancreatic islets (115) were sometimes seen in the alpha cells of the principal islets of Tilapia mossambica. Secretory granules were also seen in close association with cisternal membranes of endoplasmic reticulum. The alpha granules were considered also to be derived from mitochondria (304). No evidence was found which could suggest the formation of secretory granules in the alpha cell but it may be that synthesis of alpha granules is similar to that assumed to be happening in the beta cells or of the type hypothesised in mouse pancreatic alpha cells (115).

The mechanism by which secretory granules are released from the islet cells to the blood stream is not known with certainty (269). It was postulated (288) that a process termed 'emiocytosis' occurring in the beta cells of pancreatic islet is responsible for the release of the secretory granule. It involves margination of the membranous sacs containing the beta granule towards the cell membrane, contact with the cell membrane followed by fusion between the limiting membrane of the beta granule and the cell membrane which results in ejection of the content of the beta granule by rupturing at the site of the contact. In the present study, both in normal principal islets and tolbutamide treated principal islets some beta granules and sacs with different amounts of osmiophilic

material were often observed near the cell membrane. On very rare occasions pit-like structures as described previously (128, 288) were also observed. This was more so in the incubated islets which showed an obvious difference from the normal islets in demonstrating margination of the beta granules as a result of tolbutamide stimulation. Similar observations have also been found in rabbit pancreatic islets following tolbutamide administration (129). It appears that there is a general agreement for this type of beta granule release mechanism, as has been indicated in the pancreatic islets of a variety of species including lamprey (70, 87), normal and obese-mice (64, 115, 182), rat (113), rabbit (76, 110, 116) and Chinese hamster (269). Recently emiocytosis type of insulin release has also been observed in rabbit pancreas pieces incubated in vitro (299). The release of the secretory product has also been studied during augmented beta cell secretory activity induced experimentally (128, 129, 201, 288, 293).

It was also suggested while studying the pancreatic islets of synthaline treated rabbits that the alpha granules disintegrate in the cytoplasm forming small secretory particules and thus is liberated in a particulate form (115). Ultrastructure studies in lizard (108), rabbit (by another worker) (227) and many other species (73,243) failed to see such secretory particles in the alpha cells. Although in the present study clusters of very small-sized particles were occasionally found in the principal islets of Tilapia mossambica which could be the reminiscent of pro-secretory granule as described in rabbit pancreatic islets (115), but alpha granules were also found close to, and sometimes attached to, the cell membrane and it cannot be said whether they migrated to the cell membrane and then disintegrated or if their dissolution occurred in the cytoplasm before they were secreted. On the other hand, clusters of small

granules were observed in the alpha cells (a_2) of the guinea pig (81) and it was thought that at least for this cell type, this type of secretory mechanism (115) may be correct. This type of alpha granule liberation has not been observed in the principal islet of bullhead fish (72). The limiting membranes of some alpha granules may be continuous with the plasma membrane (72), as observed in the present investigation. The alpha granules in bullhead fish islet have occasionally been seen free outside the cell membrane. In the Chinese hamster (269) in the beta and a_1 cells discontinuity of the granule membrane was sometimes observed suggesting an intracytoplasmic release of the granular substance but as the authors themselves pointed out, obviously the mechanism remains to be clarified. Discontinuity of the individual membranes enclosing the secretory granule was observed in some other species (140) as well and similarly was suggested to represent an intracellular release of the secretory material. The existence of this type of secretory process involving intracellular solubilisation of the granule has also been suggested in toadfish islet (95). In another study (241) occasionally granules similar to islet cell granules have been noted in the endothelium of vessels adjacent to islet. The rarity of such events was thought to suggest that either the granule is dissolved by interstial fluids (110, 116), is solubilised within the islet cell prior to release, or that release of granule is episodic. In the principal islets of bullhead fish (72), the scarcity of empty sacs was considered so as to suggest that liberation of beta cell granule may be different from the 'emiocytosis' type observed in other species.

In the tolbutamide-incubated islets sometimes sacs similar to the limiting membrane of the beta granule were observed in the cytoplasm. Whether they really represent granule lacking sacs is not known. Light microscopically, similar sacs have been

observed to give intense staining of aldehyde fuchsin even though the insulin granule presumably has been lost. If so, it is probable that dissolution of the granule has taken place intracellulary. In some cases, similar sacs were seen outside the beta cell membrane as observed in mammalian pancreatic islets. Whether the secretory product along with the limiting membrane travels through the plasma membrane and on coming in contact with blood capillary squeezes out its inner content is not clear from the present study. Similarly, on rare occasions finger-like projections were observed at the cell surface but there was no evidence for them to be formed as a result of granule liberation. Such projections were considered (72) as a device for enlargement of cell surface. Microvilli which are formed during 'emiocytosis' by the protruded cytoplasm do not seem to have common agreement regarding their formation.

If margination of the beta granules is the first step of the emiocytosis process, how does the movement of the granules towards the plasma membrane take place? Hyperglycemia or stimulation of insulin release suggests quick margination of the granules. It is possible that any of the morphological structures which insulin granule has to pass before it is secreted - basement membrane associated with the capillaries, a space, and a plasma membrane and cytoplasm of endothelium - may have certain specific sites which could serve as potential impediment to the rate of transfer of insulin from the beta cells into the blood stream (304). In none of the studies so far has evidence been presented to indicate actual continuity between the interior of a granule and the outside of a cell. [illegible] observed. It has been pointed out (309) very recently that such a finding would be required to support the theory of insulin release by emiocytosis. An alternative to the theory of insulin release by emiocytosis was considered - the possibility of release from granules within the beta cell, but no evidence was found in support

of this theory. On the other hand, Lacy has very recently strengthened his 'emiocytosis' theory by suggesting a working hypothesis of events which occur between biochemical stimulation and the liberation of secretory granule (306). The presence of microtubules within the beta cells and in a few instances a filamentous structure linking the sac around the beta granule to the plasma membrane was observed and it was hypothesised that an internal cytoskeleton of microtubular system might have been involved in the translocation of the beta granules within the cells. Evidence has been presented (306) that colchine, shown previously to bind microtubules and interfere with the movement of melanin granules in melanophores, was found to inhibit insulin secretion in response to high concentration of glucose in studies performed on isolated pancreatic islets of rats. It was rather difficult to see the association of any filamentous structures with the granule and cell membrane, although sometimes microtubules were observed during the present study but were not found regularly. Nothing can, therefore, be said about this working hypothesis in the light of present observations. However, it appears that the mechanism of release of most of the secretory granules in the principal islets of Tilapia mossambica is probably similar to the 'emiocytosis' seen in most of the higher vertebrates.

Chapter 5. SUMMARY

Three cellular types (alpha, beta and agranular) have been demonstrated light microscopically in the isolated principal islets of Tilapia mossambica and Cottus scorpius, and have been shown to correspond to the respective cells present in mammalian islet tissue.

Electron microscopy of the principal islets of Tilapia mossambica revealed, in addition to the alpha, beta and agranular cells, the presence of another granular cell type - identical to D-cells (and probably to a_1 cell, by another group of workers) present in the islet tissue of higher vertebrates. Cellular composition of the principal islet tissue, particularly the controversy over a_1, a_2 and D cell types, has been discussed in the light of present findings.

Beta cell survival in in vitro up to 4 hours of the incubation period has been achieved. Light microscopy of the islets incubated in the presence of alloxan and tolbutamide separately for different periods of time revealed definite cytological changes in the beta cell indicating degenerative effect in the former case and stimulatory effect in the latter case. Insignificant or no changes were observed in the islet tissue when incubated with either growth hormone or hydrocortisone.

Electron microscopy of the tolbutamide-incubated islets showed conspicuous changes in the ergastoplasm and in the secretory granules of the beta cells. Vesiculization of the ergastoplasm as well as margination of the secretory granules were clearly observed. Endoplasmic reticulum probably plays an important role in the synthesis of insulin and beta granules are secreted probably by the emiocytosis process. Mechanism of release of secretory granules from the islet cells has been discussed and importance of in vitro morphological study in this connection as well as to study the mechanism of action of certain hormones or drugs has been emphasised.

SECTION III. METABOLIC INVESTIGATIONS ON ISOLATED PANCREATIC ISLETS

SUB-SECTION A. NUCLEIC ACID STUDIES ON PANCREATIC ISLETS OF FISH AND MICE

Chapter 1. INTRODUCTION

Protein synthesis in animal tissues is presumably controlled by the nucleus (310), the specificity of the template being determined by the nucleotide sequences in the RNA (311) and the protein itself being released from the RNA template. Insulin synthesis in islet tissue appears to be similar to protein synthesis in other tissues (136).

Sandritter, in 1964, using quantitative histochemical methods to study the mechanism of insulin synthesis/secretion demonstrated morphological observations on stimulated pancreatic beta cells and suggested (312) that the activity of cell nucleus due to changed concentration of amino acid and probably RNA is directly linked to the insulin concentration. Using UV cytophotometry, measurement of nucleic acid content in the beta cells was attempted (312) to find the RNA involvement in the secretional process of insulin, but with no success.

It could be possible to determine quantitatively the amount of nucleic acids in the islets by applying microchemical method on the homogenates of isolated pancreatic islets which have been shown to be metabolically active (35). As more islet tissue would be available from teleost fish (Cottus scorpius, Tilapia mossambica) it was decided to make nucleic acid determinations on these islets as well. Alloxan is well known to be specifically toxic for beta cells (197) and any change in RNA content of islet tissue would probably reflect largely ~~of~~ beta cell activity.

It was therefore decided to apply a microchemical method for the determination of RNA in isolated pancreatic islets and to perform a comparative study of RNA determination in mammalian and teleostean

islet tissue. Obese-hyperglycemic mice, with their raised functional activity of the beta cells, alloxan-treated and tolbutamide stimulated islet tissue would be utilized for RNA determinations. By studying the changes in the concentration of RNA in the beta cells of pancreatic islets under the influence of different stimuli, and coupled with parallel histological observations wherever possible, useful information could be obtained regarding the RNA involvement in the insulin secretional process, as well as the mechanism of action of certain diabetogenic or hypoglycemic agents.

Chapter 2. MATERIALS AND METHODS

i. Animals

Cottus scorpius, Tilapia mossambica and normal albino as well as obese-hyperglycemic mice (as described under Chapter 2 of Section II) were used in the present study. The obese-hyperglycemic mice used were always 10 - 16 weeks old.

ii. Preparation of islet tissue material for microchemical analyses

a. Micro and Macro-dissection techniques:

Freehand microdissection (34) was carried out to sample the pancreatic islet tissue of both strains of mice for microchemical purposes. An apparatus (Plate 34) was designed in this connection by Mr. H.W. Noble in this laboratory which maintains the suspending medium contained in a very small plastic dish (size 6 x 4 cm) at a constant temperature (+2 to + 3°C) controlled by a thermistor immersed in the medium. This apparatus kept the tissue and the medium cold during the whole procedure of dissection and hence ensured viability of islet cells. The inner surface of the plastic dish was black and this makes easier the identification of the pancreatic islets against a dark background.

After killing the animal by hyperextension of its neck the fresh pancreas was rapidly removed and after a quick wash in 0.25 M chilled sucrose solution was placed in the dish containing modified Kreb's Ringer bicarbonate supplemented with bovine plasma albumin in a concentration of 2 mg/ml.(50)

The pancreatic islets were identified by examining under a dissecting microscope (Olympus Stereoscopic Zoom Microscope Model SZ) as yellowish white homogenous areas dispersed in the more pink and slightly translucent acinar tissue and were isolated from the surrounding exocrine parenchyma as described by Hellerstrom (34), using watchmaker forceps (No.3) and 20 gauge hypodermic needles fixed to glass rods

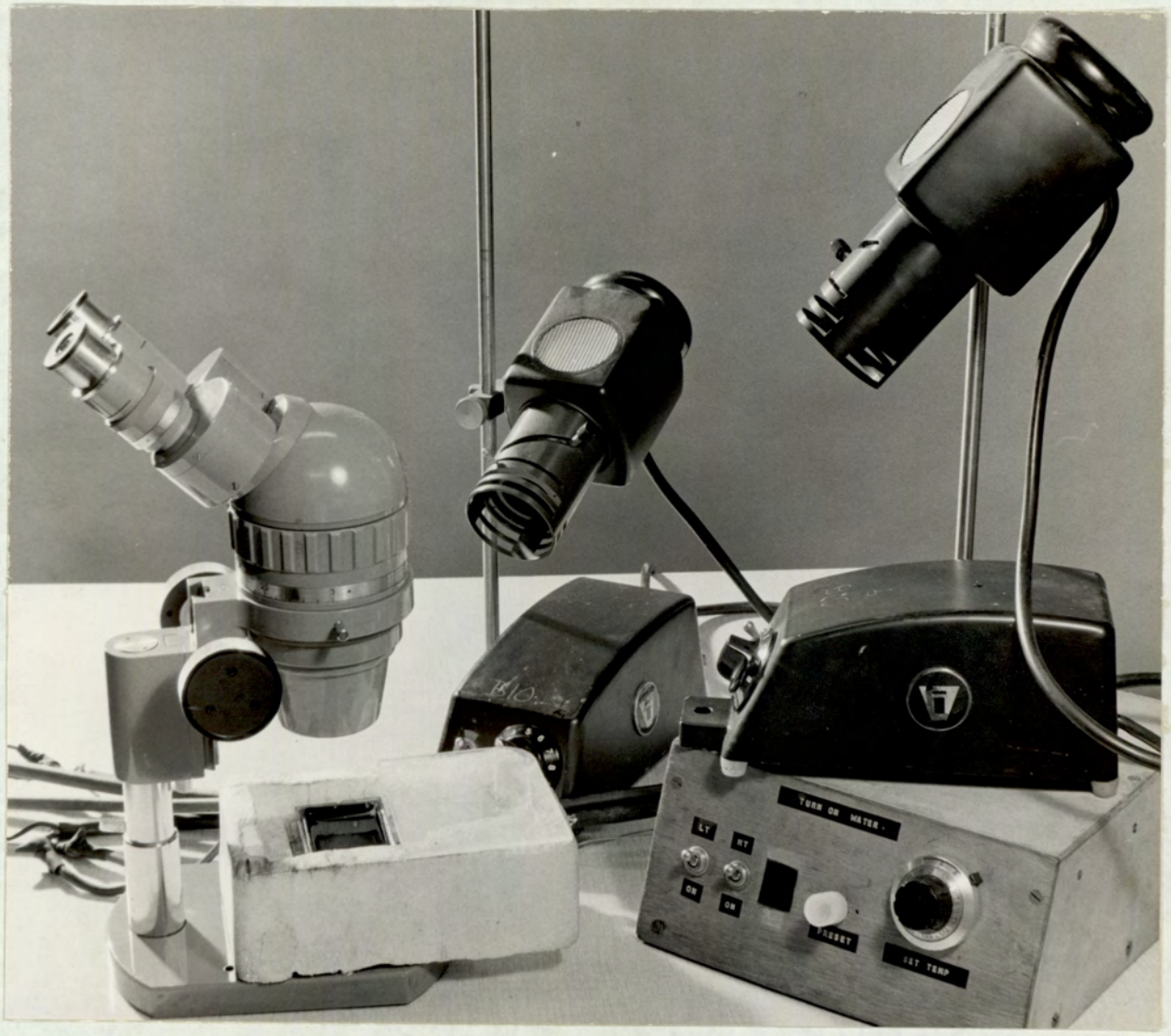


PLATE 34. Apparatus used for micro-dissection of the Pancreatic islets. It consists of, besides an ordinary binocular and illuminating lamps, a dissecting dish (A) having a thermocouple under the black plate and a thermister (B) kept immersed in the contained solution, both of which are controlled by a system of batteries placed in box C.

at one end. Dissection of an islet was completed within a few minutes. With some practice it was possible to isolate 8 - 10 islets within 12 - 15 minutes. The individual pancreatic islets were picked up using a small glass loop slightly larger than the diameter of an islet (1.5m.m) or by a glass needle with a flattened tip of 100 μ diameter with a particularly smooth surface. The islets were then transferred to a piece of aluminium foil kept over 'Cardice' in a special thermos flask having a wide open mouth. Special care was taken not to puncture or damage the islets during picking up or the transference operation. Dissection was completed within half an hour. Usually twenty or so pancreatic islets were isolated from each pancreas. In addition to the pancreatic islets, small samples of exocrine pancreatic tissue were also excised from particularly thin peripheral parts of the gland, obviously devoid of islet tissue. These specimens were trimmed to a size roughly corresponding to that of an islet.

In Cottus scorpius the endocrine pancreas is concentrated into two macroscopically visible structures. On opening the abdominal cavity, one of the principal islets could be seen lying in the mesentry behind the lower end of the spleen and near the cardiac stomach, as a large rounded thick and somewhat concrete body with an approximate diameter 3 - 4 mm. The second islet, much smaller in size, was situated close to the duodenal loop near the pylorus and bile duct. The surrounding connective capsule was removed from the islets, which were either still intact or dissected out from the animal body. In the latter case, the islets were placed on a glass plate kept over pieces of crushed ice or cardice and then they were isolated free from the connective capsule. While dissecting out the smaller islet (pylorus), a small piece of surrounding tissue from the intestinal wall was usually left attached to it. In such cases, use of dissecting microscope was necessary to remove the surrounding tissue and obtain pure islet tissue.

Dissection of the principal islets in Tilapia mossambica was carried out under a dissecting microscope at 10 - 20 magnification, particularly in the case of smaller fish in which principal islets are much smaller than their counterparts in Cottus scorpius. A simple and straightforward procedure was found to locate and dissect out the principal islets. The dissection was started with a median longitudinal slit in an upward direction starting from the anus to the gill arches. At the anterior end near the gill arches a transverse cut was made towards the animal's left dorsal side reaching the other end of the gill arches. The cut was continued in a downward direction along the vertebral axis and deviating near the posterior end towards the anus. The abdominal cavity was open now on the animal's left side only. On carefully lifting the gall bladder slightly upwards two, three and sometimes four principal islets could be seen lying close to each other in the mesentry between the liver and cardiac portion of the stomach. They appeared as a whitish mass of soft tissue usually spherical in shape. The portion of mesentry containing these islets was removed and placed in the plastic dish of the cooling apparatus (used for dissecting mouse islets). The islets were separated from the surrounding tissue by cutting and tearing, making use of micro-scalpels and dissecting needles.

Small pieces in the same range of weight as the principal islets were also taken from other tissues, e.g. liver, kidney and exocrine pancreas.

b. Weighing:

After carefully draining off the water completely the isolated islets in groups of 4 - 5 from normal and obese hyperglycemic mice as well as from Tilapia mossambica were weighed on an ultra micro balance (with a sensitivity of 0.1 µg ; UM6: E. Mettler, Zurich). A very small boat of aluminium foil with a lid on top to protect the tissue material from drying out was used to carry the islets on to the balance. This boat was re-weighed after taking out the tissue

material to obtain the latter's wet weight. On certain occasions, Cahn's microbalance was also used. The principal islets of Cottus scorpius and other tissues were weighed either on a torsion balance or on a Stanton balance, after carefully draining off the moisture completely within the folds of a filter paper.

c. Homogenization:

Homogenization of the fresh pancreatic islets or other tissues and the subsequent microchemical procedure was carried out in 0.3 ml round bottomed test-tubes (7 x 40 mm; inner diameter 4 mm.). These pyrex-glass micro test-tubes were specially made in the glass blowing section of the Physics Department in our University. Glass beads attached to thin glass rods were matched with the micro test-tubes by grinding with paste, thus forming very small homogenizers which served also as reaction and centrifuge tubes. Such micro test-tubes proved to be very useful in avoiding loss of material during homogenization and transference of homogenates. For RNA determinations the weighed tissue was dropped directly into these micro test-tubes containing the appropriate reagent (0.3N PCA) and could be kept in a small steel rack placed in the cooled thermostat bath maintained at $+2^{\circ}C$.

When using bigger pieces of tissues homogenization was carried out in small sizes glass Potter-type homogenizers (obtained through Gallenkamp) and not in micro test-tubes.

iii. Ultramicrochemical method for RNA determination

a. Apparatus:

The reagents were delivered mostly with Levy or Kirk constriction pipettes calibrated to 0.5%. An 'Agla' micrometer syringe (Burroughs Wellcome and Co., London) also was used for handling the liquids.

Stirring was accomplished by means of a Mickle shaker (Comshall, Surrey). Centrifugation was carried out in a high speed microcentrifuge (Measuring and Scientific Equipment Ltd., London). Quartz

microcuvettes (10 mm. light path) or liquid microcells (10 mm. light path: No.97260) were used in conjunction with the Beckman Spectrophotometer (Model DB) provided with its Scale Expansion Accessory and Linear and Log Recorder. The equipment for micro-spectrophotometry was obtained from Beckman Instruments Ltd. (Great Britain). On some occasions, spectrophotometric analysis was also carried out in a Unicam SP.800.

b. Reagents:

1. 1.2N Perchloric acid (PCA)(7.092 ml. of 70% PCA made to 100 ml. with glass distilled water.)
2. 0.3 N PCA
3. 0.2 N PCA
4. 0.3 N KOH (1.683 gms. KOH/100 ml. glass distilled water).
5. Standard RNA : stock solution (1 mg/ml) of yeast RNA (from B.D.H) was diluted appropriately to make a working standard solution (1 µg/ml - 30 µg/ml) in 0.2 N PCA

c. Procedure for determination of RNA:

RNA in the isolated pancreatic islets of mice (normal as well as obese-hyperglycemic) and the principal islets of both species of fish was determined using a modified procedure, adapted from Munro & Fleck's method (314).

Tissue weighing about 100 µg. was homogenized directly in ice cold 0.3 N PCA (50 µl). After thorough homogenization, the micro test tubes were left in a cooled thermostatic bath (Gallenkamp) 5 - 10 minutes. These were centrifuged in a cold atmosphere for about 5 minutes at 3,000 r.p.m. and the supernatant was discarded. The precipitate was then washed twice with 50µl of cold 0.2 N PCA, and centrifuged as above, discarding the supernatant each time. Complete draining of acid was done by placing a small roll of filter paper carefully into the tubes. The rack containing these tubes remained

in cold water bath until the hydrolysis step was started.

To each tube 50µl. of 0.3 N KOH was added and the tissue residue after stirring for some time (using a Mickle shaker) was kept in an incubator set at 37°C. After one hour of incubation the tissue digest was cooled down by placing the rack in crushed ice and then 25µl. of 1.2 N PCA was added to each tube. The tubes, after stirring, were left in the cold water bath for 10 minutes and then centrifuged at 3,000 r.p.m. for five minutes or until the supernatant was clear. The supernatant was removed in a pipette. The precipitate was washed with 50µl. of cold 0.2 N PCA and centrifuged as previously. These washings and the supernatant from the pipette were taken in a 200 µl.micropipette which was then filled up to the volume with 0.2 N PCA.

This final, RNA fraction, solution was transferred to the micro-cuvette and its absorbency was measured at 260mµλ or its UV absorption spectras was obtained (200mµλ - 300mµλ) in the U.V. spectrophotometer against a blank of 0.2 N PCA solution. The absorbency of the final solution was stable over a few hours.

The absorbency of standard RNA solutions of strengths varying from 0.1 µg./100µl to 3 µg /100µl was also measured at the same wavelength against the same blank solution of 0.2 N PCA as for RNA fraction solutions.

The method described above was used for pancreatic islets of mice and Tilapia mossambica. It was scaled up ten times for liver, kidney and exocrine pancreas and also for principal islets of Cottus scorpius. In pieces of liver, kidney and exocrine pancreas, RNA was also determined by macrochemical procedure (314)[a] for comparison.

Experiments were always carried out on duplicate samples and their mean was taken for one determination.

iv. Experimental Treatment:

Injections were made with a Record all-glass 1-ml. syringe and a 26 gauge needle.

Alloxan monohydrate was dissolved in citrate phosphate buffer at PH 4.0 (17) as 5% w/v solution and used within 15 - 20 minutes. It was given intramuscularly to fish (17) and intraperitoneally to overnight starved mice in doses of 310 mg/kg and 230 mg/kg body weight respectively. Fish were killed at 2, 4, 6, 12, 24, 48 and 72 hours after the injections while mice were sacrificed after 24 hours.

Controls received citrate buffer injections alone.

v. Statistical methods:

Statistical treatment of data aimed only at establishing the significance of the difference between uncorrelated means. Firstly, the Standard Deviation (S.D) was calculated. Secondly, student 't' test was applied to most of the results.

All calculations were done on a calculator (Archimedes Diehl Machine Co.Ltd., London) while S.D. and 't' tests were obtained using the electronic desk computer (Olivetti, London).

vi. Histological Procedure:

For histological study principal islets of Cottus scorpius, pieces of pancreas, liver and kidney of both Cottus and mice (normal and alloxan treated) were fixed in Bouin's fluid. Pancreatic paraffin embedded sections were stained with Aldehyde fuchsin (as described under Chap.2. Section II). Routine hematoxylin-eosine staining was applied to the rest of the tissue sections.

Chapter 3. EXPERIMENTAL RESULTS

i. Microdissection:

Plate 35 shows an isolated pancreatic islet dissected from mouse pancreas. When completely dissected out, these tiny islets appeared as free, round or ovoid structures with a greyish white to slightly reddish brown colour which on close observation seemed to be due to the blood capillaries present in the islet. Sometimes it was rather difficult to identify the islets in fresh pancreas of fed mouse as the translucency of the tissue was decreased due to its greyish colour and the contrast between the islets and the exocrine tissue was minimized to a great extent.

It was found that pancreatic islets of obese-hyperglycemic mice were rather easy to identify in the mass of fresh pancreas, due to their much enlarged size as compared to normal mouse islets. They were also very much more conspicuous in the thin pancreas because of their bright colour owing to the numerous distended blood capillaries. It was, therefore, comparatively easy to detach exocrine tissue and dissect out islets in pancreas obtained from obese hyperglycemic mice in comparison to normal mouse.

The pancreatic islets were observed to be surrounded by a delicate capsule of connective tissue. The capsule was found to be very fragile and if it was damaged even minutely, it got punctured and islet cells found their way through it in to the surrounding medium.

During the preliminary studies, the size of the freshly dissected islets of both normal and hyperglycemic mice was measured directly by an eye-piece micrometer and is given in Table 1. It was possible to ensure measurement of the maximum optical surface in each islet. The maximum diameter of that optical surface and the diameter perpendicular to it were both measured. The average values for the isolated islets are based on observations from at least four islets for each individual mouse.

PLATE 35. A completely isolated pancreatic islet dissected from fresh pancreas of mouse. The delicate connective capsule surrounding the islets tissue is distinct and very clearly seen.

(X 55)

TABLE 1

Measurements of Isolated Islets Size

Species	Number of observations made	Maximum diameter of the islet		Diameter perpendicular to the maximum diameter	
		Range observed	Average value	Range observed	Average value
Normal mouse	40	80μ - 250μ	160μ	70μ - 220μ	135μ
Obese hyperglycemic mouse	40	100μ - 340μ	190μ	85μ - 330μ	175μ

The average wet weight of a number of isolated islets, singly or in groups was also determined and is given in Table 2.

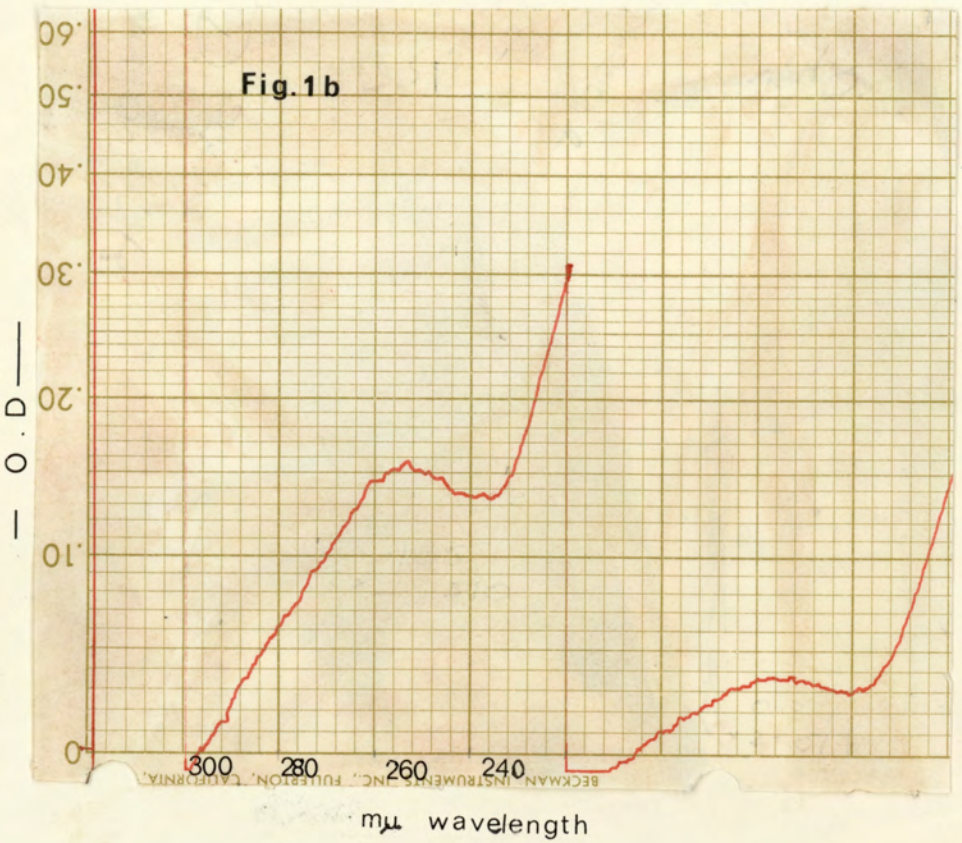
TABLE 2

Determination of wet weight of Isolated Islets

Species	Number of islets	Mean weight (μg.)	Range observed (μg.)
Normal mouse	205	22.7	9.3 - 44.3
Obese hyperglycemic mouse	115	65.4	30.7 - 165.3

ii. Specificity and accuracy of ultramicrochemical procedure

There were two micro-methods, one of Scott (313) and the other of Munro and Fleck (314), available for RNA determination in milligram quantities. Both these methods, particularly Scott's for fresh tissue sections were applied to determine the amount of RNA in the isolated pancreatic islets and other tissues of mouse. Reasonably good results were obtained in the case of liver, exocrine pancreas and kidney homogenates, as suggested by their respective ultra-violet absorption spectra (Fig. 1a showing maximum absorption at 260 mμ). It was, however, not possible to obtain pure RNA fraction from pancreatic islet homogenates by these methods. These methods appeared either to be



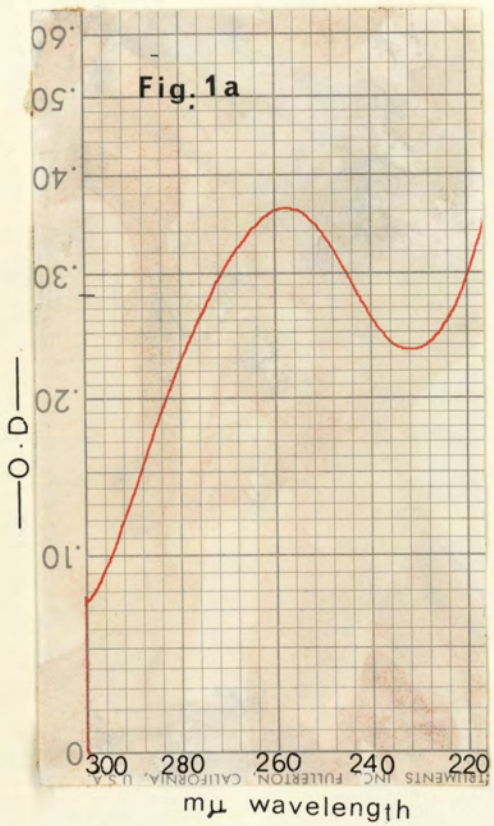
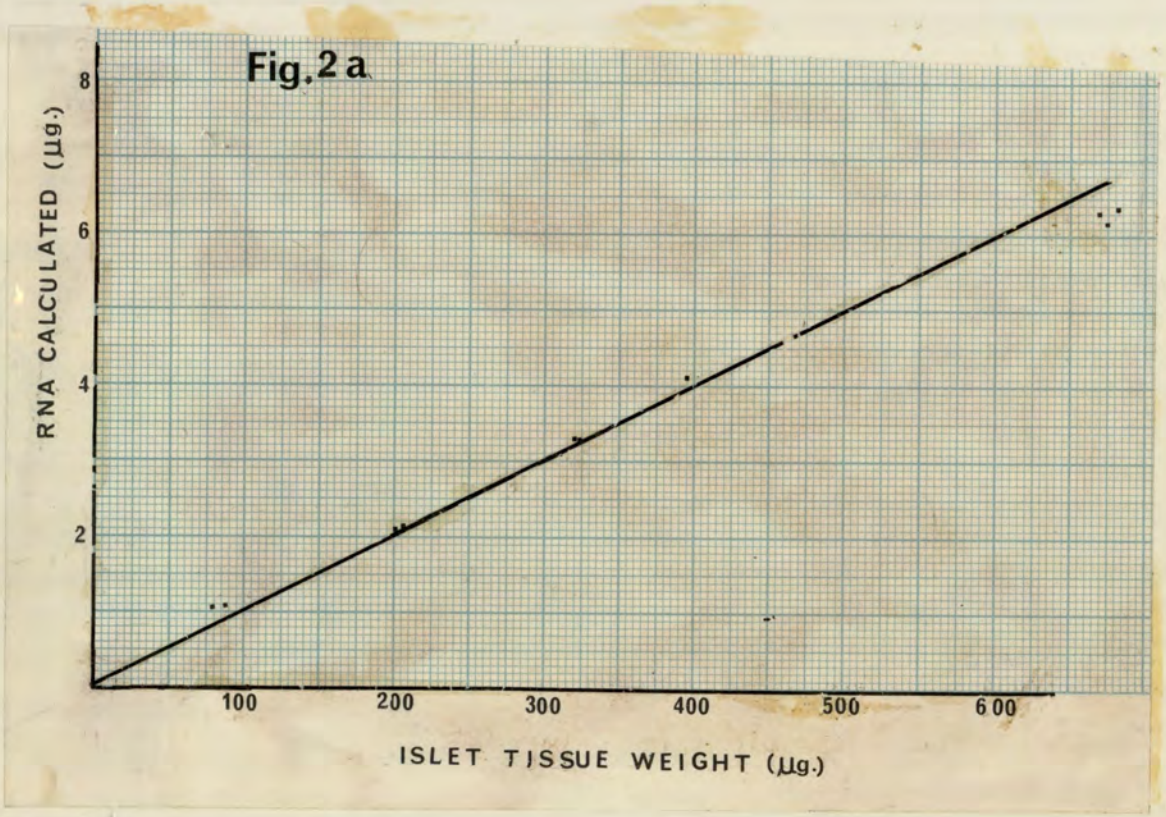


Fig. 1a. UV absorption spectra of RNA fraction obtained from mouse exocrine pancreas by Scott's method

Fig. 1b. UV absorption spectra of islet RNA fraction showing interference due to degradation products, (Scott's method)

insensitive when the final solution was read in the spectrophotometer or they gave very inconsistent results. The RNA fraction obtained from homogenates of pancreatic islets (islet RNA fraction) never showed maximum absorption at 260 mμλ . The UV absorption spectrum of islet RNA fraction (Fig.1b), on the other hand, mostly showed interference, probably due to NaOH or certain degradation products, which ruled out any possibility of accurate determination of the amount of RNA.

It was possible to develop an ultramicrochemical procedure which was used in the present study for RNA determination in μg. quantities of pancreatic islet tissue. Pancreatic islet homogenates of varying concentration when subjected to this modified procedure produced consistently good and uniform results to a great extent. The result of such an experiment is shown in Fig.2a. The complete UV absorption spectra obtained from RNA fractions of isolated pancreatic islets are illustrated in Fig.2b. It shows that these RNA fractions exhibited characteristic UV absorption spectra similar to those obtained normally from pure yeast RNA (Fig.3a) and mouse pancreas RNA (Fig.3b) but altogether different from that of islet RNA fractions obtained by Scott's micromethod (Fig.1b). The UV absorption spectra seen in Fig.3a were obtained when yeast RNA, used as standard RNA in the present study, with varying strengths, was subjected to the present modified procedure. Obviously, owing to the very small amount of pancreatic islet tissue available it was not possible to prepare pure RNA from it to use as the standard RNA, but a successful attempt was made to prepare RNA from normal mouse pancreas according to the method of Kirby (315) and when its absorbency was read in the spectrophotometer, a characteristic UV absorption spectra showing the maximum absorption at 260 mμλ for RNA was obtained as shown in Fig.3b. The UV absorption spectra of yeast RNA (Fig.3a) and of islet RNA fraction (Fig.2b) likewise show the maximum absorption nearly at the same wavelength characteristic for RNA.



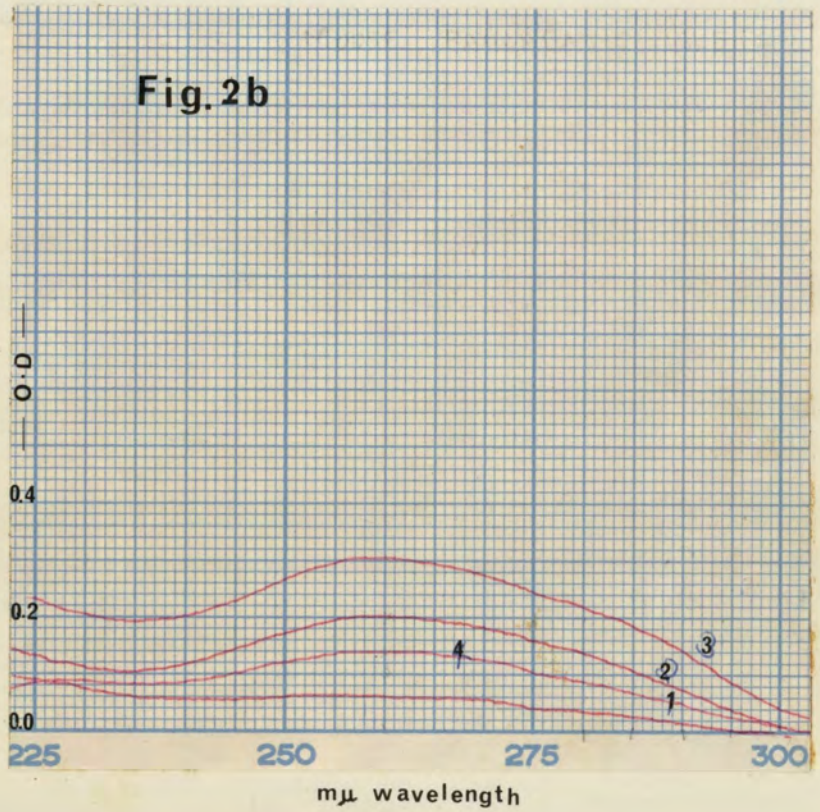
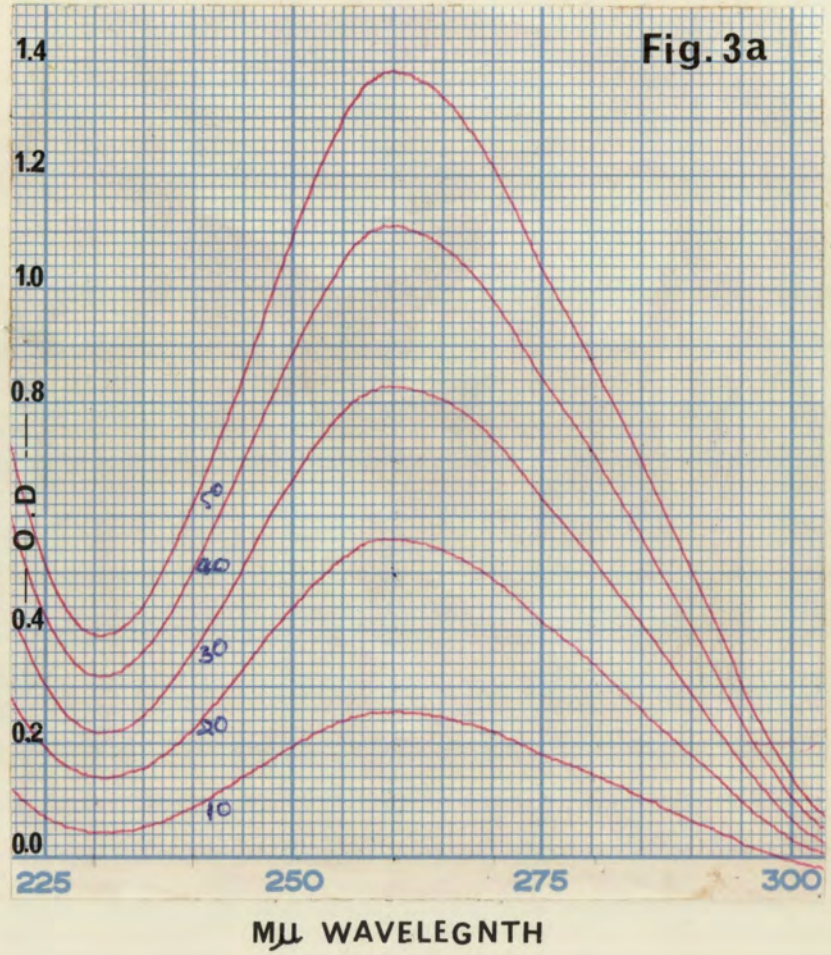


Fig.2a. Relationship between islet tissue weight and the amount of RNA (µg) calculated

Fig.2b. Characteristic UV absorption spectra of RNA fraction of mouse pancreatic islet (2,3,4 in Fig.b) as obtained by modified ultra micromethod



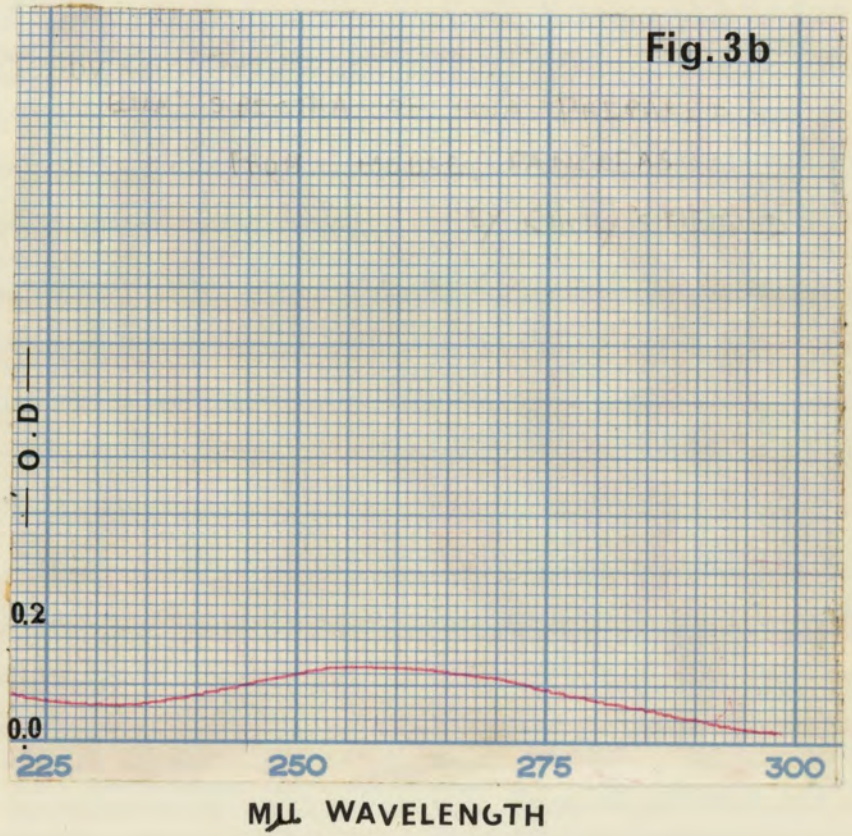


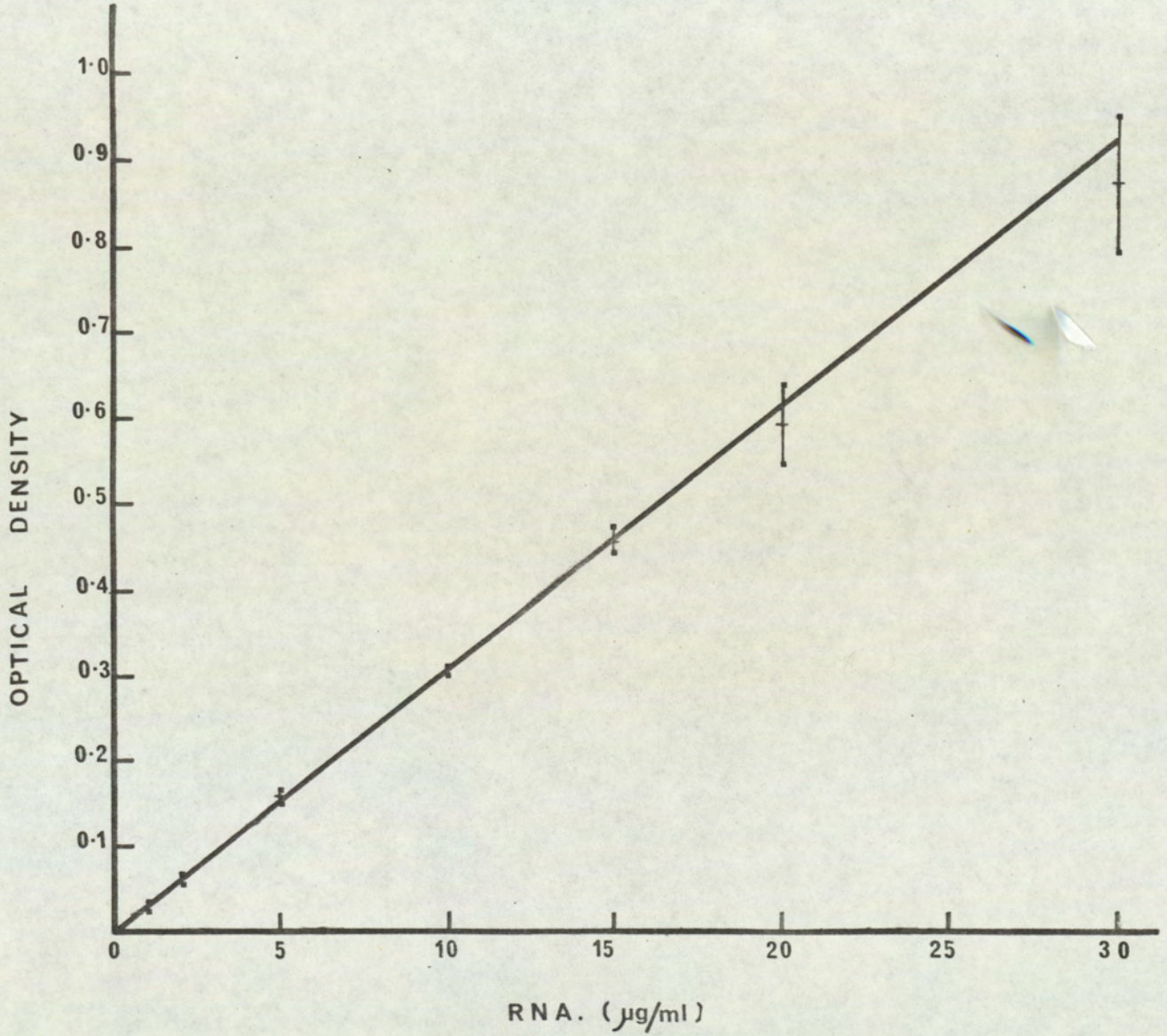
Fig. 3a and 3b: Characteristic UV absorption spectra of different concentrations of yeast RNA (3a) and of mouse pancreas RNA (3b obtained by Kirby's method).

It could be seen that the complete spectra of islet RNA fractions neither showed occurrence of any sort of interference due to NaOH nor any degradation of ribomononucleotides and proteins due to prolonged alkaline digestion. It was also apparent that complete precipitation of DNA and protein was carried out during this procedure. When 232 mμ absorption/260mμ absorption and 260mμ absorption/280mμ absorption ratios, calculated from the complete UV absorption spectra of tissue RNA fractions were compared with those obtained from yeast RNA to detect significant amounts of polypeptides, no difference was found. The presence of a prominent peak (maximum absorption) at 260mμ or very close to this wavelength indicated that it would be possible to determine the amount of RNA in accurate quantities.

Standard curve: Fig.4 shows a typical standard curve for yeast RNA. The curve is seen linear up to a sample concentration of about 25 μg/ml When the straight line was fitted to the points it was found that 10 μg /ml RNA had an optical density (O.D) of 0.32. The range of optical densities at each concentration of RNA is represented by the line drawn between the two large points while the smaller points represent the average optical density of all the determinations carried out at each concentration. Since for tissue digest the best results were obtained at optical densities above 0.08 and below 0.40 and when the wet weight of the tissue digest was approximately between 100 and 500 μg (Fig.2a), it (100 - 500 μg) was found to be the usual range of fresh islet tissue used for RNA determination.

As a rule the determinations were carried out on the same day as the tissue sampling. On certain occasions, the tissue was frozen and kept in the deep-freeze (at -25°C) for a day or so but no appreciable loss of RNA quantity was found. The tissue RNA quantity appeared to decrease gradually as the duration of tissue or its homogenates kept in the deep-freeze increased.

Fig.4 STANDARD CURVE FOR RNA. DETERMINATION



iii. Comparison of macro and micro methods for RNA determinations

Results obtained from experiments involving determination of RNA quantity in liver, kidney and exocrine pancreas homogenates of mouse by Munro's macro method and Scott's micro procedure were compared with those obtained by using the present modified ultra micromethod and are shown in table 3. It could be seen from this table that RNA quantities (μg/mg. wet weight of tissue) for different tissue obtained by ultramicrochemical procedure are nearly the same or very close to those determined by macro method. On the other hand, when Scott's micro method was applied lower values (approximately 3 to 13% less) resulted in different tissue homogenates.

Certain ratios, O.D. at 232mμλ/O.D. at 260mμλ and O.D. at 260 mμλ/O.D. at 280mμλ were calculated from complete U.V. absorption spectra for each tissue, and are also shown in Table 3. These ratios were about the same for liver RNA fractions obtained by any of these methods. There was not much difference in these ratios for RNA fractions of both kidney and exocrine pancreas when the macro and ultramicrochemical methods were applied, except that one ratio (O.D. at 232mμλ /O.D. at 260mμλ) for kidney RNA fractions was slightly higher using the latter method. The same was the case when Scott's micromethod was used but this resulted in higher ratio (O.D. at 260 mμλ/O.D. at 280mμλ) for RNA fraction of exocrine pancreas homogenates as well.

iv. RNA determination in mammalian tissues

a. RNA content:

The amount of RNA was determined in the homogenates of isolated pancreatic islets of both normal and obese-hyperglycemic mice as well as in those of liver, kidney and exocrine pancreas of normal mice. The average calculated RNA content (μg/mg. wet weight of tissue) in the pancreatic islets and other tissues of normal mouse is shown in Fig. 5.

TABLE 3

COMPARISON OF RNA ANALYSIS ON MOUSE TISSUES BY MACRO AND MICRO METHODS

TISSUE	MACRO METHOD (munro)			MICRO METHODS					
				(modified)			(SCOTT)		
	A	B	RNA (µg/mg)	A	B	RNA (µg/mg)	A	B	RNA (µg/mg)
EXOCRINE PANCREAS									
MEAN	0·33	1·33	26·88	0·34	1·34	26·65	0·355	1·39	25·6
N			10			15			10
%			100			99·14			97·0
LIVER									
MEAN	0·34	1·38	6·25	0·34	1·39	6·10	0·36	1·38	5·75
N			8			8			8
%			100			97·6			92·0
KIDNEY									
MEAN	0·34	1·35	4·30	0·39	1·35	4·28	0·38	1·34	3·74
N			8			8			8
%			100			99·2			86·9

A = RATIO $\frac{\text{O.D. } 232}{\text{O.D. } 260}$ CALCULATED FROM COMPLETE SPECTRA

B = " $\frac{\text{O.D. } 260}{\text{O.D. } 280}$ " " " "

N = NUMBER OF DETERMINATIONS CONTRIBUTING TO THE MEAN

% = VALUES AS PERCENTAGE OF MACRO DETERMINATIONS

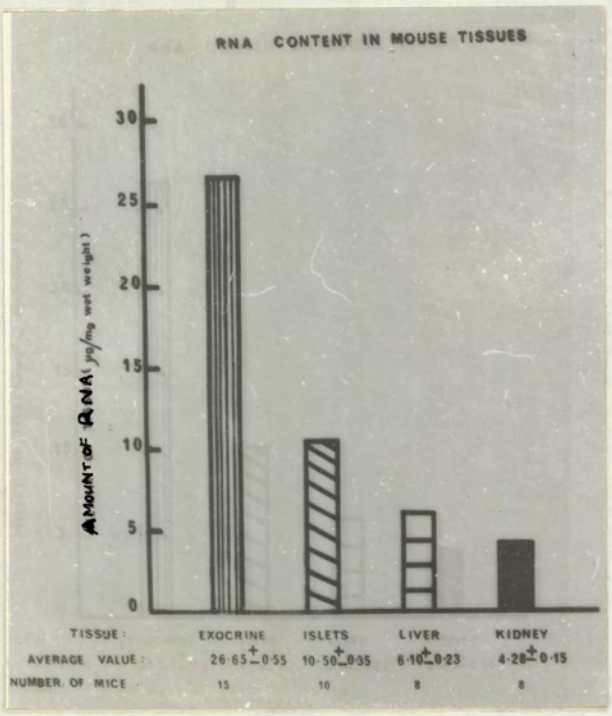


Fig. 5. The average RNA content (ug/mg wet weight of tissue) in the pancreatic islets and other tissues of normal mouse.

These values are based on 15 determinations each for isolated pancreatic islets and exocrine pancreas and 10 determinations each for liver and kidney. Usually one determination was carried out in one animal. It was quite obvious to note that in a tissue the the amount of RNA determined falls within a certain range because it can vary from locus to locus within the same tissue. The results showed that the islet tissue contains higher amounts of RNA (μg/mg wet weight of tissue) than liver and kidney. It was about 42% higher than in liver and about 59% higher than in kidney. On the other hand, RNA quantity (μg./mg. wet weight of tissue) in islets was approximately two and a half times less than in exocrine pancreas, the secretory organ apparently rich in RNA.

The average value of RNA quantity (μg /mg wet weight of tissue) in the pancreatic islets obtained from obese-hyperglycemic mice was found to be 11.20 $\pm$0.25. This average value is based on 12 determinations carried out on homogenates of isolated pancreatic islets obtained from 8 animals (Table 4). No significant difference in RNA content was found between unit islet tissue of normal mouse and that of obese-hyperglycemic mouse ($P < 0.4$).

b. U.V absorption spectra of tissue RNA fractions:

The complete UV absorption spectra of RNA fraction of normal mouse islet homogenates is illustrated in Fig.2b. Similar characteristic maximum absorption was also observed at 260 mμ in the RNA fractions of isolated islet homogenates obtained from obese-hyperglycemic mice. The U.V.absorption spectra of RNA fractions of both strains of mice showed minimum absorption at wavelength 234 mμ. In some cases there occurred a shift in the maximum absorption wavelength from 260mμ to 259.5mμ . Likewise the minimum absorption wavelength varied from 232mμ to 234mμ and very rarely even to 236mμ.

The U.V. absorption spectra of RNA fractions obtained from mouse liver, kidney and exocrine pancreas clearly showed maximum and minimum absorption at wavelengths 260mμ and 232mμ respectively. The kidney RNA fraction, however, sometimes showed minimum absorption at wavelengths 234mμ or 236mμ . The average absorption ratios, O.D. at 232 mμ /O.D. at 260mμ and O.D. at 260mμ /O.D. at 280mμ as shown in table 3 were found to be 0.34 and 1.34 for exocrine pancreas, 0.34 and 1.39 for liver and 0.39 and 1.35 for kidney RNA fractions.

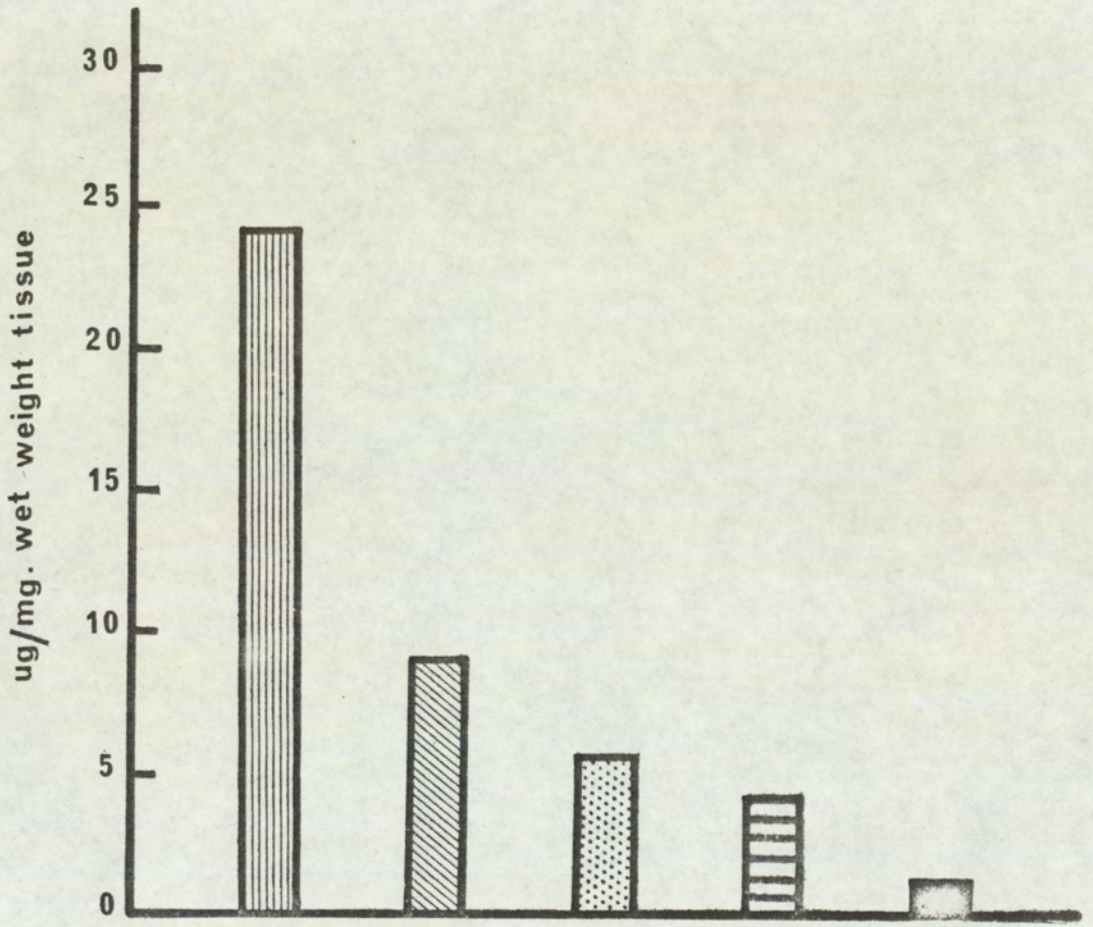
v. RNA determination in tissues of teleost species

a. RNA content:

The amount of RNA was determined in the homogenates prepared from principal islets of Cottus scorpius and Tilapia mossambica. For comparative study, the RNA quantity was also measured in the exocrine pancreas, liver, kidney and muscle. The average calculated RNA (μg /mg wet weight of tissue) in the principal islets and other tissues of Cottus scorpius is shown in Fig.6. At least two determinations were carried out on tissues obtained from each animal. As in mouse tissues, islet tissue of Cottus scorpius was also found to contain more RNA (μg./mg. wet weight of tissue) than liver (35% less) or kidney (54% less). The quantity of RNA was approximately 85% higher in unit amount of islet tissue than in muscle. Also, it was approximately 2.65 times less in islet tissue than in exocrine pancreas. In general the average values of RNA for different tissues in Cottus scorpius appeared to be in the same range, though slightly lower, when compared with those in mouse tissues. The islet tissue of mouse, however, showed about 15% more RNA content (μg./mg. wet weight of tissue) than the principal islets of Cottus scorpius (Table 4).

Fig. 6

RNA CONTENT IN COTTUS TISSUES



TISSUE :	EXOCRINE	ISLETS	LIVER	KIDNEY	MUSCLE
AVERAGE VALUE:	24·1	9·1	5·9	4·2	1·28
NUMBER OF FISH :	8	15	8	8	8

TABLE 4

Comparison of average RNA content (μg/mg. wet weight of tissue) and the absorption ratios in the RNA fractions obtained from pancreatic islets of both mammalian and teleost species.

ANIMAL SPECIES	RATIOS A	RATIOS B	RNA CONTENT	%
Normal Mouse	0.41	1.37	10.5 ± 0.32 (15)	100
Obese-Hyperglycemic Mouse	0.43	1.39	11.2 ± 0.25 (12)	106.6
Cottus scorpius	0.40	1.36	9.1 ± 0.19 (26)	86.6
Tilapia mossambica	0.43	1.37	9.4 ± 0.21 (12)	89.6

A = Absorption ratio O.D. at 232 mμλ/O.D. at 260 mμλ calculated from U.V. absorption spectra

B = Absorption ratio O.D. at 260 mμλ/O.D. at 280 mμλ calculated from U.V. absorption spectra

Number of determinations contributing to the mean (mean result of duplicate samples in each experiment was taken for one determination) are given within parentheses.

% = values as percentage of result obtained from mouse islets

When analysed separately, pylorous and splenic principal islets of Cottus scorpius were found to contain the same amount of RNA (μg /mg. wet weight of tissue).

The average calculated amount of RNA in the principal islets and other tissues of Tilapia mossambica is given in Table 5.

TABLE 5

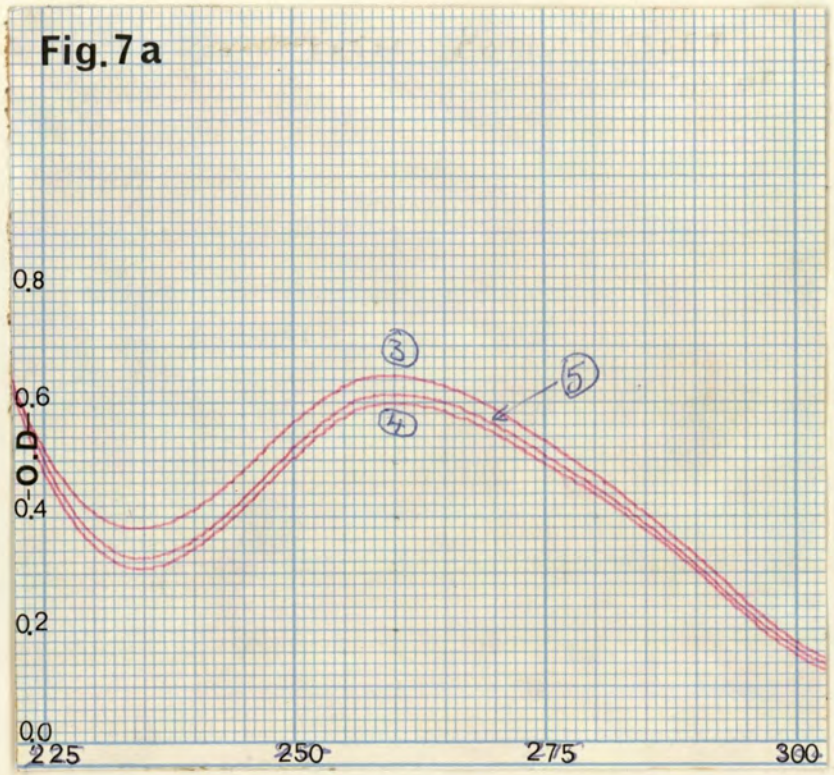
Amount of RNA (μg /mg. wet weight of tissue) in Principal islets and other tissues of Tilapia mossambica

	Principal islets	Exocrine pancreas	Liver	Kidney
Mean value	9.4 ± 0.21	22.90 ± 0.52	5.70±0.48	4.23 ±0.31
Number of determinations	12	8	8	8
Number of animals	6	4	4	4

Again, two determinations were carried out from tissues of each animal. It was apparent that the average amount of RNA for different tissues corresponded very closely to those of their counterparts in Cottus scorpius. There appeared to be no difference in the RNA content (μg./mg. wet weight of tissue) between the principal islets of two teleost species.

b. U.V. absorption spectra of tissue RNA fractions :

The complete U.V. absorption spectra of RNA fractions obtained from the principal islet tissue of Cottus scorpius and of Tilapia mossambica are shown in Fig. 7 (a) and 7 (b) respectively. As in the case of mammalian tissue RNA fractions these spectra exhibited the same characteristic prominent peak (maximum absorption) at wavelength 260 mμ as those observed in yeast RNA (Fig. 3a) and mouse pancreas RNA (Fig. 3b). However, in some cases the wavelength for maximum absorption shifted to 259.5 or 260.5. They also showed the minimum absorption at wavelengths between 232 mμ and 234 mμ.



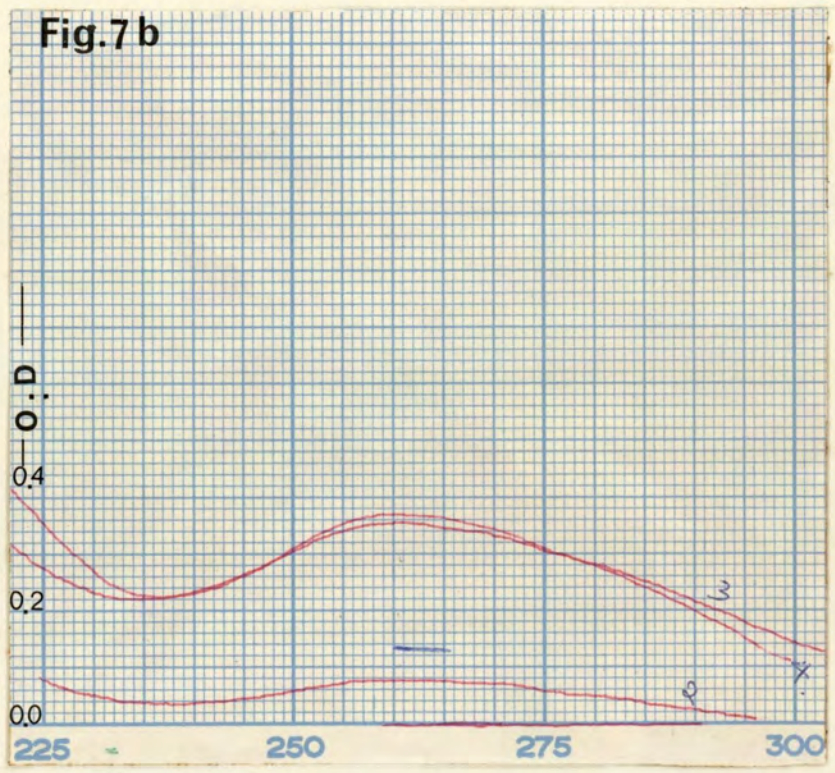


Fig.7a.b. UV absorption spectra of islet RNA fractions of Cottus scorpius (3,4,5 in Fig.7a) and Tilapia mossambica (3,4 in Fig.7b) (Modified ultra micro method).

The RNA fraction of liver, exocrine pancreas, kidney and muscle of both teleost species showed the normal characteristic U.V. absorption spectra. The maximum absorption was clearly recorded at wavelength 260 mμ in all these tissue RNA fractions but the minimum absorption at wavelength 232 mμ was observed only in the case of liver and exocrine pancreas RNA fractions. The kidney and muscle RNA fractions, however, showed the minimum absorption mostly at wavelengths 234 mμ or 236 mμ.

vi. Absorptivities and absorption ratios

The solution of ribomononucleotides in the mole ratios (316) for preparation of mammalian RNA was not prepared and analysed spectrophotometrically to get the absorption spectrum for comparative purposes. Instead, two absorption ratios - the ratio between absorptions at wavelengths 232 mμ and 260 mμ and that between absorptions at wavelengths 260 mμ and 280 mμ were calculated from such a spectrum of ribomononucleotides solution prepared by Scott (313). These absorption ratios, denoted in this text as A (O.D. at 232 mμλ /O.D. at 260 mμλ) and B (O.D. at 260 mμλ/O.D. at 280 mμλ) are 0.34 - 0.35 and about 1.35 respectively. These values were taken as standard absorption ratios for the present series of experiments. Scott's reported (313) the absorption ratio B to be 1.33 for liver and 1.37 for kidney in rat.

When the absorption ratios A and B, calculated from the absorption spectra of yeast RNA, normalized to the optical density at 260 mμλ for the nucleotide mixture, were compared with these standard absorption ratios, no significant difference was found.

Absorption ratios A and B were calculated for each absorption spectra of tissue RNA fractions to test the validity of the spectra. The average absorption ratios for RNA fractions of mouse tissue are given in Table 3 and those for islet RNA fractions in both mammalian and teleost species are illustrated in Table 4. These ratios were not constant in all the experiments, either for different tissue

TABLE 6

COMPARISON OF AVERAGE RNA CONTENT ($\mu g/mg$) AND ITS PERCENTAGE DECREASE IN MOUSE AND COTTUS TISSUES AFTER ALLOXAN TREATMENT.

	COTTUS SP.			MOUSE		
TISSUE	CONTROL	TREATED	DECREASE %	CONTROL	TREATED	DECREASE %
EXOCRINE						
MEAN	23.50 ± 0.45	22.30 ± 0.58	5.25	25.85 ± 0.25	23.95 ± 0.66	7.90
N	3	4		2	6	
ISLETS						
MEAN	9.05 ± 0.20	6.48 ± 0.93	28.3	10.30 ± 0.12	6.50 ± 0.62	36.90
N	8	12		4	12	
LIVER						
MEAN	5.80 ± 0.15	4.5 ± 0.95	22.4	5.95 ± 0.10	5.15 ± 0.31	13.4
N	3	4		2	6	
KIDNEY						
MEAN	4.20 ± 0.18	3.45 ± 0.67	17.8	4.12 ± 0.32	3.45 ± 0.58	16.2
N	3	4		2	6	

N = NUMBER OF ANIMALS USED FOR RNA DETERMINATIONS

RNA fractions or for various RNA fractions of the same tissue in one species but varied to some extent, though insignificantly.

It could be seen from Table 4 that the absorption ratio B calculated for islet RNA fractions was generally found to be close or nearly close to the standard one (1.35), but sometimes slightly higher ratios (normal mouse 1.42; obese hyperglycemic mouse, 1.41; Cottus scorpius, 1.40; Tilapia mossambica, 1.41) were also calculated. The absorption ratio A was usually found to be higher (occasionally as high as 0.48, as in Cottus scorpius) than the standard ratio (0.35) but in certain cases it was calculated to be even lower (0.31, usually in all species) than it.

vii. Effect of Alloxan administration on the RNA content of mouse tissues.

The amount of RNA in the pancreatic islets, exocrine pancreas, liver and kidney of mouse three days after alloxan injection was determined. The results are illustrated in Table 6. Two determinations were carried out for each tissue in control animals (total determinations in islets were 8 and in other tissues 4 each) whereas only one determination for each tissue was done in treated animals (total determinations in islets were 24 and in other tissues 12 each). The amount of RNA in pancreatic islets of treated animals was found to vary from 5.5 μg to 7.7 μg/mg. (average 6.4) wet weight of tissue and these values were significantly ($P < 0.001$) different from those obtained in control animals. The average percentage decrease in the RNA content of pancreatic islet after alloxan administration was found to be about 37% (Table 6). In one of the alloxan treated mice this decrease in RNA content was as much as about 46%.

A significant decrease in the RNA content (μg/mg. wet weight of tissue) was also found in liver ($P < 0.02$) and kidney ($P < 0.02$) of treated animals. The average percentage decrease of RNA content in

these tissues was about 13% in the former and about 16% in the latter case. One of the treated mice which showed the highest percentage decrease in its islet RNA content showed about 33% decrease in its liver RNA content. Although there occurred a decrease (average decrease about 8%) in the RNA content (μg/mg. wet weight of tissue) of exocrine pancreas of treated animals it was not significant ($P < 0.5$).

Although separation of RNA nucleotides was not attempted during the present investigation it seemed that when the complete U.V. absorption spectra and the absorption ratios $\frac{A}{A}$ (O.D. at 232 mμ/ O.D. at 260 mμ) and B (O.D. at 260 mμ/O.D. at 280 mμ) of tissue RNA fractions obtained from treated animals, were compared with those from the control animals, no qualitative difference could be seen.

vii. Effect of alloxan administration on RNA content of tissues of Cottus scorpius.

In the preliminary experiment, 12 fish were injected with alloxan intramuscularly (17) and the RNA content was determined in the principal islets and in the exocrine pancreas, liver and kidney, five to six hours after the injection. Two determinations each were carried out on tissues of one control animal (total determinations for each tissue, 6). In treated animals, 12 determinations for principal islets and 6 determinations each for liver, kidney and exocrine pancreas, at least one sample from each animal, were performed in all. The results showed a fall of RNA content in the principal islets, liver and kidney of treated animals.

The average content of RNA (μg/mg. wet weight of tissue) in different tissues of Cottus scorpius after alloxan administration is given in Table 6. It could be seen that a considerable decrease occurred in the amount of RNA in all the tissues studied, particularly in the principal islets, except in exocrine pancreas. Table 6 also shows the average percentage decrease in RNA content of different

tissues in comparison with that observed in different tissues of alloxan treated mice. It shows that decrease in RNA content was highest in the principal islets (about 28%) followed by that in liver (about 22%) and kidney (about 18%). It was observed that the percentage decrease in RNA content of principal islets fell mostly within a certain range - from about 20% to 35% of the control values but it was always marked and significant. On the other hand, RNA content in liver and kidney of a couple of treated animals did not appear to be appreciably changed. In these cases, the decrease in their RNA content was very low; about 9% in liver and about 4% in kidney. In most of the experiments, however, a definite and very pronounced decrease in the RNA content of both liver and kidney, as much as 36% and 32% respectively was recorded.

Characteristic histological changes described elsewhere were observed in the principal islets of both mice and fish after alloxan treatment. Studies involving RNA determination were then extended so as to follow the change in RNA content in the principal islets and other tissues of Cottus scorpius at different periods of time following the administration of alloxan. The amount of RNA (µg/mg. wet weight of tissue) in the principal islets, liver, kidney and muscle of Cottus scorpius was determined at 2, 4, 6, 12, 24, 48 and 72 hours after the administration of alloxan and the average values obtained in these tissues for each experiment are illustrated in Table 7. Only two animals served as controls for each tissue in each experiment but two determinations of duplicate samples were carried out on each animal. Four treated animals were used for each tissue in each experiment except that in the case of principal islets for experiments done at 2, 4, 6 and 12 hours after the alloxan administration, 8 treated animals were used and for those performed at 24, 48 and 72 hours after alloxan treatment, 6 treated animals were used. In most of the cases at least one determination of duplicate sample of each tissue per one animal was carried out. The

TABLE 7

RNA Content (μg/mg. wet weight tissue) in the Principal Islets, Liver, Kidney and Muscle of Cottus Scorpius at Different Periods of time following Alloxan Administration

TISSUE		RNA CONTENT (Hours after Alloxan Administration)						
		2	4	6	12	24	48	72
ISLETS	C	9.15 ± 0.16 (2)	9.02 ± 0.14 (2)	8.87 ± 0.11 (2)	8.94 ± 0.19 (2)	9.21 ± 0.18 (2)	9.04 ± 0.18 (2)	9.09 ± 0.16 (2)
	T	7.55 ± 0.16 (8)	6.35 ± 0.09 (6)	5.89 ± 0.17 (6)	5.70 ± 0.07 (7)	5.17 ± 0.11 (6)	5.20 ± 0.08 (8)	6.20 ± 0.12 (7)
LIVER	C	5.93 ± 0.11 (2)	5.81 ± 0.09 (2)	5.89 ± 0.12 (2)	5.81 ± 0.15 (2)	5.70 ± 0.15 (2)	5.74 ± 0.11 (2)	5.89 ± 0.15 (2)
	T	3.42 ± 0.11 (4)	3.60 ± 0.15 (4)	4.02 ± 0.29 (4)	4.22 ± 0.14 (4)	4.20 ± 0.11 (4)	5.60 ± 0.16 (4)	5.80 ± 0.11 (4)
KIDNEY	C	4.19 ± 0.14 (2)	4.27 ± 0.24 (2)	4.23 ± 0.12 (2)	4.06 ± 0.15 (2)	3.99 ± 0.02 (2)	4.13 ± 0.06 (2)	4.24 ± 0.07 (2)
	T	2.40 ± 0.29 (4)	2.60 ± 0.13 (4)	3.00 ± 0.11 (3)	3.15 ± 0.06 (3)	3.20 ± 0.06 (4)	3.50 ± 0.08 (4)	4.15 ± 0.11 (3)
MUSCLE	C	1.26 ± 0.11 (2)	1.29 ± 0.11 (2)	1.25 ± 0.02 (2)	1.21 ± 0.09 (2)	1.33 ± 0.04 (2)	1.30 ± 0.05 (2)	1.24 ± 0.04 (2)
	T	0.80 ± 0.03 (4)	0.81 ± 0.03 (3)	0.88 ± 0.02 (3)	0.95 ± 0.08 (3)	1.10 ± 0.01 (3)	1.12 ± 0.01 (3)	1.17 ± 0.05 (3)

The figures denote the mean values; ± standard deviation. Number of animals used are given within parentheses. (Usually two determinations were done from each control animal whereas only one from treated animals.)

total number of determinations done on the principal islets in each experiment were sometimes less than the number of animals used due to the death of a certain number of animals during the experiment. In all, 8 fishes died after alloxan administration between the period of 3 hours to 65 hours (four between $3\frac{1}{2}$ and 5 hours, 1 at 7 hours, 2 at about 18 hours and 1 at 65 hours after alloxan administration).

It appears from Table 7 that the amount of RNA (µg/mg. wet weight of tissue) was much lower than controls in all the tissues at 2 hours after the alloxan injection. The average values of RNA content for different tissue were plotted against hours after the injection and are illustrated in Fig.8. It is apparent from Fig.8 that there is a sudden fall in the RNA content per unit weight of tissue, in all the tissues studied. In the case of principal islets this decrease continued gradually until 24 hours after the injection and there seemed no further decrease afterwards. Instead the principal islets showed higher amounts of RNA (µg/mg. wet weight of tissue) though insignificant at 72 hours after the injection than that at 48 hours after the injection but it was still significantly lower than the controls (Table 7.). Percentage decrease in RNA content of different tissues was also calculated for each experiment and is shown in Table 8. It appears from this Table that the decrease in islet RNA content is very much pronounced (about 44%) at 24 hours after the alloxan administration. Also, though the decrease at 72 hours after the alloxan administration is not as much as in the former case, it is still significant ($P < 0.01$).

Liver, kidney and muscle also showed a decrease in their RNA content (Table 7) at 2 hours after the alloxan administration but it was apparent (Fig.8) that afterwards their RNA content appeared to rise and approach the normal value of the respective tissue (Table 7). Liver, though, showed lower values of RNA content

Fig. 8

EFFECT OF ALLOXAN ON RNA CONTENT OF COTTUS TISSUES

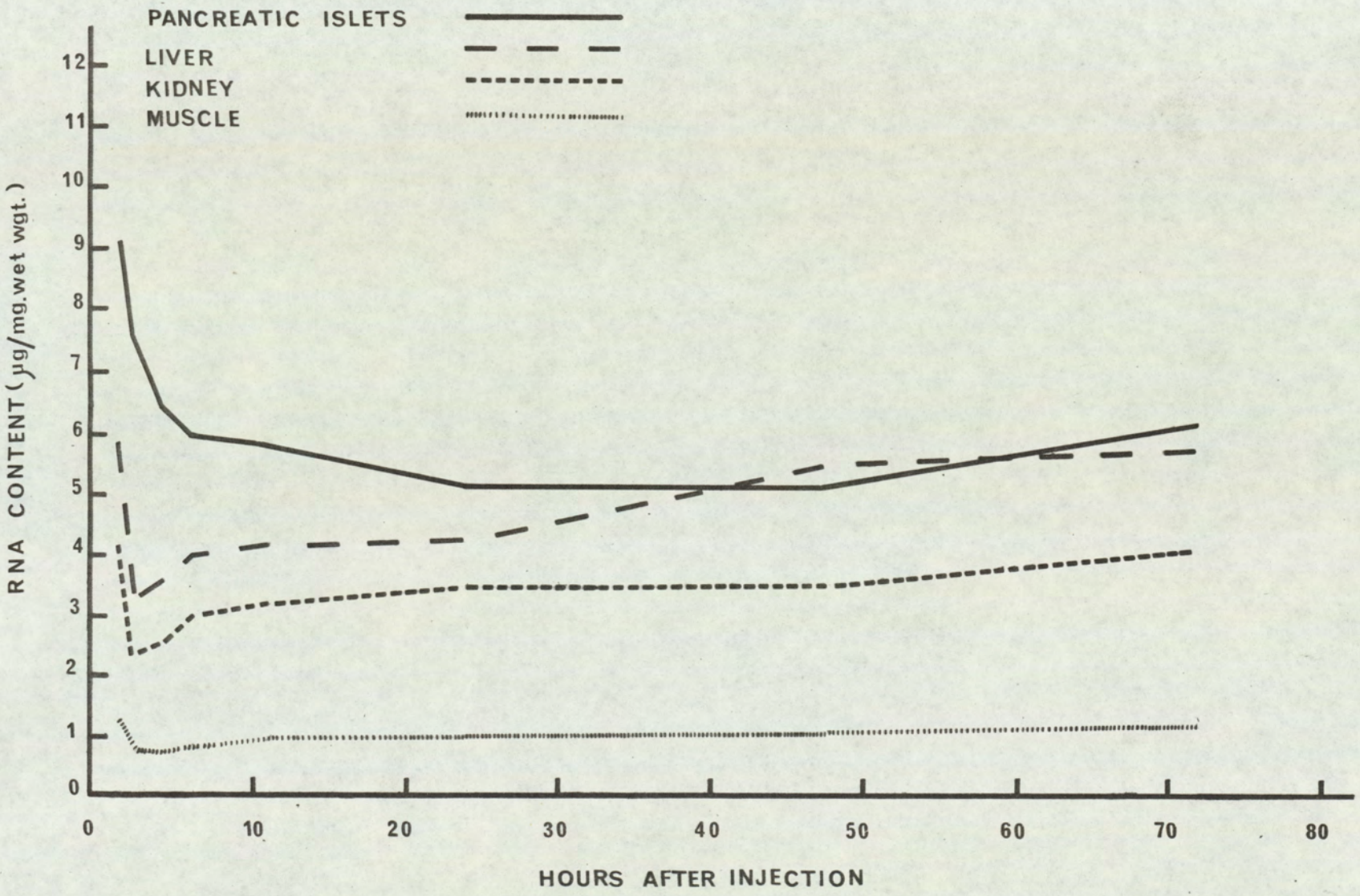


TABLE 8

PERCENTAGE DECREASE IN RNA CONTENT OF COTTUS TISSUES AFTER ALLOXAN TREATMENT

HOURS AFTER INJECTION	ISLETS	LIVER	KIDNEY	MUSCLE
2	17·50	42·30	42·80	37·50
4	29·67	38·90	38·00	37·50
6	33·51	32·20	28·50	29·60
12	36·26	28·80	23·80	21·80
24	43·90	25·50	19·40	17·90
48	42·70	1·60	16·60	14·00
72	31·80	1·60	2·30	6·20

(Table 7) at 48 hours after the alloxan administration but the average percentage decrease was only about 2%. Kidney and muscle, on the other hand, showed significantly lower values at 24 or 48 hours after the alloxan injection (Table 7) and about 17 - 20% and 14 - 20% respectively decrease in their RNA content 72 hours after the alloxan administration. Liver, kidney and muscle all contained fractionally but insignificantly lower values for their RNA content (Table 7) and the percentage decrease was negligible (Table 8).

The U.V. absorption spectra and the absorption ratios A and B for RNA fractions of the principal islets, liver, kidney and muscle obtained from alloxan treated animals exhibited the characteristic features of pure RNA fractions and when compared with those obtained from the untreated ones, there seemed to be no qualitative difference.

viii. Parallel histological observations.

Pancreatic islets of both fish and mice analysed for determining the amount of RNA were also studied histologically to observe the effect of alloxan administration on their morphology and to correlate the change in RNA content with any particular cell type. Histological study in certain cases was also done on the liver and kidneys after alloxan administration to observe morphological changes, if any, occurring in these tissues.

Alloxan-induced changes of degenerative type were evident in the principal islet of Cottus scorpius about 4 hours after the injection. At that time in certain places within the beta cell area of the principal islet a slight shrinkage of the beta cells with an early loss of cohesion was visible. In very few cases it was possible to detect alloxan-induced degenerative changes, though in a very early stage, in the beta cells, even two hours after the injection. Similar changes but more clearly visible and occupying most of the central region were found mostly 6 hours after the injection. At that time pycknosis of the beta cell nuclei was noticed and a lack of cohesion between the cell trebeculae in the central region as well as empty

spaces containing aldehyde-fuchsinophil debris were observed.

More than half of the beta-cell area of the islet was found out to be damaged about 10 hours after the injection. Beta-cell necrosis in the islets was widespread and practically all beta cells of the islet were destroyed in islets studied at 24 hours after the injection. At that time a peripheral rim of unaltered other-than-beta-cells' structure remained. The beta cell area was found to contain remnants of connective tissue, hyperemic vessels, cellular debris and possibly hemorrhages.

In all this study anamolous behaviour was shown by four out of ten pylorous islets. These islets contained a normal amount of RNA. Where the material was available for histological study (in two out of four cases) alloxan-induced degenerative changes were not observed. In these cases the pylorous islets were taken for study six hours after the injection. The beta cells were morphologically intact and RNA content remained apparently unchanged (9.0 ± 0.25).

Only very few samples of liver and kidney obtained from alloxan treated fish (6 hours after the injection) were studied histologically. Kidney clearly showed the degenerative changes. In these specimens the renal tubuli were observed to be damaged to some extent showing a patchy appearance. The tubular epithelium could be seen more or less swollen. The cell boundaries were rather indistinct. Pyknosis of the nuclei, having a coarsely granular cytoplasm, was usually observed. Liver showed irregularly affected areas of necrosis, though sometimes specimens from normal animals were also observed to show slightly degenerative changes probably due to varying degrees of the fatty nature of the organ. In general, the histological changes observed in both liver and kidney after alloxan treatment were rather similar to those described by earlier workers both in fish (17) and mammals (317). It has not been possible to study the histological changes in these organs for each experiment in relation to those carried out for RNA determinations.

In the alloxan treated mice only the pancreatic islets were studied histologically. There were great similarities with the alloxan degenerative changes described by various investigators in various species (197,317). In these islets beta cells were clearly the part of the islet damaged by alloxan. The islets were observed to have granular debris in the centre, probably occupied by the beta cells. It was apparent that the beta cells had degenerated into masses of debris containing fragmented nuclei. Some necrotic beta cells could occasionally be spotted. The cytoplasm obviously was disintegrated with the collapse and co-alescence of beta cells. The aldehyde fuchsin usually stained the central debris mass indicating the presence of some aldehyde-fuchsin positive granules, probably the membranous sacs.

ix. Effect of Tolbutamide treatment on islet RNA content

Six experiments were carried out to see the change in the RNA concentration of mouse islets following their stimulation by treatment with tolbutamide injected in the doses of 0.25 mg/kg. body weight intramuscularly for three days before killing the animal. One determination was performed on each of six treated animals and of four of the control group. The average calculated RNA (μ g/mg. wet weight of tissue) of islet tissue in control and treated animals is given in Table 9.

TABLE 9

Effect of Tolbutamide Treatment on Islet RNA Content (μ g/mg. wet weight of tissue) in Mouse

Control	Tolbutamide	% change	P
10.03 $\pm$ 0.62 (4)	11.14 $\pm$ 0.62 (6)	about 11% increase	< 0.05

The figures denote the mean values, $\pm$ standard deviation.

Number of animals used given within the parentheses.

It could be seen from Table 9 that the average RNA content in the pancreatic islets after tolbutamide treatment is fractionally higher

than that of controls. There was a degree of variability in islet RNA content to some extent, as observed throughout the studies of RNA determination. One sample obtained from treated animals showed about 18% increase in their RNA content but the average percentage increase was found to be about 11. (Table 9). The islet RNA fractions obtained from treated animals displayed absorption spectra having normal characteristic features of islet RNA absorption spectra observed in the earlier study (part of this section); maximum absorption was at 260 mμ and the absorption ratios A and B were in the same range as those of islet absorption spectra in untreated animals.

x. Effect of insulin on the RNA content of Plaice tissues

During the period of studies being carried out in St. Andrews, it became possible to perform a few experiments on another teleost, the Plaice. The study of RNA determination was extended to Plaice tissues (liver and muscle) and, furthermore, the change in their RNA content was studied after treatment with insulin. The results of these experiments are reported briefly here.

Twelve fish (body weight between 70 and 100 g) were given intramuscularly injections of insulin (dose 47.2 U/ Kg.) in Cottus Ringer (Chap. II) daily for three days before killing the animals. Six fishes were administered Cottus ringer only and they served as controls.

The amount of RNA (μg/mg wet weight of tissue) was determined in liver and muscle tissues of ten insulin treated animals and of six control fishes, one determination performed for each tissue in one animal. Two fishes died during the course of the experiment; one was dead a day or so after the first injection, the second died during the second day of the experiment.

The average calculated amount of RNA in liver and muscle

tissues of control as well as treated animals is given in Table 10.

TABLE 10

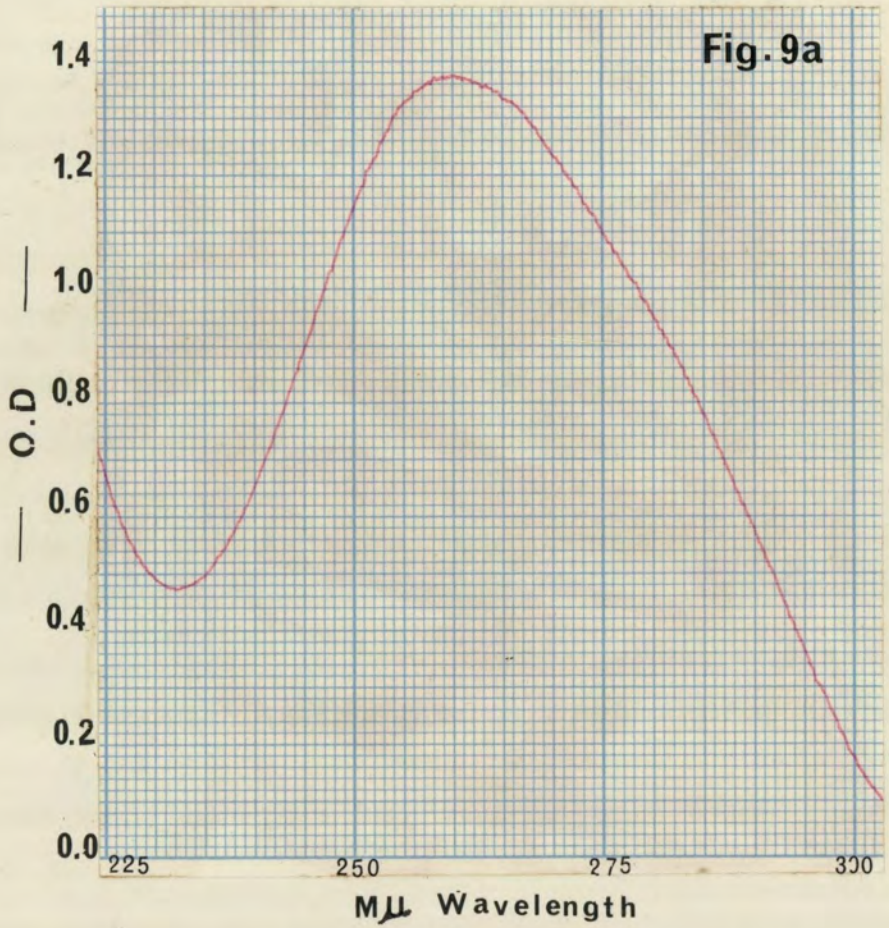
Effect of insulin on RNA content (μ g/mg wet weight of tissue) and muscle tissues of Plaice

Tissue	Control	Treated	% decrease	P
Liver	6.58 $\pm$ 0.70 (6)	4.99 $\pm$ 0.62 (10)	25.6%	< 0.01
Muscle	1.15 $\pm$ 0.13 (6)	0.92 $\pm$ 0.15 (10)	20%	< 0.02

The figures denote mean values $\pm$ standard error

Number of animals given within parentheses.

It could be seen from Table 10 that the RNA concentration in both liver and muscle is lower in treated animals than in controls. The decrease in the RNA content in liver after insulin treatment was found to be about 25% whereas that in muscle tissue average about 20%. Fig.9a and 9b shows the absorption spectra of RNA fractions obtained from Plaice liver and muscle tissues respectively. It shows characteristically maximum absorption at 260 m μ.



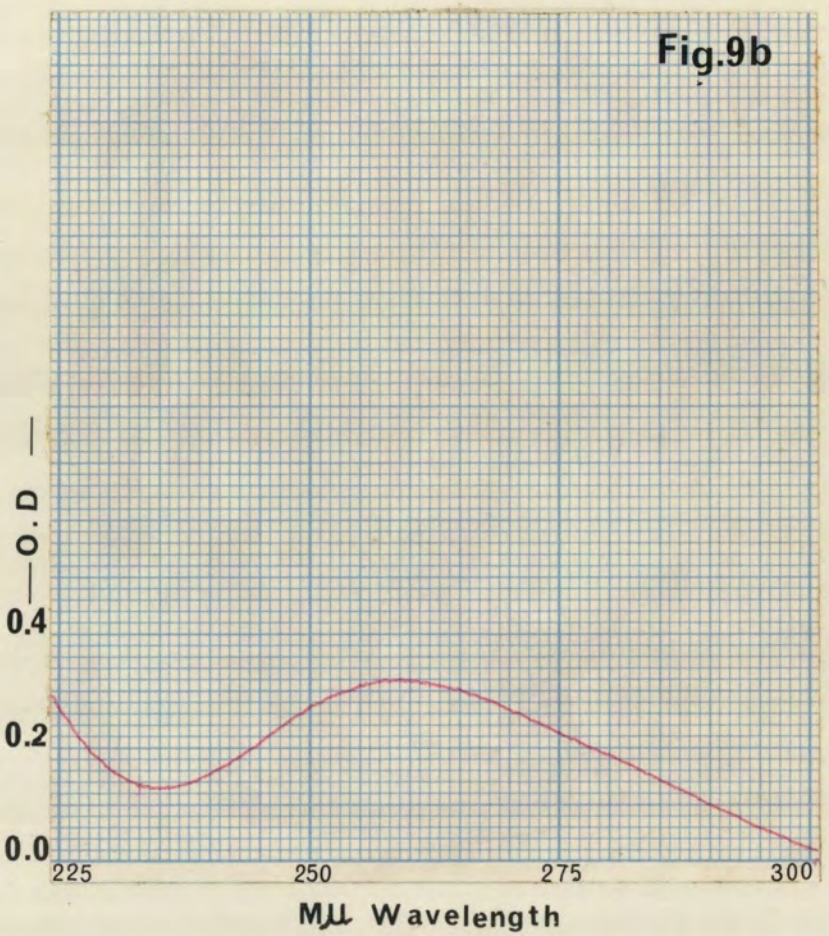


Fig.9.(a.b): Characteristic UV absorption spectra of RNA fractions obtained from Plaice liver (Fig.9.a) and muscle (Fig.9.b). (Munro's macro method).

Chapter 4. DISCUSSIONS

Microdissection

It was soon established during the present study that freehand microdissection of isolated pancreatic islets from fish pancreas following Hellerstrom's technique (34) proved very good to obtain enough tissue material for microchemical analyses.

Previously tissue samples of mammalian pancreatic islets had been obtained from unstained frozen-dried sections of the pancreas of known thickness by microdissection, the sample area of the dissected islets being measured to determine the sample volume in order to correlate the microchemical data obtained, e.g. phosphorus content in the endocrine and exocrine pancreas of mouse, with the unit amount of tissue, but studies involving this technique resulted in only rough quantitative information. Later on, Lowry developed remarkable micro-technique for obtaining µg. quantities of the tissue samples for bio-chemical analyses (318). Lacy and his associates (188, 189) afterwards made significant progress when they applied Lowry's techniques to mammalian pancreatic islets. They used frozen-dried pancreatic section of rabbit and dissected the β cell samples in the range of 0.02 - 0.2 µg localising the islets in the unstained thick frozen-dried section by their characteristic colour and abundant vascularisation (188). Assays for the insulin content (190) as well as for a variety of enzymes both in normal and in experimental conditions were thus carried out (188 - 190). Lazarow and his associates (23) used the same technique to do enzymatic studies on rat pancreatic islets.

Recently Hellerstrom (34) elaborated in detail Benseley's method (6) of mechanical isolation for pancreatic islets from guinea pig pancreas. Hellerstrom's method involved separating the islets from the surrounding exocrine pancreas under the control of a dissecting microscope. This method has two main advantages over Lacy's method of collecting the islet sample. Firstly, the amount of islet tissue

dissected from the fresh pancreas was considerably larger and secondly, fresh islets should be the first choice for any metabolic study as freeze-drying destroys the metabolism of most animal tissues (319). Pancreatic islets have been isolated by this method in rat, guinea pig and obese-hyperglycemic mice and have been shown to be metabolically active (35 - 39). In the present study, the suspended media used was Kreb's modified bicarbonate buffer instead of sucrose solution, to ensure viability of tissue for longer periods during the dissection. In vitro viability of tissue in the former suspended media has been fully confirmed (149).

Moskalewski (320) isolated the pancreatic islets by treating the fresh pancreas with collagenase which is known to digest the collagenous framework and hence was thought to facilitate and speed up greatly the separation of the islets. Keen's attempt (321) to obtain islet tissue from rat pancreas involved ligation of one of the main pancreatic ducts, and then two to four weeks later, dissection of the pancreatic islets from the atrophied pancreas. It is apparent, however, that the material available for dissection would be pathologic and unsuitable for metabolic studies. During the course of the present study, Lacy, in 1967, in an attempt to use Hellerstrom's technique found it difficult to perform on rat pancreas and instead modified (322) Moskalewski's procedure (320). He obtained intact pancreatic islets from rat pancreas which had been treated with collagenase, by sedimentation or by centrifugation method, as he called the two procedures (322). The centrifugation method for isolating the pancreatic islets seemed to be unsatisfactory for in vitro metabolic studies, because it is quite possible that hyperosmolar sucrose might have damaged the islet or the centrifugation and prolonged incubation intervals required for their separation might have initiated damage which was not evidenced by light microscopy (322). It has not been demonstrated yet whether the pancreatic islets isolated by the

sedimentation method using collagenase are suitable for enzymatic studies or not. It would be quite useful to perform parallel studies on pancreatic islets isolated from exocrine pancreas both by freehand dissection and by collagenase sedimentation technique, in order to compare and establish viability of the islets, particularly so as the present day investigations regarding insulin synthesis/secretion mostly involved metabolic studies on the isolated pancreatic islets.

Accuracy of the microchemical method.

To use conventional methods for RNA determination, many animals were needed to get an adequate amount of islet tissue for each experiment. This is obviously so in the case of mammals as the weight of islet tissue obtained is in the μg. range. There are a number of methods available for analysis of nucleic acids (314,323,324) in mg. amounts of tissue but very few have been further modified for RNA determination in the microgram range (313,325).

The methods are based either on extraction of nucleic acids with hot PCA or TCA in the earlier stages, as initiated by Schneider (324) or on the separation of RNA and DNA by the digestion in alkali, as suggested by Schmidt-Thannhauser (323). The former method has been shown (314) to give erroneously low values of DNA because of inadequate extraction from the tissue or because of destruction of deoxyribose (326), the latter has been regarded basically as more reliable when a satisfactory method of estimating RNA was obtained (314). To the latter category belonged Scott's method, designed for RNA determination in microgram amounts of tissue (313) and based on UV absorption of ribomononucleotide mixture possibly without interference by contaminating peptides. Scott's method was utilised during the present study but despite the fact that it produced good results when applied on liver, kidney or exocrine homogenates, it either appeared insensitive for islet RNA determination or showed interference due to NaOH used for alkaline digestion step. Scott himself (313)

experienced difficulty with IN NaOH reagent which has become known for the ease with which it becomes contaminated with ultraviolet-absorbing impurities. Although Scott (313) after reducing the period of digestion in IN NaOH at 22°C to one hour, observed the spectrum of the acid soluble fraction to approach that of pure RNA, the data, as was indicated later on (326) do not allow one to decide whether errors arising from UV absorption of peptides are eliminated or merely reduced by his procedure. Moreover, this method did not produce satisfactory results when used by other workers (314a) and again these failures might have been due to NaOH interference in the UV absorption. Munro and his workers modified (314) Schmidt-Thannhauser's method (323) to obtain a satisfactory procedure for RNA separation but it was not found suitable for islet RNA determination when used during the present study, again due to the small amount of tissue available for each experiment.

It was, therefore necessary to adapt a method to an ultramicro-scale. It has been possible to develop an ultramicrochemical procedure based on Scott's modification (313) of Schmidt and Thannhauser's method (323) and the adaptation has been carried out under the light of a macromethod, modified by Munro and his co-workers (314) for RNA determination. The principle, as in the case of Schmidt-Thannhauser's method, is based on the fact that RNA and DNA are separated by digestion in alkali which hydrolyses RNA to mononucleotides making it acid soluble. DNA and most of the protein of the cell, on the other hand, are precipitated on acidification of the digest. The principle of optical density reading in the ultraviolet spectrophotometer in place of colour reaction or phosphorus estimation has much to recommend it, on the grounds of simplicity, speed and specificity. As it has been reported (326) that other substances become acid soluble during alkaline digestion and in consequence the phosphorus content of acid soluble fractions is usually considerably greater than the amount of RNA.

Also, it is known that certain other carbohydrates are released into the acid soluble fraction and can therefore cause interference in the orcinol reaction (314). On the other hand, the error in UV assay of RNA is apparently due to products of protein degradation (326). The purines and pyrimidines can be determined by measuring the extinction of their solutions in the UV spectrophotometer.

The main exceptions from Scott's method are cold precipitation of tissue constituents (326), omission of lipid extraction as it leads to considerable losses of RNA (314) and RNA hydrolysis by KOH instead of NaOH, at 37°C for one hour (314). PCA is a useful extracting and precipitating agent and it is preferable to TCA since it has a lower extinction in the UV Absorption than TCA which absorbs powerfully at wavelength 260 mμ (314). Previous studies (314) have established that preliminary treatment with cold acid, to remove free nucleotides and to precipitate protein and nucleic acids, followed by incubation of the precipitate with 0.3 N KoH for one hour at 37°C renders RNA acid-soluble. Such treatment has been shown to produce satisfactory results in rat liver (326) with complete hydrolysis of RNA and without its degradation of proteins. During this procedure a negligible amount of acid soluble polypeptide material is extracted (326) and thus an accurate estimate of RNA content can be obtained from the absorbency of the acid-soluble (RNA) fraction at 260 mμ . NaOH was not used in the present study as it is known to interfere with the UV absorption. The present procedure allows the determination of RNA in very small quantities (100 - 500 μg) of fresh islet tissue (Fig. 2a). Results obtained by this method in mouse liver, kidney and exocrine pancreas have been compared with the results obtained on the same tissues by either Scott's (313) or Munro's (314) method of analysis for tissue nucleic acid, and approximately similar values were obtained particularly in relation to the latter method.

Usually the characteristic UV absorption of any tissue RNA

is subject to error when appreciable amounts of protein degradation products are released by prolonged alkaline digestion. When the complete UV absorption spectra of RNA fractions were compared with those of pure RNA (either yeast or mouse pancreas RNA) it appeared that no such degradation occurred and that complete precipitation of DNA and protein seemed to be carried out during this procedure. Furthermore, comparison of UV absorption spectra and certain absorption ratios (O.D. at wavelength 230 mμ /O.D. at wavelength 260 mμ and O.D. at wavelength 260 mμ /O.D. at wavelength 280 mμ) with those obtained from pure RNA showed the procedure to be specific to a great extent as no significant amounts of polypeptides (327) seemed to be detected.

Islet RNA in normal and different functional states of pancreatic islets

The present study shows a new approach of metabolic studies on the isolated pancreatic islets. No study so far has been done to measure RNA content in this tissue of either fish or mammals, though previously histochemical methods indicated a feeble reaction for RNA (312) which was confirmed during the present investigation as well. This study demonstrates that the determination of RNA in the pancreatic islets can be carried out even if approximately 100 μg. of tissue is available. Sandritter (312) on the other hand, using ultraviolet cytophotometrical technique was not able to measure RNA content in the islet beta cells and it was said that unlike in the exocrine pancreas, nucleic acids cannot be found in measureable quantity in the cytoplasm of beta cells. A small amount of RNA, however, was found in the beta cell nucleoli, after stimulation with tolbutamide, as seen after gallocyanin-chromalum staining and treatment with ribonuclease (70). Applying microchemical methods it became possible to determine quantitatively the amount of RNA content in the islet tissue and the quantity of RNA in the beta cell can be calculated to approximation as the loss of amount of RNA by islet tissue after alloxan treatment reflects that contributed by the beta cells. The present parallel microchemical and histological study showed that the damage done to the pancreatic

islets with reference to decrease in RNA is due to the damage of the beta cells. The average decrease of RNA in principal islets of Cottus scorpius after alloxan administration is approximately 29% and this coincides roughly with the planimetric analysis carried out on the islets of this species (17) that aldehyde fuchsin positive cells constitute roughly a quarter of the total amount of isolated islet tissue. Quantitative morphological studies (57) have shown beta cells of mouse pancreatic islets to constitute about 40% of the total cellular mass. The present studies indicated that the RNA amount lost after alloxan administration (about 37%) reflects that contributed by the beta cells.

The amount of RNA calculated in the pancreatic islets of all the four species studied was found to vary slightly from each other, though in each case it was within a certain range. It was significantly different from the RNA amount determined in other tissues of the same species. It is known (327) that there are differences in ribonucleic acid mononucleotide composition of the same tissue from different species and likewise, the ribonucleic acids of various tissue from one species differ in composition and as the calculation of amounts of nucleic acids are dependent upon the purine and pyrimidine compositions of the nucleic acid, it can vary from tissue to tissue, and conceivably from locus to locus within the same tissue. The pancreatic islets were found to have approximately 2.5 - 2.7 times less amount of RNA than in the exocrine pancreas and higher than in any of the other tissues studies (liver, kidney or muscle). It was rather difficult to explain why the histochemical reaction indicated very low amount of RNA in pancreatic islets as compared to that in exocrine pancreas. On the other hand, it was not surprising to find its amount higher than in liver, kidney or muscle, as it is known that cellular components of the islet tissue, i.e. alpha and beta cells, secrete peptide hormones, glucagon and insulin respectively (23) and probably the delta cells

as well secrete a hormone undiscovered yet (62). It is a well established fact that RNA is a template for protein synthesis in other tissues (310) and that insulin biosynthesis in the pancreatic islet tissue is probably similar to protein synthesis in other tissues.

The U.V. absorption spectra of islet RNA fractions obtained from the four species under study was similar in characteristics to that of pure RNA. RNA study has not been carried out previously on the islet tissue of either mammalian or teleostean species, the two absorbency ratios (O.D. at 230mμλ/O.D. at 260mμλ and O.D. at 260mμλ/ O.D. at 280 mμλ) calculated from the U.V. absorption spectra of islet RNA fractions were compared with those obtained in other tissues e.g. liver, by other workers (313).

Further analysis of hydrolysed RNA obtained from pancreatic islet homogenates was not attempted as no method was available at the time of the present study which could be specific and sensitive enough for micrograms of islet tissue. During the final sequence of protein synthesis in other tissues, as has been suggested (311), the specificity of the template is determined by the nucleotide sequence in the RNA, and the various amino acids are assembled on the template in the proper sequence and after all the peptide bonds have been formed, the protein is presumably released from the RNA template and folded into its final specific molecular configuration. Useful information regarding insulin biosynthesis in relation to RNA could be obtained by separating and measuring the four constituents of RNA in pancreatic islet tissue of both normal and experimentally treated animals as, very recently during the course of the present study, a procedure based on thin-layer chromatography for analysis of hydrolysed RNA in microgram quantity of tissue, has been reported (329).

The role of cell nucleus in various stages of endocrine function, the correlation between nucleus and cytoplasm and the role of RNA in

secretional processes was stressed during a recent study of islet tissue (312) by the present day optic physical methods of quantitative histochemistry. A working hypothesis that the nucleus and cytoplasm of islet cells form a functional unit and act jointly during the process of insulin synthesis was postulated (312). In the present approach to study islet tissue by biochemical analyses only the RNA involvement in its functions was emphasized. RNA of islet tissue in tolbutamide treated animals was found to be slightly increased and this showed direct involvement of RNA in the process of insulin synthesis as a result of stimulation of insulin secretion by tolbutamide. Tolbutamide is known to stimulate the secretion of insulin (148). It seems from the present study that increased secretion of insulin is probably preceded by increased synthesis of insulin. Though cytophotometric (visible and UV) study involving measurement of RNA and DNA on islet tissue was not successful (312), it was revealed that distinct enlargement of nucleoli after stimulation with tolbutamide occurred, followed by extrusion of a substance seen as a chromatin indentation in the nuclear membrane. An enlargement of nucleoli and extrusion of nucleolar substance has been observed in many organs after a stimulation of protein synthesis (330).

A considerable increase in the quantity of RNA in the adrenals, vascular wall and kidney of rabbit has been reported due to an effect of cortisone (331). In the adrenals and vascular wall an increase in the amount of DNA was also noted (331). In the present study, determination of DNA was not performed. Whether the increase in RNA quantity in islet tissue after tolbutamide treatment is associated with an increase in the content of DNA is, therefore, not known. Similarly, the amount of RNA as well as that of protein was found to increase significantly twelve hours after a single intraperitoneal injection of estradiol-benzoate to ovariectomized rats (332). A correlation of this increase with increase of enzyme activities

associated with various metabolic pathways may be possible (332). The precise manner in which stimulation of beta cells by sulfonylureas releases insulin remains obscure, but it appears from the present study that probably there occurs an effect on DNA which results in increased synthesis of RNA, as it is known that RNA synthesis is dependent on DNA (333), and that RNA is involved in protein synthesis in other tissues (136). The increase in the amount of RNA may probably be associated with the increase in RNA particles observed in the electron micrographs after an in vitro effect of tolbutamide (Section II). It was shown conclusively (334) that sites of active protein synthesis were also rich in RNA and that the increase in RNA particles may be attributed to the increased synthetic activity of the cell (334). In the light of messenger hypothesis of Jacob and Monod (310) it was indicated that regulation of protein biosynthesis by growth hormone could be explained in terms of the ability of the hormone to stimulate the biosynthesis of RNA in general, and of messenger RNA in particular. Whether tolbutamide stimulates the nucleolar fraction of RNA which appears to be the precursor of other fractions of RNA (333) and exerts a direct control over the rate of synthesis of nucleic acids, which in turn control the rate of protein synthesis, is not known.

Both mouse and Cottus scorpius species have been shown to be alloxan sensitive animals (137). The present study clearly shows that in both these groups of animals alloxan produces cytotoxic effects on all the tissues studied in the fact that it reduces the level of their RNA. Pancreatic islets were the only tissue to have a prolonged marked effect on their RNA content by alloxan administration, thus showing its specificity towards alloxan. The change in the RNA concentration is very significant in the case of liver and kidney during the first two hours of alloxan treatment but it is less significant afterwards. Alloxan, it seems, after entering the body has an initial effect on all those tissues it reaches and passes through but in the

later stages owing to its specificity towards islets it is only the islets which get affected as the decrease in their RNA content becomes progressively marked. This is also evident from histological study as damage done to the beta cells in the pancreatic islets increases with the increase of duration of alloxan treatment. Parallel histological and microchemical study on the pancreatic islets of fish and mice therefore indicates that the decrease in their RNA level due to alloxan administration went parallel with the selective damage of islet beta cells.

Decreased level of RNA has been shown in the liver by the studies of a number of workers (335 - 337), as a result of hypophysectomy in rats, but this could be restored to normal level by administration of growth hormone. In the present study, though the islet tissue remained affected by alloxan during prolonged treatment with alloxan, liver, kidney and muscle tissues tended to approach their normal values of RNA content. It is not known whether decreased RNA level in the pancreatic islets and other tissues could be restored by the administration of growth hormone. Lower levels of liver RNA as found in some experiments (338) might have been due to shock, since shock during handling was shown to lower the liver RNA level (339). The cause of this is not known. Similarly, it has also been observed (340) that levels of both RNA and DNA in liver, kidney and lung tissues of normal males of Swiss strain of mice were significantly decreased after a single injection of urethane, known to cause lung adenomata and skin tumors (341). No explanation was given for this significant (75 - 80%) decrease in RNA content of these tissues (340).

Despite extensive studies on the pancreatic islet tissue, the precise mechanism of action of alloxan remains unknown (162). It cannot be said whether the action of alloxan, indicated by the decrease of islet RNA, is due to its direct action on the cell nucleus or any of the cytoplasmic organelle. A significant decrease of the RNA level occurred

in the nuclei obtained from the liver of α-aminitin-intoxicated mice (342). α-aminitin has been shown to have lethal effects in mice, with necrosis of liver and kidney. These lesions were observed both by light and electron microscope and it was concluded (343) that the nucleus is the target of cytopathogenic action of α-aminitin. The change in RNA appeared as early as one hour after the injection of α-aminitin and became progressively more marked during the 24-hour period considered (342). The decrease in islet RNA level may be a consequence of a decrease in RNA synthesis and if so it may result in decreased levels of protein, as has been pointed out in the studies on an insect (344). It may be mentioned that a possible effect of urethane, which also decreased tissue RNA level (341), on nucleic acid metabolism was suggested (345) i.e, that urethane inhibits DNA synthesis at the site of thymine biosynthesis. There could be a possibility that alloxan does have a direct action on the cell nucleus and not on any of the other cytoplasmic organelle. None of the previous studies on the pancreatic islet tissue to study the mechanism of action approached this aspect.

More recent studies postulate that the primary action of alloxan is to change the permeability of the beta cell membrane (346). Experiments done by Lazarow and his co-workers (23,162) provided strong evidence that tracer doses of alloxan did not penetrate the cell membrane but remained in the extracellular compartment. On the other hand, quantitative enzymatic studies on the isolated pancreatic islets (35) may point out that selective effect of alloxan on the beta cells appears to be associated with intracellular enzymes. The changes in the islets' metabolic activities after alloxan administration (35) in these studies and RNA decrease in the present investigations, is probably not the result of an indirect effect as Lazarow and his co-workers have suggested (23,346). Though the present study involving an attempt to follow the course of labelled alloxan in the mouse

pancreatic islets (Appendix I) failed to give any conclusive results, evidence was presented during the course of this investigation by the Uppsala group (347) applying water soluble autoradiographic technique that, in fact, alloxan does enter the beta cell. It may be, therefore, possible that alloxan has an early effect on the nucleus, as the decrease in the RNA content may be due to decreased activity of DNA failing to synthesize more RNA. The present study, therefore, coupled with the observations by the Uppsala group, suggests that it is the intracellular metabolism (possibly the nuclear) which is first affected by alloxan and not that its effects occur after its action on the permeability of beta cell membrane.

Increased RNA/DNA ratios have been described under stress conditions (348) and as a result of hormonal action (349). The level of RNA has also been shown to increase progressively in the liver after the effect of growth hormone treatment (350) to a teleost fish.

Pylorous islets in Cottus scorpius did not appear to be affected by alloxan as shown by both microchemical and histological study. This finding confirms previous (17) histological observations on this species that two principal islets were seldom affected to the same degree by the alloxan damage and also that islets near the spleen apparently were more severely damaged by the alloxan than the islets near the pylorous. As it has been pointed out (17), circulatory conditions might play a role in alloxan distribution throughout the body. It could be said that there is much slower absorption of alloxan after intramuscular administration in the case of islets as compared to liver and kidney (17) which results in significant damage to liver and kidney in the early phase after alloxan treatment. This shows that the change in RNA content in other tissues does not occur as a result of an indirect effect of alloxan or as a consequence of the establishment of diabetic state.

Chapter 5. SUMMARY

Freehand microdissection technique to isolate the fresh pancreatic islets for metabolic studies has been successfully accomplished in normal and obese-hyperglycemic mice. Their average weight and size were measured to be 22μg and 135μ in diameter in the former, and 65 μg and 175 μ in diameter in the later strain of mice.

Determination of RNA was initiated in the islet tissue in relation to beta cell function. An ultramicromethod was adapted to measure RNA spectrophotometrically in μg. quantities of isolated pancreatic islets. Determination of RNA in islets, liver, kidney and exocrine pancreas of mouse has been carried out. Comparative studies of RNA determinations in islets and other tissues of obese mouse as well as of two teleost species have also been performed. UV absorption spectra of islet RNA fractions showed characteristic features of pure RNA. Comparison of certain absorption ratios of RNA fractions have been presented among tissue RNA fractions of different species. RNA content in islet tissue of all the species studied was found to be higher than in liver and kidney, but approximately 2.5 - 2.8 times less than in exocrine pancreas. Pyloric and splenic islets of Cottus scorpius contained the same amount of RNA.

A significant decrease in the RNA content of islet tissue, as well as that of liver and kidney but not of exocrine was found in mouse and Cottus scorpius made diabetic with alloxan. Necrosis of beta cells have been observed in the islets of treated animals.

Parallel histological and RNA determination studies have been performed on islets and other tissues of Cottus scorpius at different periods of time (2 - 74 hours) after alloxan administration. The decrease in RNA content of islets was continuous and gradual whereas that of kidney and liver, after initial depression, tended to be less.

A close relationship between decrease in RNA content of islet tissue and beta cell damage was postulated. Tolbutamide treatment to mouse resulted in significant increase in the islet RNA content, probably stimulating insulin synthesis.

A few experiments on the effect of insulin on liver and muscle tissues of Plaice have been reported. Moderate decrease (20 - 25%) in their RNA content was found.

Significance of these findings in relation to RNA involvement in insulin synthesis/secretion and to the mechanism of alloxan action have been discussed and emphasis of such studies involving RNA/DNA changes in different functional states of pancreatic islets, in relation to insulin synthesis/secretion has been laid.

SUB-SECTION B (SECTION III) GLUCOSE-6-PHOSPHATASE STUDIES ON THE PANCREATIC ISLETS OF FISH AND MICE

Chapter 1. INTRODUCTION

The fact that the beta cell activity of insulin synthesis and/or secretion is responsive to blood sugar concentration (148) and that the output of insulin by the beta cells is regulated by the concentration of blood sugar (142 - 151) is now well established. Despite extensive studies (22, 23, 141 - 151, 162) which have been carried out in this connection the precise mechanism by which glucose governs or stimulates the insulin output is unknown. The study of intracellular metabolism of glucose which it undergoes within the beta cells is probably of great importance in elucidating the mechanism of insulin secretion (22, 147, 149, 151, 162 - 164, 168). With regard to this aspect of the study, a number of enzymes taking part in the metabolic pathways in the beta cells have been detected histochemically (170 - 180) and measured microchemically (23, 35 - 39, 122, 162, 189 - 192) mostly in the pancreatic islets of mammalian species.

Histochemical investigations demonstrated a pronounced activity of glucose-6-phosphatase (D-glucose-6-phosphate phosphorylase) (G-6-pase) in the islets of Langerhans of guinea pigs, rabbit and dog pancreas (170). It has been suggested that this enzyme may be a controlling factor of insulin secretion from the beta cells (170). It has been shown that mannoheptulose both acts as a blocker of glucose phosphorylation and inhibits the insulin release (150) and this presented a strong evidence for the assumption that the glucose stimulation of insulin secretion involves phosphorylation of the sugar. G-6-P hydrolysis may, therefore be regarded as a possible competing pathway, acceleration or inhibition of which affects the production of intermediary metabolite of glucose triggering the insulin release. G-6-pase is known to release glucose in mammalian liver (353-355) and kidney (206). Lazarow and his co-workers failed to detect any

specific G-6-pase in the principal islets of toadfish even when assaying microchemically (162, 193).

If the pancreatic beta cells could be shown to contain this enzyme specifically and the changes in its activity could be correlated to different functional states of pancreatic islets, it might be possible to say that G-6-pase releases glucose in the pancreatic beta cells which in turn controls the insulin secretion and that G-6-pase functions in relation to the mechanism of insulin secretion. It was obvious that histochemical techniques were not quantitative (170) and the specificity of the enzyme demonstrated is questionable. Hence, it was proposed to study the properties of the specific G-6-pase by micro-enzymatic method in isolated pancreatic islets obtained from normal mouse and compare its activity in isolated pancreatic islet obtained from fish (Cottus scorpius and Tilapia mossambica) as well as from obese-hyperglycemic mice, and then to study its behaviour under different experimental conditions, e.g. fasting, alloxan treatment or stimulation by tolbutamide so as to assess the role of this enzyme in the function of pancreatic beta cells.

Chapter 2. MATERIALS AND METHODS

The animal species used in the present investigations were the same as for other studies (described under Chapter 2, Section II), i.e. normal and obese-hyperglycemic mice, Cottus scorpius and Tilapia mossambica. Obese-hyperglycemic mice used in this study weighed between 60 - 80 g. whereas the body weight of normal mice was about 25. g.

Untreated animals were given free access to food and water (normal mice and obese-hyperglycemic mice).

Six normal mice were allowed only water for 3 days before being killed (starved mice).

Six normal mice were starved for 12 hours before they received a single intraperitoneal injection of 230 mg/kg. body weight of alloxan monohydrate (5% w/v solution). The animals were sacrificed 24 hours after the injection (alloxan-treated mice). Alloxan solution was prepared in citrate buffer (17). The control animals received citrate buffer injections only.

Tolbutamide was administered to 12 normal mice intraperitoneally daily for three days in the doses of 0.25 g/kg. body weight, before killing the animal (tolbutamide treated animals). The controls received physiological saline only.

For microchemical purposes pancreatic islet tissue from mice as well as from fish was obtained by micro and macro-dissection respectively as described in Chap. 2. Section II. In addition to the pancreatic islets small samples of exocrine pancreas, liver and kidney were also excised from the animal body for enzyme determination. In fish, enzyme determination was also done on heart and gill tissues.

Homogenates in micro test-tubes (as described under Chapter 2, Section II) or in microhomogenizer were made always when placed in test-tube racks, kept in thermostat cold bath (temperature +2°C to + 4°C). Lucite microhomogenizer

Microenzymatic Method

The apparatus made use of in the present study, i.e. micropipette, micrometer syringe, U.V. spectrophotometer, has been mentioned already (Chapter 2, Section II).

Reagents:

1. Buffer. 0.067 M Maleic acid buffer (Maleic acid analar grade) (final concentration 0.04M), containing 0.0017 M of ethylene diamine-tetra acetate (final concentration 0.001 M). The buffer was adjusted to PH 6.5 with 0.1 N NaOH. Maleic acid buffer solutions were also prepared in the same concentration but adjusted to PH 9.0 and PH 5.0.

2. Substrates. D-Glucose-6-Phosphate (G-6-P) Dipotassium salt, Sigma Chemical Company, U.S.A., was used as specific substrate. It was dissolved in glass distilled water in the concentration of 0.2 M (its concentration in the final reaction being 0.04 M). Sodium β-glycerophosphate (Glycero-P) was used as non-specific substrate and substituted G-6-P in equimolar concentration, for comparative studies parallel to those with the specific substrate.

3. Trichloro acetic acid : 10% (TCA)

4. H_2SO_4 : 10 N

5. Ferrous sulphate-ammonium molybdate reagent (made up freshly before use). A stock solution of:

1% ammonium molybdate in 1.15 N H_2SO_4 was prepared by dissolving one gram of ammonium molybdate in 11.5 ml. of 10 N H_2SO_4 and then

diluting to 100 ml. with distilled water.
To obtain the ferrous sulphate-ammonium molybdate reagent, 0.4 g. of Ferrous sulphate was dissolved in 10 ml. of the above stock solution.

6. Standard solution of phosphorus:

Stock solution was prepared by dissolving 574 mg. of $Na_2HPO_4 2H_2O$ in 100 ml. distilled water to obtain the concentration as 1 mg. of phosphorus per ml. solution.
Stock solution was diluted x 10 with distilled water. Then working standard solutions of phosphorus of strengths varying from 1 µg/ml to 20 µg/ml. were prepared for each experiment before use, by taking 0.01 ml., 0.02 ml. and so on and diluting to one ml.

Assay procedure.

An ultramicrochemical procedure was adapted successfully from Bonting's modification (351) to assay G-6-pase enzyme quantitatively in the present study. In principal the enzymatic activity was determined by spectrophotometric measurements of inorganic phosphorus, produced by the reactions during the incubation. The method employed to determine the liberated inorganic phosphorus was that of Tausky and Schorr's (352). The assay procedure scaled down to ultramicro proportions was as follows:-

1. The buffer solution (30µl. in each micro test tube) was warmed up for about 5 minutes at 37°C. 10µl. of the specific substrate was added to it. 10µl. of the tissue homogenate under study was then added to the reaction mixture.
2. The tubes were incubated at 37°C in a shaking water bath for 30 minutes.
3. The reaction was stopped by the addition of 100µl. of 10% TCA.

4. The reaction tubes were brought to the room temperature. These were then centrifuged in a high speed centrifuge at 5,000 r.p.m. for 5 - 7 minutes or until the clear supernatant was obtained.
5. 0.1 ml. of the supernatant was accurately pipetted out and transferred to small test tubes containing accurately measured 0.1 ml. of freshly prepared ferrous sulphate - ammonium molybdate reagent.
6. Similarly 0.1 ml. of working standard solution was also added to equal volume of the ferrous sulphate-ammonium molybdate reagent.
7. The optical density of the resulting solutions from (5) and (6) was read in SP.800 at 700 mμλ using silica microcells (light path 10 m.m.) to measure the liberated inorganic phosphorus.

The enzyme activity calculated using the standard curve for phosphorus, shown in Fig. 9 was expressed as μg. of phosphorus (P) liberated per mg. of fresh tissue.

The enzyme activity was assayed always in duplicate samples and their mean values were considered as one determination. Occasionally when one of the samples was lost during the experimental procedure the other was taken as one determination. A reagent blank containing everything but the specific or non-specific substrate and a tissue blank again containing everything except the tissue homogenate were included in most of the experiments to serve as controls and appropriate corrections were applied wherever necessary. Experiments with non-specific substrate for each tissue were always carried out parallel to those with specific substrates. Standard solution absorptions were made for individual experiments.

Chapter 3. EXPERIMENTAL RESULTS

i. Analyses of optimal assay conditions and enzyme properties

a. PH optimum

The G-6-P and glycerophosphate-splitting activities of whole homogenates of mouse pancreatic islets as well as of exocrine pancreas were measured at three differing PH levels: PH 5.0, PH 6.5 and PH 9.0. The results are illustrated in Table 11. It appears from Table 11 that inorganic phosphorus liberation due to G-6-P in pancreatic islets at PH 6.5 is about 15% and about 22% higher than at PH 5.0 and PH 9.0 respectively. These values obtained for islets in the presence of glycerophosphate were, on the other hand, lower at PH. 6.5 than at either of the other two PH's tested. Pi liberated in this case at PH 6.5 was about 11% and about 33% lower than that at PH 5.0 and PH 9.0 respectively.

A series of similar experiments was also carried out on pancreatic islets and exocrine tissue homogenate prepared from obese-hyperglycemic mice. The results, also illustrated in Table 11 show that pancreatic islet at PH 6.5 has about 18% and 28% higher inorganic phosphorus liberated due to G-6-P than that at PH 5.0 and PH 9.0 respectively, whereas there appeared to be no difference at all in Pi liberation at these PH levels in the presence of glycerophosphate.

Table 11 also shows that in exocrine pancreas of both strains of mice, Pi liberation due to G-6-P at PH 6.5 is not higher, but lower than that at PH 5.0 (about 14 - 16%) or at PH 9.0 (about 5% in the case of obese-hyperglycemic mice but as low as 24% in normal mouse).

The relative enzyme activities towards G-6-P and glycerophosphate calculated as percentage of the values obtained for pancreatic islets at PH 6.5 are shown in Fig.10 (normal mouse) and Fig.11 (obese-hyperglycemic mice). It becomes distinctly clear from the representative PH curves that the enzyme hydrolizing G-6-P in the pancreatic islets of

TABLE 11

Inorganic Phosphorus (Pi) liberated (µg/mg. fresh tissue/30 minutes) in Pancreatic Islets and Exocrine Pancrease of normal and obese-hyperglycemic mice due to G-6-P at Differing PH levels

Animal	Tissue	Pi liberated at PH 5.0	6.5	9.0
Normal Mice	ISLETS	0.96 ± 0.09 (4) 85%	1.14 ± 0.12 (12) 100%	0.89 ± 0.11 (4) 78%
	EXOCRINE	0.81 ± 0.08 (2) 116%	0.70 ± 0.10 (6) 100%	0.87 ± 0.09 (2) 124%
Obese-hyper-glycemic mice	ISLETS	1.02 ± 0.14 (4) 82%	1.24 ± 0.14 (12) 100%	0.90 ± 0.13 (4) 72%
	EXOCRINE	0.71 ± 0.09 (2) 114%	0.62 ± 0.22 (5) 100%	0.65 ± 0.16 (2) 105%

The figures denote the mean values; ± standard deviation.
Number within parentheses express the number of determinations done
% values expressed as % of the activities in islets at PH 6.5

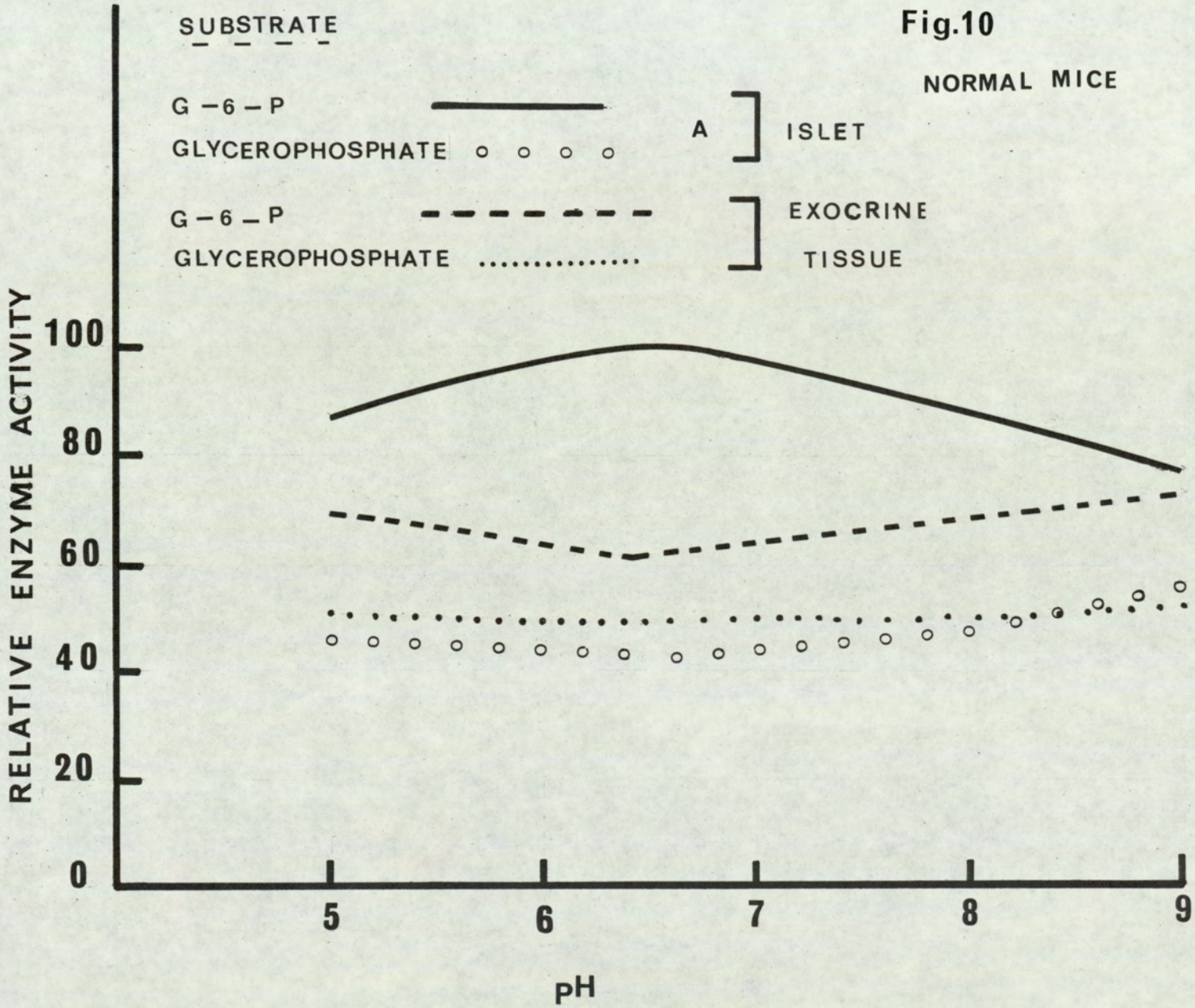
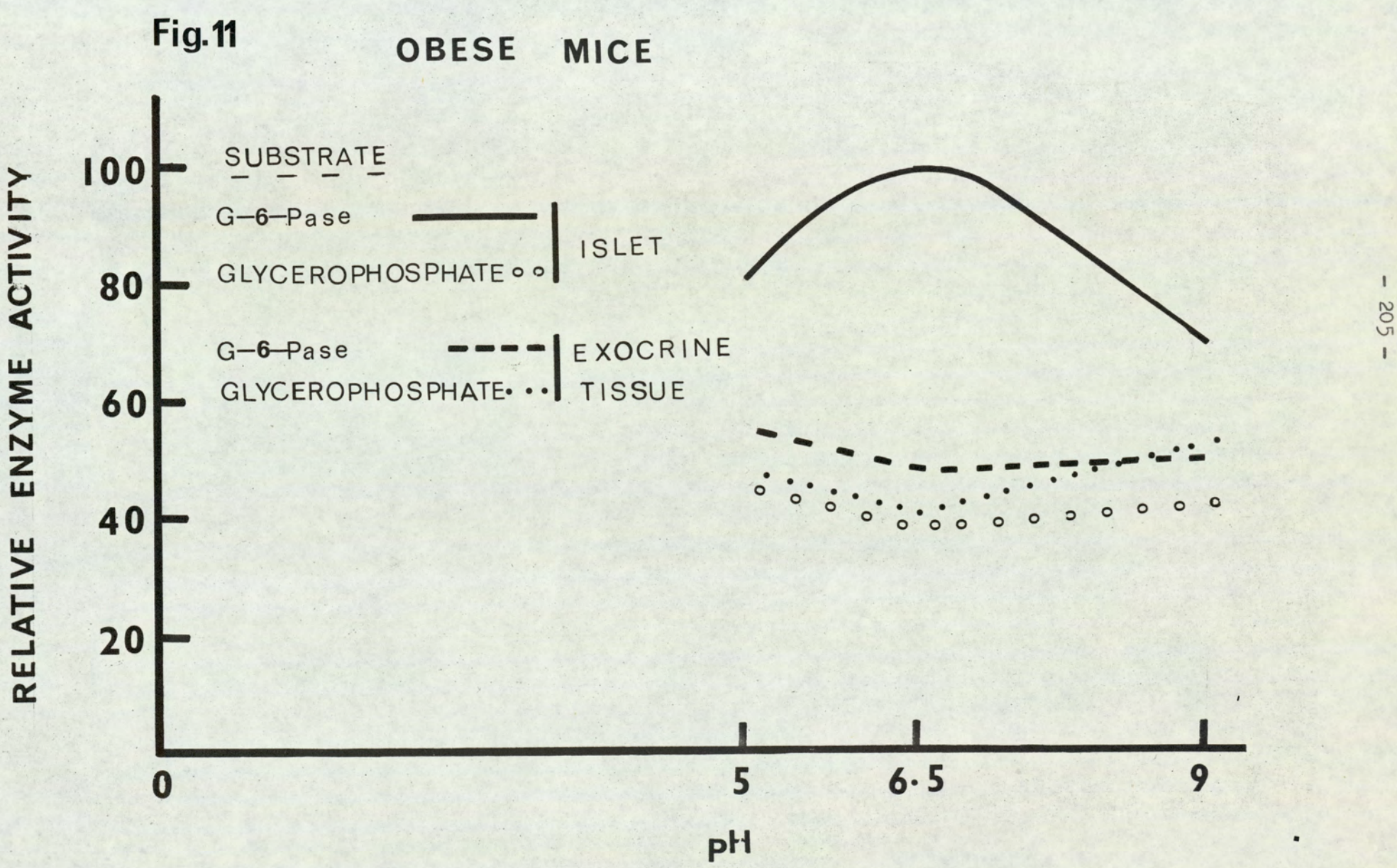

SUBSTRATE
NORMAL MICE
G – 6 – P
GLYCEROPHOSPHATE
A
ISLET
G – 6 – P
GLYCEROPHOSPHATE
EXOCRINE
TISSUE
RELATIVE ENZYME ACTIVITY
100
80
60
40
20
0
5
6
7
8
9
pH

Fig.10

Fig.11


OBESE MICE
RELATIVE ENZYME ACTIVITY
100
80
60
40
20
0
5
6·5
9
pH
SUBSTRATE
G—6—Pase
GLYCEROPHOSPHATE
ISLET
G—6—Pase
GLYCEROPHOSPHATE
EXOCRINE
TISSUE

both normal and obese-hyperglycemic mice displays maximum activity at PH 6.5. By contrast, the activity towards G-6-P in exocrine pancreas of both strains of mice does not show any difference at differing PH levels.

The ratios of G-6-P and glycerophosphate-splitting activities at different PH levels were also calculated and are shown in Table 12.

For comparative purposes a few experiments were also done to determine the enzyme activities towards G-6-P and glycerophosphate at differing PH levels in homogenates in the homogenates of liver as well as kidney of both normal and obese-hyperglycemic mice. The results are shown in Table 13. It is apparent that these tissues clearly show significantly higher Pi liberation due to G-6-P at PH 6.5 than either at PH 5.0 or PH 9.0. As in normal mouse, the Pi liberated at PH 5.0 and 9.0 is about 25% and about 27% lower respectively in liver, and about 13% and about 17% respectively lower in the case of kidney than the values obtained for respective tissues at PH 6.5. Similarly, high G-6-P-splitting activity was found in kidney of obese mouse at PH 6.5 when compared with that at either PH 5.0 or PH 9.0.

Experiments on principal islets of Cottus scorpius.

Six experiments were performed to determine G-6-P and glycerophosphate splitting activities in the principal islets as well as in liver at PH 5.0 and PH 9.0. Similar experiments, sixteen on islet homogenates and then on liver homogenates, were carried out at PH 6.5. The results are shown in Table 14. It could be seen from this table that G-6-P-splitting enzyme activity in principal islets at PH 6.5 is about 32% and 43% higher than that at PH 5.0 and PH 9.0 respectively. Likewise, the same activity in liver at PH 6.5 was about 29% and 36% higher than the corresponding values at PH 5.0 and PH 9.0 respectively.

Ratios calculated for G-6-P/glycerophosphate-splitting activities in the principal islets at PH 5.0 and PH 9.0 are shown in Table 15.

TABLE 12

Effect of PH on Ratios of G-6-P/Glycero-Phosphate Splitting Activities in Mouse Islet Homogenates

EXPERIMENT	PH 5.0	PH 9.0
1	2.3	1.4
2	1.5	1.2
3	1.8	1.6
4	1.7	1.7
Mean	1.82	1.47

As compared to the above ratios, the mean ratio of G-6-P/Glycero splitting activities at PH 6.5 was calculated to be 2.35 (Table 17)

TABLE 13

Pi liberated (µg/mg. fresh tissue/30 minutes) in Liver and Kidney of Normal and Obese-hyperglycemic mice due to G-6-P at differing levels

ANIMAL	TISSUE	Pi liberated at PH		
		5.0	6.5	9.0
Normal Mice	LIVER	9.50 ± 0.57 (4) 75%	12.68 ± 1.4 (11) 100%	9.26 ± 1.4 (4) 73%
	KIDNEY	8.15 ± 0.35 (2) 87%	9.24 ± 1.32 (8) 100%	7.74 ± 0.08 (2) 83%
Obese-hyper-glycemic mice	LIVER	9.87 ± 0.35 (4) 67%	14.82 ± 1.30 (8) 100%	9.68 ± 0.17 (4) 66%
	KIDNEY	7.94 ± 0.26 (2) 80%	9.71 ± 1.0 (6) 100%	7.80 ± 0.16 (2) 80.5%

The figures denote the mean values; ± standard deviation.
The number of determinations done are given within parentheses.
% values expressed as% of the activities in islets at PH 6.5

TABLE 14

The Effect of PH levels on G-6-P and Glycerophosphate Splitting Activities in Principal Islets and Liver of Cottus Scorpius

SUBSTRATE	TISSUE	Pi (µg /mg. fresh tissue/30 min. sets) liberated at PH 5.6	6.5	9.0
G-6-P	ISLETS	0.90 ± 0.11 (6) 68%	1.37 ± 0.18 (16) 100%	0.78 ± 0.08 (6) 57%
	LIVER	8.34 ± 0.24 (6) 71%	11.8 ± 0.78 (10) 100%	7.48 ± 0.16 (6) 64%
Glycero-phosphate	ISLETS	0.61 ± 0.10 (6) 180%	0.45 ± 0.16 (16) 100%	0.86 ± 0.18 (6) 191%
	LIVER	1.06 ± 0.16 (6) 129.2%	0.82 ± 0.12 (10) 100%	1.07 ± 0.11 (6) 130%

The figures denote the mean values; ± standard deviation
The number of determinations done are given within parentheses
% values expressed as% of the activities measured in islets at PH 6.5

TABLE 15

The Effect of PH Level on Ratio of G-6-P/Glycerophosphate-splitting Activities in Principal Islets of Cottus Scorpius

Experiment	Ratio at PH 5.0	9.0
1	1.46	1.23
2	1.24	0.95
3	1.21	0.92
4	1.09	0.89
5	0.99	0.85
6	1.16	0.99
Mean	1.19	0.96

Mean ratio at PH 6.5 was 3.0 (Table 18)

This ratio, mean of 16 determinations was found to be much higher (3.0) at PH 6.5 (Table 18.).

b. Substrate specificity

Relative degree of cleavage of G-6-P, glycerophosphate and glucose-1-phosphate (G-1-P) was determined in homogenates of the pancreatic islets, exocrine pancreas and liver of normal mouse, when incubated at PH 6.5. The results are illustrated in Table 16. It could be seen from Table 16 that the enzymic liberation of inorganic phosphorus in all the three tissues under study was highest when G-6-P was the substrate. It is also clear that the hydrolysis of the substrate G-6-P was more in the pancreatic islets than in the exocrine pancreas. The hydrolysis of all the three substrates used in the present study was essentially of the same order in the case of exocrine pancreas. It showed therefore that the pancreatic islets and liver display a much higher degree of specificity towards G-6-P and the exocrine pancreas had no such specificity for this substrate.

To demonstrate clearly the differences, the activity on G-6-P and glycerophosphate in different tissues, the ratios between the rate of hydrolysis of G-6-P and glycerophosphate in these tissues were calculated. The differences and the ratios between G-6-P and glycerophosphate-splitting activities in the pancreatic islets, liver, kidney and exocrine pancreas of normal as well as obese-hyperglycemic mice are illustrated in Table 17. It seems quite clear that the difference is apparently pronounced in all the tissues except in the exocrine pancreas, in both groups of animals. Pancreatic islets of both normal and obese-hyperglycemic mice showed much higher difference in the activities towards G-6-P and glycerophosphate. G-6-P hydrolysis in the pancreatic islets is about 2 - 2.2 times higher than that in the exocrine pancreas.

Experiments on principal islets of Cottus scorpius and Tilapia mossambica

Differences between the phosphorus liberated due to G-6-P and glycerophosphate and their ratios were calculated from their respective

TABLE 16

Pi Liberated due to Different Substrates at PH 6.5 in the Pancreatic Islets, Exocrine Pancreas and Liver of Normal Mice

SUBSTRATE	TISSUE		
	ISLETS	EXOCRINE PANCREAS	LIVER
G-6-P	1.14 ± 0.12 (12)	0.70 ± 0.10 (6)	12.68 ± 1.45 (11)
G-1-P	0.62 ± 0.11 (4)	0.68 ± 0.08 (4)	1.03 ± 0.12 (4)
Glycero-phosphate	0.49 ± 0.07 (12)	0.57 ± 0.12 (6)	0.84 ± 0.09 (11)

The figures denote the substrate-splitting activities (Pi liberated µg./mg fresh tissue/30 minutes). ± standard deviation.

Number of determinations carried out are given within parentheses.

TABLE 17

The Differences in G-6-P- and -Glycerophosphate-Splitting Activities at PH 6.5 and their Ratios in the Pancreatic Islets and Other Tissues of Normal and Obese-hyperglycemic Mice

Animal		T I S S U E			
		Islets	Exocrine Pancreas	Liver	Kidney
Normal Mice	Difference	0.65	0.13	11.84	8.51
		(12)	(6)	(11)	(5)
	Ratio	2.32	1.22	15.09	12.45
Obese-hyperglycemic Mice	Difference	0.77	0.10	13.85	9.78
		(12)	(5)	(6)	(6)
	Ratio	2.63	1.19	15.13	12.44

Number of determinations done are given within the parentheses.

TABLE 18

The Differences in G-6-P and Glycerophosphate-Splitting Activities at PH 6.5 and their Ratios in the Principal Islets and other Tissues of Teleost Species

Tissue	COTTUS SCORPIUS		TILAPIA MOSSAMBICA	
	Difference	Ratio	Difference	Ratio
ISLETS	0.92 (14)	3.0	0.93 (10)	3.6
EXOCRINE PANCREAS	- 0.10 (4)	0.83	0.06 (4)	1.11
LIVER	10.92 (6)	14.38	9.96 (8)	14.46
HEART	- 0.11 (5)	0.89	- 0.6 (3)	0.94
GILLS	-	-	0.63 (2)	1.4

Numbers given within parentheses denote the number of determinations contributing to the mean

activities determined in the principal islets, exocrine pancreas, liver and heart of both Cottus scorpius and Tilapia mossambica. Similar calculations were also done for homogenates of gills prepared from Tilapia mossambica. The results are shown in Table 18.

It appears from Table 18 that as in the case of mammalian islets the principal islets of these teleost species also show much higher difference in their activities towards G-6-P and glycerophosphate when compared with the respective activities in the exocrine pancreas. Such difference was distinct in the case of liver. The ratios between the activities towards G-6-P and glycerophosphate were about 3 - 3.75 times higher in the principal islets of both teleost species, when compared with those obtained in exocrine pancreas. Liver, on the other hand, showed about 15 times higher activities towards G-6-P than towards glycerophosphate. Though gills of Tilapia mossambica showed a significant difference between the activities towards G-6-P and glycero-phosphate when compared to those in exocrine pancreas, the calculated ratio between G-6-P and glycero-phosphate-splitting activities was only fractionally higher.

c. Effect of tissue and enzyme substrate concentration.

Serially diluted homogenates of pancreatic islets of normal mouse were incubated with the specific substrate at PH 6.5 and the relative activity towards the substrate was determined. The result of such an experiment is shown in Fig.12. It appears from Fig.12 that under the present assay conditions the hydrolysis of G-6-P is linearly related to the concentration of tissue homogenates.

It was not possible to determine G-6-P splitting activity under these conditions in the pancreatic islet homogenates weighing about 50 µg or less.

Fig. 12

G – 6 – P HYDROLYSING ENZYME AND ISLET TISSUE WEIGHTS.

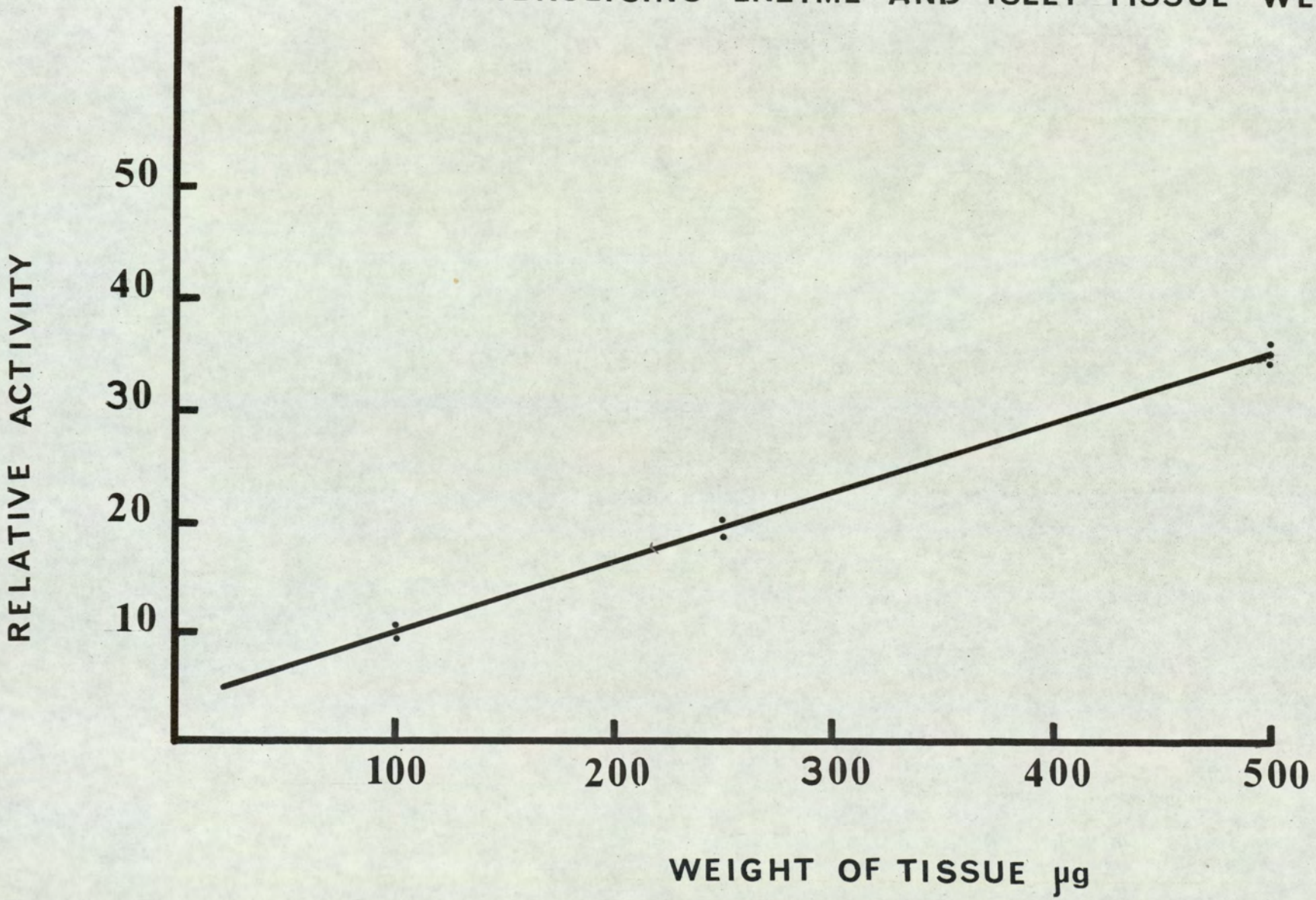


TABLE 19

G-6-P and Glycerophosphate-Splitting Activities in Pancreatic Islets and Liver of normal Mice, at Varying Concentrations of the Substrate

Tissue	Substrate	Pi Liberated (µg /mg. fresh tissue/30 minutes)at concentration		
		0.01 M	0.02 M	0.04 M
ISLETS	G-6-P	0.31 ± 0.06 (4)	0.59 ± 0.08 (4)	1.14 ± 0.12 (12)
	Glycerophosphate	0.22 ± 0.06 (4)	0.29 ± 0.06 (4)	0.49 ± 0.11 (12)
LIVER	G-6-P	3.42 ± 0.23 (4)	6.79 ± 0.49 (5)	12.68 ± 0.14 (11)
	Glycerophosphate	0.38 ± 0.13 (4)	0.50 ± 0.21 (5)	0.84 ± 0.13 (11)

Number of determinations contributing to the mean values are given within parentheses

The figures denote mean values; ± standard deviation

A few experiments were performed on liver and pancreatic islet homogenates of normal mouse, using varying concentrations of the specific substrate. The G-6-P and glycerophosphate-splitting activities of these tissues when incubated with 0.01 M and 0.02 M concentration of the substrate were compared with those obtained when the concentration was 0.04 M. The results are illustrated in Table 19. It shows that increasing the substrate concentration, hydrolysis of G-6-P in both pancreatic islets and liver, with respect to that of glycero-phosphate, increases. The ratio between the activities towards G-6-P and glycerophosphate was highest when the concentration was 0.04 M. Though the activity towards glycero-phosphate in the pancreatic islets also increased with increase of concentration to 0.04 M, no significant difference was observed between the glycero-phosphate-splitting activities with concentrations 0.01 M and 0.02 M whereas a significant difference existed at these concentrations towards G-6-P substrate.

The relation between substrate concentration and G-6-P splitting activities in the pancreatic islets and liver of normal mouse is illustrated in Fig.13. The hydrolysis of G-6-P appears to be directly proportional to the concentration of the enzyme substrate.

d. Effect of time of incubation:

Four experiments were performed on pancreatic islets and liver homogenates to determine the effect of incubation time on G-6-P-splitting activity. Pancreatic islet and liver homogenates were incubated for 15 and 60 minutes with both specific and non-specific substrates and Pi liberated was measured. The results of these experiments along with those obtained from these tissues after incubating for 30 minutes are given in Table 20. It is apparent that G-6-P hydrolysing activity in pancreatic islets and liver increased about 84% and 94% respectively when the time of incubation was prolonged

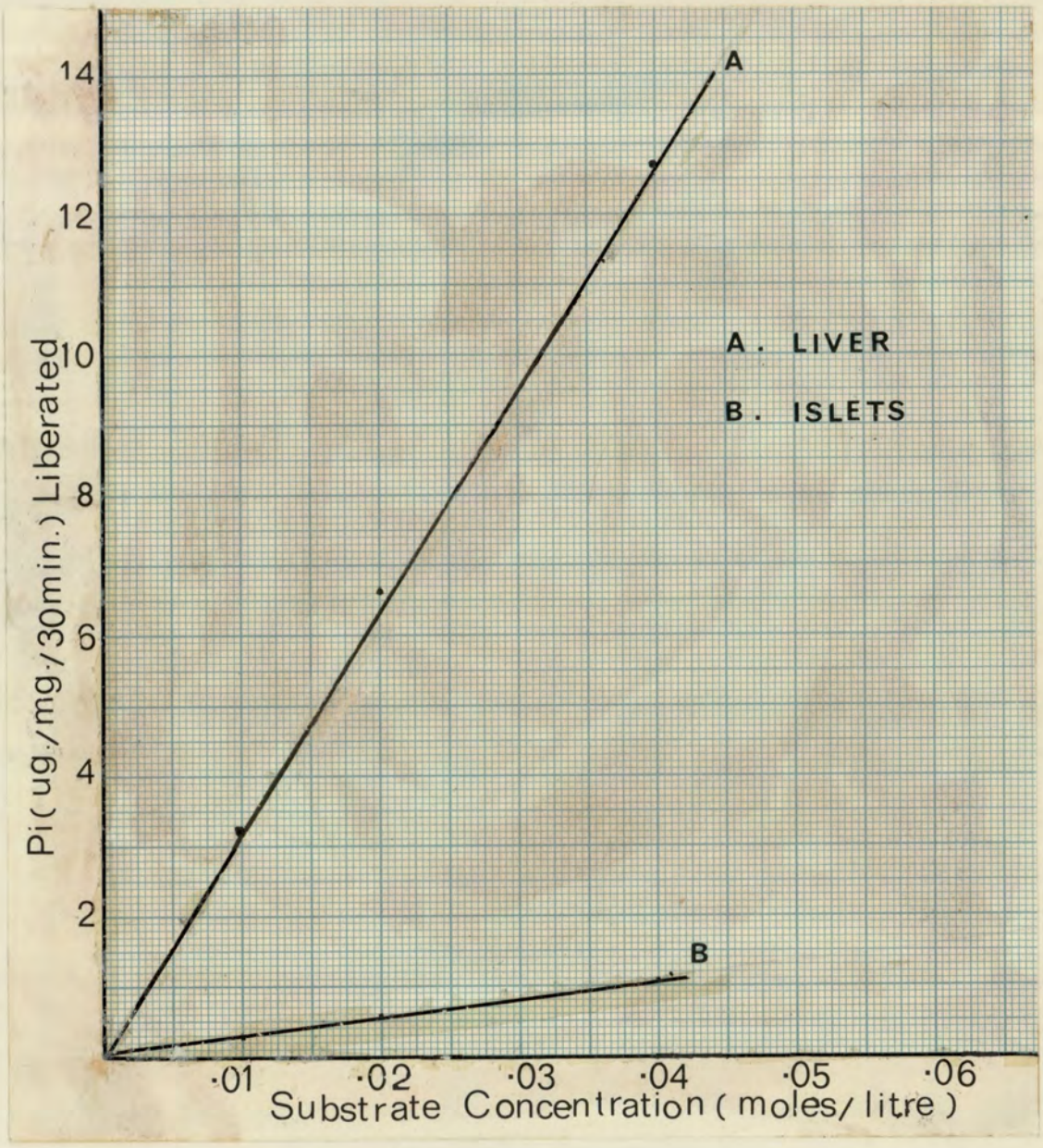


Fig.13

Fig. 13: Relation between substrate (G-6-P) concentration and the enzyme activity determined in Liver and the Pancreatic Islets.

TABLE 20

G-6-P- and Glycerophosphate-Splitting Activities in Pancreatic Islets and Liver of Normal Mouse, after incubation at Different Periods of Time

TISSUE	SUBSTRATE	Pi liberated (µg/mg. fresh tissue/incubation time)		
		15 minutes	30 minutes	60 minutes
ISLETS	G-6-P	0.62 ± 0.06 (4)	1.14 ± 0.12 (12)	1.68 ± 0.09 (4)
	Glycero-phosphate	0.21 ± 0.03 ?	0.49 ± 0.11 (12)	0.76 ± 0.15 (4)
LIVER	G-6-P	6.54 ± 0.12 (4)	12.68 ± 0.14 (11)	17.4 ± 0.35 (4)
	Glycero-phosphate	0.51 ± 0.16 (4)	0.84 ± 0.13 (11)	1.44 ± 0.17 (4)

The figures denote mean values determined when incubation was carried out at PH 6.5 with substrate concentration 0.04 M. ± Standard deviation. Numbers within parentheses express the number of determinations contributing to the mean values.

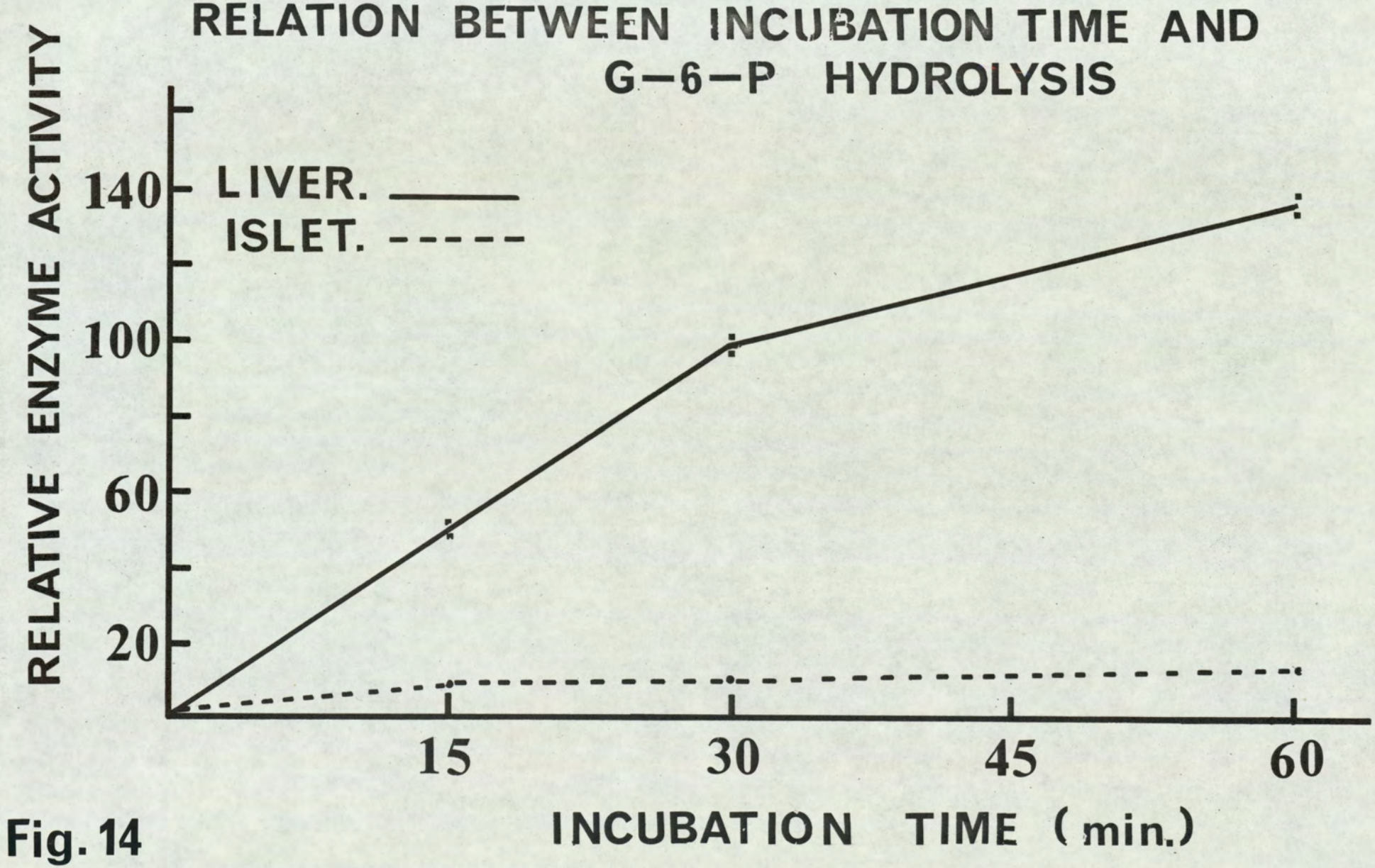

RELATION BETWEEN INCUBATION TIME AND
G—6—P HYDROLYSIS
RELATIVE ENZYME ACTIVITY
140
100
60
20
LIVER.
ISLET.
15
30
45
60
INCUBATION TIME (min.)

Fig. 14

from 15 minutes to 30 minutes. But, on incubating the tissues for one hour, increase in the G-6-P-splitting activity in the pancreatic islets is only 47% when compared to that obtained with 30 minutes incubation. Similarly the increase in the activity towards G-6-P in liver with 1-hr. incubation as compared to 30 minutes' incubation is only 37%.

Relative enzyme activities towards G-6-P in the pancreatic islets and liver of normal mouse incubating the tissues for different periods of time are shown in Fig.14.

It is evident, therefore, from Table 20 as well as from Fig.14 that G-6-P hydrolysis in both the tissues, particularly in the case of pancreatic islets, is linearly related to the incubation time for at least 30 minutes.

e. Thermal Stability

The G-6-P hydrolyzing activity in mouse pancreatic islets was demonstrated to be thermally unstable. It was done by determining the G-6-P and glycero-phosphate-splitting activities in pancreatic islets after pre-incubating the homogenates at 37°C. Only two experiments in all were performed. The effect of pre-incubation of the tissue homogenates for 5 and 15 minutes on the substrate-splitting activities was determined. The result of these experiments is shown in Table 21. These relative enzyme activities towards G-6-P are shown in Fig.15. It appears from these experiments that pre-incubation of islet homogenates significantly decreased the activity towards G-6-P while glycerophosphate-splitting activity was instead slightly increased. The inactivation of G-6-P was ~~instead~~ slightly increased. The inactivation of G-6-P-splitting activity after 5 minutes of pre-incubation was about 24% of the original value obtained without any pre-incubation. After 15 minutes of pre-incubation, the G-6-P splitting activity was markedly inactivated and the decrease was

TABLE 21

The Effect of Pre-incubation of Pancreatic Islet Homogenates for Different Periods of time on the G-6-P- and Glycero-phosphate Splitting Activities

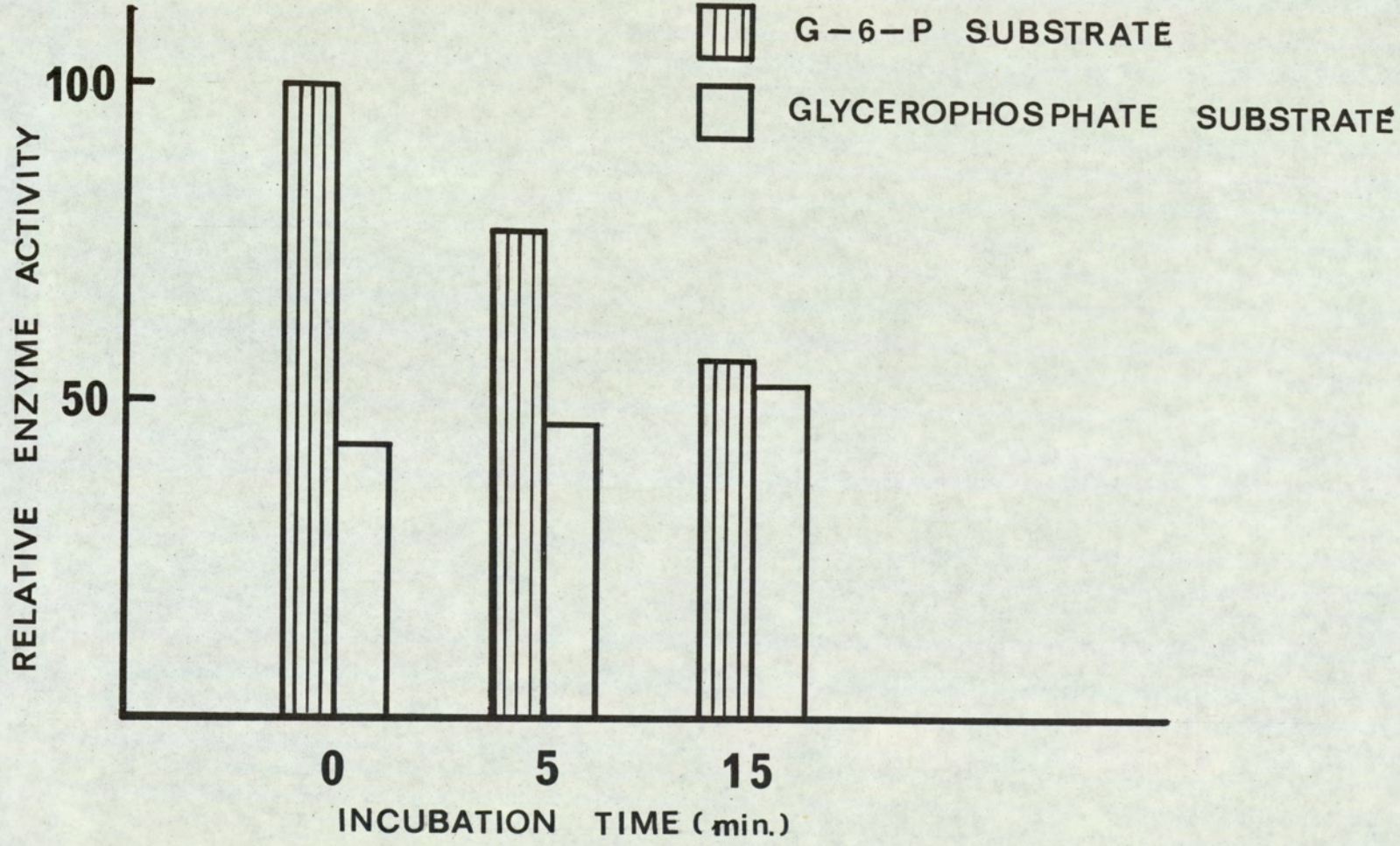
Time of pre-incubation (min)	Pi Liberated (µg/mg. fresh tissue/30 mins. due to	
	G - 6 - P	Glycerophosphate
0	1.14	0.49
	100%	100%
5	0.86	0.52
	76%	106.1%
15	0.61	0.58
	54%	118.4%

% values expressed as percentage of activities determined without any preincubation

Only two determinations contribute to the average values determined after preincubation for 5 and 15 minutes each.

Fig. 15

INACTIVATION OF G–6–Pase ACTIVITY BY PREINCUBATION OF ISLET HOMOGENATES AT 37°C



about 46%. On the other hand, the activity towards glycerophosphate remained apparently unaffected (the increase in activity was slight; 6% after 5 minutes and 18% after 15 minutes of pre-incubation of the homogenates).

Parallel experiments were also performed on liver and exocrine pancreas. It was found that results obtained from experiments on liver homogenates were similar to those observed in pancreatic islets. On the other hand, no inhibitory effect of pre-incubation could be established on the G-6-P hydrolyzing activity in homogenates of exocrine pancreas.

f. Effect of inhibitor

Ammonium molybdate was tested to see the inhibitory effect on G-6-P hydrolyzing activity of the pancreatic islets of normal mice. Only two experiments were carried out, determining the G-6-P-splitting activity in the homogenates after addition of ammonium molybdate (in concentrations 3.2×10^{-4} and 3.2×10^{-3}) to the incubation medium. The results are presented in Table 22.

It appears from Table 22 that addition of ammonium molybdate to the incubation medium, at a concentration of 3.2×10^{-4} resulted in about 27% decrease and at a concentration of 3.2×10^{-3} produced almost complete inhibition of the G-6-P-splitting activity in the homogenates of the pancreatic islets. It is evident, therefore, that a drastic effect of ammonium molybdate was established on G-6-P hydrolyzing activity in mouse pancreatic islets.

ii. G-6-P and Glycerophosphate-splitting activities in tissues of normal and obese-hyperglycemic mice.

The activity towards both G-6-P and glycerophosphate was determined in the pancreatic islets and exocrine pancreas homogenates of normal and obese-hyperglycemic mice. The tissue homogenates were incubated for 30 minutes at PH 6.5 . The relative enzyme

TABLE 22

The Effect of Ammonium Molybdate on G-6-P Splitting Activity of the Pancreatic Islets of Mouse

Ammonium Molybdate added (Moles/litre)	Relative Enzyme Activity
NONE	100
3.2×10^{-4}	73
3.2×10^{-3}	Negligible

Relative enzyme activity is average of two determinations each for 3.2×10^{-4} and 3.2×10^{-3} concentration.

Determination of G-6-P-splitting activity carried out at PH 6.5 with 0.04 M substrate concentration and incubating for 30 minutes.

activity for these two substrates in pancreatic islets and exocrine pancreas of both strains of mice is illustrated in Fig. 16. It is evident from Fig. 16 that for neither group of animals there was any appreciable difference between pancreatic islets and exocrine pancreas with regard to the rate of glycerophosphate hydrolysis. The activity towards G-6-P was, however, greater in the pancreatic islets than in the exocrine pancreas for both normal and obese-hyperglycemic mice. It also clearly shows that a significant difference exists between the two activities in the pancreatic islets whereas no such difference of activities is apparent in exocrine pancreas of both strains of mice. Pancreatic islets of obese-hyperglycemic mice display G-6-P splitting activity slightly greater (about 11%) than that determined in the islets of obese-hyperglycemic mice, but the difference was not significant ($P < 0.1$).

Results of all the experiments carried out on pancreatic islets and exocrine pancreas as well as on liver and kidney homogenates of normal and obese-hyperglycemic mice are given in Table 23. It shows that G-6-P hydrolyzing activity of the pancreatic islets is approximately 11 - 12 times less than that of liver in both groups of animals. Likewise, the activity towards G-6-P in the kidney is about 34% less than that of liver in obese-hyperglycemic mice whereas it is about 27% less in case of normal mice when compared with liver. The G-6-P-splitting activity of kidney, however, is approximately 7 - 8 times higher than that of pancreatic islets in both groups of animals.

The activity towards G-6-P in kidney of normal mouse did not differ significantly ($P < 0.1$) from that of the corresponding tissue of obese-hyperglycemic mice, although kidney of obese-hyperglycemic mice showed approximately 5% higher G-6-P splitting activity. On the other hand, G-6-P-splitting activity of liver homogenates prepared from obese-hyperglycemic mice was found to be about 14.5% higher ($P < 0.02$) than that of normal mouse liver.

Fig.16

G−6−Pase IN NORMAL AND OBESE MICE PANCREAS

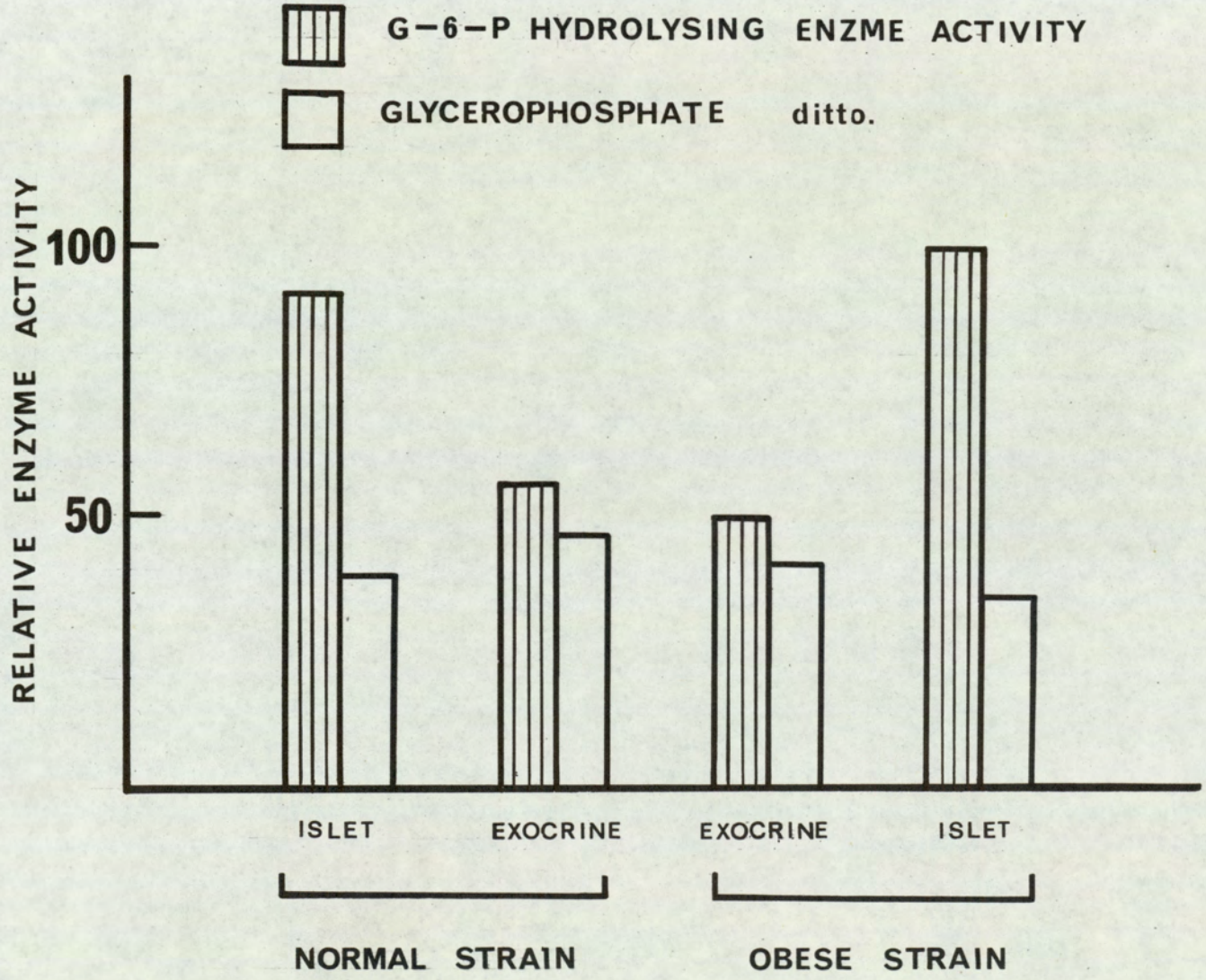


TABLE 23

Enzymatic Activities Hydrolyzing G-6-P and Glycerophosphate in the Pancreatic Islets, Liver, Exocrine Pancreas and Kidney of Normal and Obese-hyperglycemic Mice

ANIMAL	SUBSTRATE	ISLETS	EXOCRINE PANCREAS	LIVER	KIDNEY
Normal Mice	G-6-P	1.14 ± 0.12 (12)	0.70 ± 0.10 (6)	12.68 ± 1.45 (11)	9.24 ± 1.39 (8)
	Glycero-phosphate	0.49 ± 0.07 (12)	0.57 ± 0.12 (6)	(11)	(5)
Obese-hyperglycemic mice	G-6-P	1.24 ± 0.14 (12)	0.62 ± 0.22 (5)	14.83 ± 0.78 (8)	9.71 ± 1.13 (6)
	Glycero-phosphate	0.47 ± 0.07 (12)	0.52 ± 0.19 (5)	0.98 ± 0.09 (6)	0.93 ± 0.30 (6)

The figures denote mean values, determined with standard procedure (substrate concentration 0.04, PH 6.5, incubated for 30 minutes), of enzyme activities expressed as Pi liberated µg/mg fresh tissue/30 minutes.

± standard deviation; Number of determinations are given within parentheses. Average of duplicate samples results was taken for one determination.

iii. G-6-P and glycero-phosphate splitting activities in tissues of teleost species.

The enzymatic activities towards G-6-P and glycerophosphate was determined (PH 6.5 and incubation time 30 minutes) in homogenates of principal islets, exocrine pancreas, liver and heart of both Cottus scorpius and Tilapia mossambica. In addition, these activities were also determined in homogenates prepared from gills of Tilapia mossambica. The results are presented in Table 24.

It is apparent that the activity towards G-6-P in the principal islets was much higher than in exocrine pancreas for both Cottus scorpius and Tilapia mossambica. It also shows that the principal islets displayed about eight times less activity towards G-6-P than that of liver in both groups of animals.

It appears that the activity towards either of the substrate in exocrine pancreas and heart in both groups of animal does not show any appreciable difference and is apparently of the same order. The same is true for gills of Tilapia mossambica.

It also shows that liver and principal islets as compared to the rest of the tissues have much higher activity towards G-6-P with regard to that toward glycero-phosphate. There does not appear to be any significant difference between the G-6-P-splitting activity of liver homogenates obtained from Cottus scorpius and that of the same tissue homogenates prepared from Tilapia mossambica. However, the activity towards G-6-P in the principal islets of Cottus scorpius is slightly greater, though insignificant, than that determined in the principal islets of Tilapia mossambica.

The enzyme activities hydrolyzing G-6-P and glycerophosphate in mammalian tissues have been compared with those of the teleostean tissues (Cottus scorpius) and are illustrated in Fig.17. Maximum activity towards G-6-P was obtained in liver of obese-hyperglycemic mice. Liver of Cottus scorpius seemed to have about 21% less G-6-P

TABLE 24

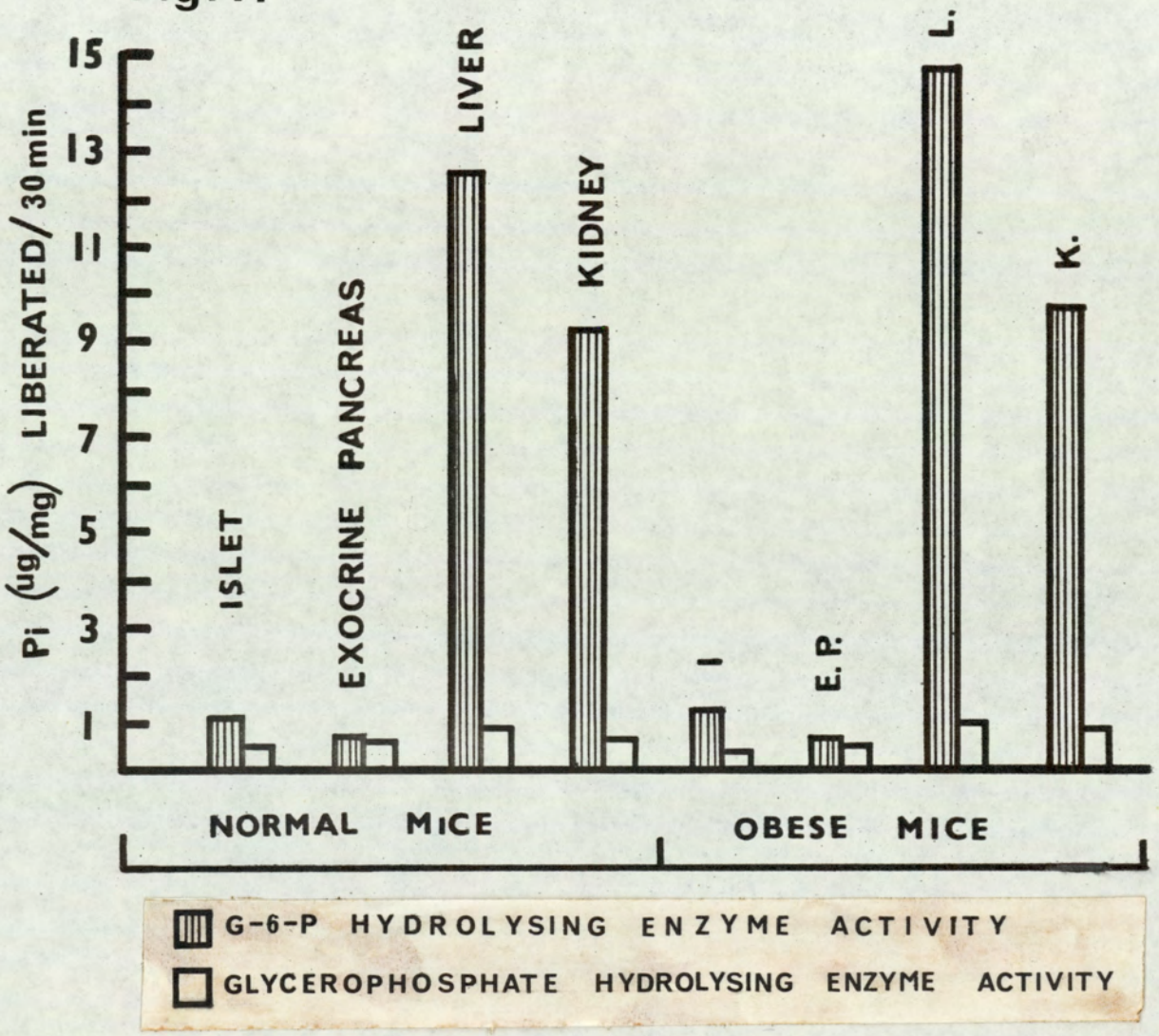
Enzymatic Activities Hydrolyzing G-6-P and Glycero-phosphate in Different tissues of Cottus Scorpius and Tilapia Mossambica

Species	Substrate	T I S S U E				
		Islets	Exocrine Pancreas	Liver	Heart	Gills
Cottus Scorpius	G-6-P	1.37 ± 0.18 (16)	0.52 ± 0.06 (4)	11.8 ± 0.70 (6)	0.96 ± 0.13 (5)	———
	Glycero-phosphate	0.45 ± 0.16 (14)	0.62 ± 0.04 (4)	0.82 ± 0.12 (6)	1.07 ± 0.08 (5)	———
Tilapia mossambica	G-6-P	1.28 ± 0.06 (10)	0.50 ± 0.08 (4)	10.70 ± 0.04 (8)	0.93 ± 0.14 (3)	1.95 ± 0.08 (2)
	Glycero-phosphate	0.35 ± 0.07 (10)	0.59 ± 0.06 (4)	0.74 ± 0.16 (8)	0.99 ± 0.10 (3)	1.32 ± 0.15 (2)

The figures denote mean values, determined with standard procedure (substrate concentration 0.04, PH 6.5, incubated for 30 minutes), of enzyme activities expressed as Pi liberated µg./mg fresh tissue/30 minutes.

± standard deviation; Number of determinations are given within parentheses. Average of duplicate samples results was taken for one determination.

Fig.17


Pi (ug/mg) LIBERATED/30 min
15
13
11
9
7
5
3
1
ISLET
EXOCRINE PANCREAS
LIVER
KIDNEY
I.
E.P.
L.
K.
NORMAL MICE
OBESE MICE
G-6-P HYDROLYSING ENZYME ACTIVITY
GLYCEROPHOSPHATE HYDROLYSING ENZYME ACTIVITY

hydrolyzing activity than that in liver of obese-hyperglycemic mice but only about 6% less than that in liver of normal mice.

iv. Effect of fasting on G-6-pase activity in Pancreatic islets, liver and kidney.

Six experiments, one per each animal were carried out to study the change in the G-6-pase activity in homogenates of pancreatic islets prepared from normal mouse after they had been fasting for three days. The enzyme activity was also determined in liver and kidney of fasting animals. The ratios between G-6-P enzyme activity and glycero-phosphate-splitting activity were also calculated for each determination. Usually two determinations were carried out for each experiment. The results of these experiments are presented in Table 25.

It appears from these results that G-6-pase activity was affected by fasting in all the three tissues studied particularly liver and kidney. On the other hand, glycerophosphate-splitting activity remained apparently unchanged in all the tissues. An insignificant 3.5% decrease in G-6-pase activity of pancreatic islets was found. In two of the fasting animals, however, a decrease up to 10% was observed. The ratio between G-6-pase activity and glycero-phosphate-splitting activity, in general fell within the same range as that calculated for pancreatic islets of the normal mouse.

It becomes distinctly clear from Table 25 that there occurs a marked increase in liver (59.0%) and kidney (31.8%) G-6-pase activities when the animals were fasted for three days. Nearly all the fasted animals showed this decrease in liver and kidney tissues. Also, the ratios between G-6-pase activity and glycerophosphate-splitting activity became significantly higher in both these tissues when compared with those for respective tissues of non-fasted animals.

TABLE 25

G-6-Pase and Glycerophosphate-Splitting Activities in Pancreatic Islets, Liver and kidney of mice after fasting for three days.

Tissue	Substrate	EXPERIMENTS 1	2	3	4	5	6	N*	Mean values	% increase/ decrease
Islets	G-6-P	1.02	1.15	1.09	1.20	1.10	1.04	12	1.10 ± 0.06	about 3.5% decrease
	Glycero-phosphate	.47	.61	.55	.59	.51	.53	12	0.54 ± 0.05	
	Ratio	2.17	1.88	1.98	2.03	2.15	1.96		2.03	
Liver	G-6-P	19.85	17.96	22.57	18.38	20.82	21.48	12	20.17 ± 1.7	about 59.0% increase
	Glycero-phosphate	0.87	-	0.76	0.69	-	0.87	8	0.79 ± 0.08	
	Ratio	22.81		29.69	26.63		24.68		25.53	
Kidney	G-6-P	12.46	9.85	14.27	-	13.82	10.52	10	12.18 ± 1.95	about 31.8% increase
	Glycero-phosphate	0.74	0.81	-	-	0.77	0.68	8	0.75 ± 0.05	
	Ratio	16.83	12.16			17.94	15.47		16.24	

The figures against the substrates denote the enzyme activity determined; ± standard deviation

At least two determinations were carried out on each tissue from each animal.

* N = Total Number of determinations contributing to the mean values

v. Effect of alloxan administration on G-6-pase activity in pancreatic islets, liver and kidney.

At the micro-dissection it was evident that alloxan injected mice had much smaller pancreatic islets than the untreated animals and that it was comparatively less easy to isolate them in the former case than in the latter. Moreover, more islets were to be dissected to get the required amount of tissue for each determination. Hence, sometimes it was not possible to perform two determinations of duplicated samples of pancreatic islets obtained from one animal. In such cases, the determinations were performed on islet homogenates pooled out from more than one animal.

G-6-pase activity and the glycero-phosphate-splitting activity was determined in pancreatic islets as well as in liver and kidney of alloxan treated mice. The results of these experiments are presented in Table 26. It shows that the pancreatic islets had their G-6-pase activity drastically reduced to significantly lower values than in the normal pancreatic islets. The lower G-6-pase activity was evident in all the experiments. The ratios of G-6-pase activity to that of glycero-phosphate-splitting activity were also calculated and are shown in the same table. This ratio in the case of pancreatic islets is much lower (average ratio 1.17) than the ratios in the pancreatic islets of untreated animals (average ratio 2.30).

It also appears from Table 26 that there occurred a marked increase of G-6-pase activity in liver and kidney after alloxan treatment. Again, this increase was apparent mostly in all the experiments performed. The glycero-phosphate-splitting activity in liver and kidney in treated animals remained apparently the same as in the untreated animals. This is also evident from the calculated ratios of G-6-pase activity to glycerophosphate splitting activity, also given in Table 26. These ratios are much higher for both

TABLE 26

G-6-Pase and Glycero-Phosphate-Splitting Activities in Pancreatic Islets, Liver and Kidney in Mice after Alloxan Administration

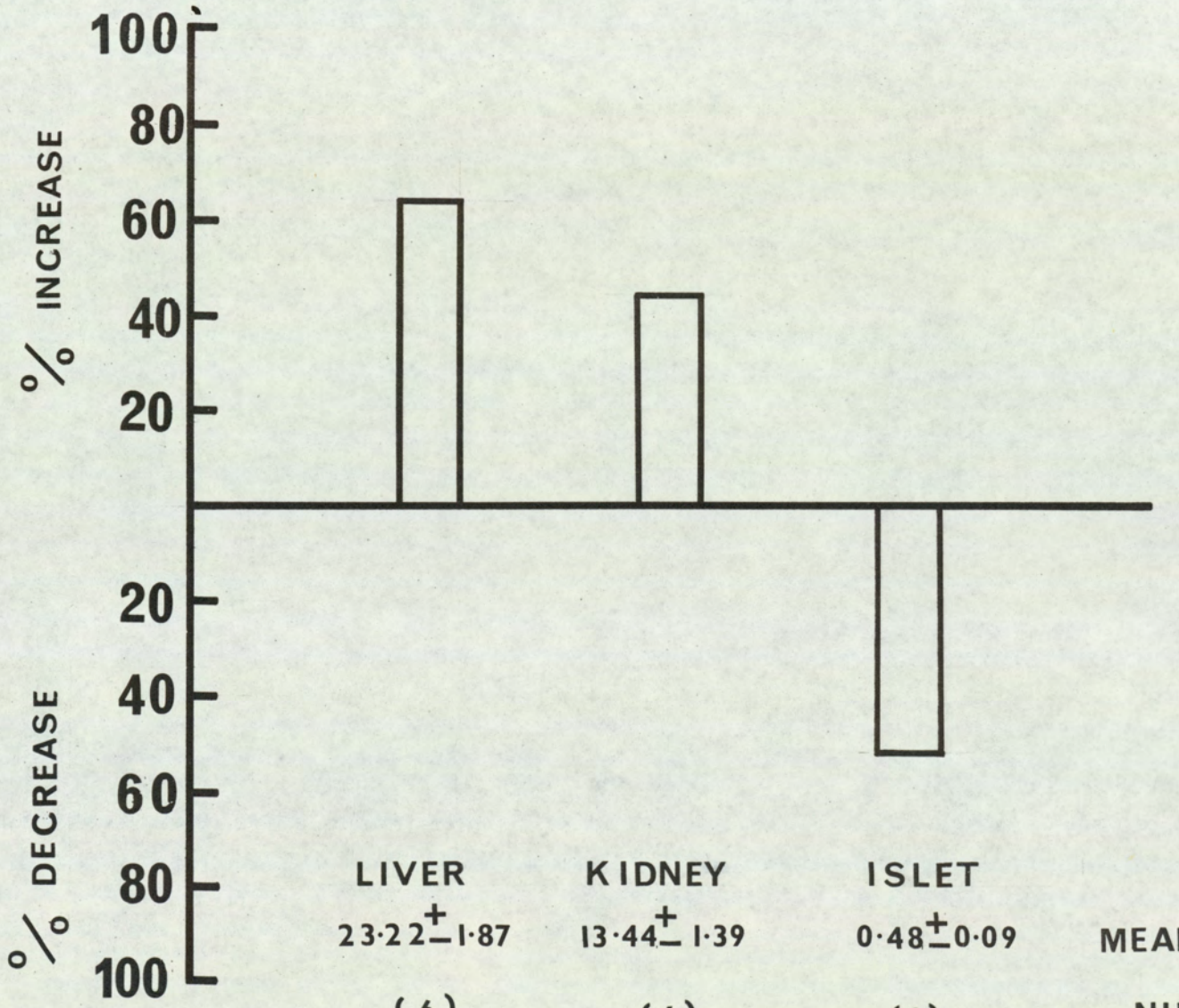
Tissue	Substrate	EXPERIMENTS 1	2	3	4	5	6	Mean Values
Islets	G-6-P	0.51	0.45	0.63	0.37	0.40	0.55	0.48 ± 0.09 (10)
	Glycero-phosphate	0.43	0.31	0.45	0.41	0.39	0.49	0.41 ± 0.06 (6)
	Ratio	1.18	1.45	1.40	.90	1.02	1.12	1.17
Liver	G-6-P	18.56	24.96	22.58	25.83	26.85	20.54	23.22 ± 1.87 (12)
	Glycero-phosphate	0.89	0.71	-	0.91	0.85	-	0.85 ± 0.07 (8)
	Ratio	20.85	35.15		28.38	31.58		27.31
Kidney	G-6-P	10.52	13.89	13.85	14.29	14.58	12.96	13.34 ± 1.39 (12)
	Glycero-phosphate	-	0.73	0.81	0.75	-	0.69	0.74 ± 0.04 (8)
	Ratio		19.02	17.09	19.05		18.78	18.02

The figures against the substrate denote the enzyme activity determined: ± standard deviation

Total number of determinations carried out for each tissue are given within the parentheses

Fig.18

EFFECT OF ALLOXAN ADMINISTRATION ON G–6–Pase



liver (average ratio 27.31) and kidney (average ratio 18.02) in alloxan treated animals as compared to those of the respective tissues in untreated animals.

The percentage increase/decrease occurring in the pancreatic islets, liver and kidney after alloxan treatment is presented in Fig.18. It becomes quite clear from Fig.18 that G-6-pase activity in the pancreatic islets was decreased by 57% of the activity of normal mouse pancreatic islets. The liver and kidney showed about 84% and 44% respectively, increase in their G-6-pase activity after alloxan treatment.

vi. Effect of tolbutamide treatment on G-6-pase activity in the pancreatic islets and other tissues of mice.

Twelve experiments on isolated pancreatic islets, seven on kidney, six on liver and only five on exocrine pancreas were performed to determine G-6-pase activity after tolbutamide treatment ~~on~~ mice. Only one determination per animal (each experiment) was carried out. In certain experiments, however, glycero-phosphate-splitting activity parallel to G-6-pase activity was not determined. The results of these experiments are presented in Table 27. The ratios of G-6-pase activity to glycero-phosphate-splitting activity for all experiments are also given in Table 27. The average G-6-pase activity in the pancreatic islets, after tolbutamide treatment was found to be slightly lower (0.98) than the value obtained for normal mouse pancreatic islets (1.14). It was evident in all the experiments but one that the G-6-pase activity was decreased very slightly. Though the ratio between G-6-pase activity and glycero-phosphate activity in the pancreatic islets of tolbutamide treated animals was lower than that obtained for untreated ones, there appeared to be no significant difference.

The G-6-pase activity of exocrine pancreas, on the other hand, remained apparently unchanged in tolbutamide treated animals.

TABLE 27

G-6-Pase and Glycero-Phosphate Splitting Activities in Pancreatic Islets, liver, Kidney and Exocrine Pancreas of Mice after Tolbutamide Treatment

Tissue	Substrate	EXPERIMENTS 1	2	3	4	5	6	7	8	9	10	11	12	Mean Values
ISLETS	G-6-P	0.98	1.05	0.89	1.00	1.10	0.90	0.92	1.06	1.01	1.02	0.88	0.95	0.98 ± 0.07 (12)
	Glycero-phosphate	-	0.51	-	-	0.63	-	0.41	0.59	-	0.55	0.43	-	0.52 ± 0.08 (6)
	Ratio		2.05			1.74		2.24	1.79		1.85		2.04	1.88
LIVER	G-6-P	-	-	8.0	-	5.40	11.03	-	-	9.19	8.60	-	9.14	8.56 ± 1.84 (6)
	Glycero-phosphate	-	-	0.74	-	0.81	0.83	-	-	0.89	0.79	-	-	0.81 ± 0.05 (5)
	Ratio			10.81		6.66	13.2			10.32	10.88			10.56
KIDNEY	G-6-P	-	-	14.5	-	14.5	9.9	-	10.70	13.6	12.0	-	13.0	12.60 ± 1.81 (7)
	Glycero-phosphate	-	-	0.83	-	-	0.74	-	0.66	0.78	0.71	-	0.84	0.76 ± 0.06 (6)
	Ratio			17.46			13.37		16.21	17.43	16.90		15.47	16.57
EXOCRINE PANCREAS	G-6-P	-	0.60	-	-	0.63	-	0.66	0.72	-	0.69	-	-	0.66 ± 0.14 (5)
	Glycero-phosphate	-	0.52	-	-	0.59	-	0.56	0.63	-	0.65	-	-	0.59 ± 0.05 (5)
	Ratio		1.15			1.06		1.17	1.14		1.06			1.11

The figures against the substrates denote the enzyme activities determined; ± standard deviation

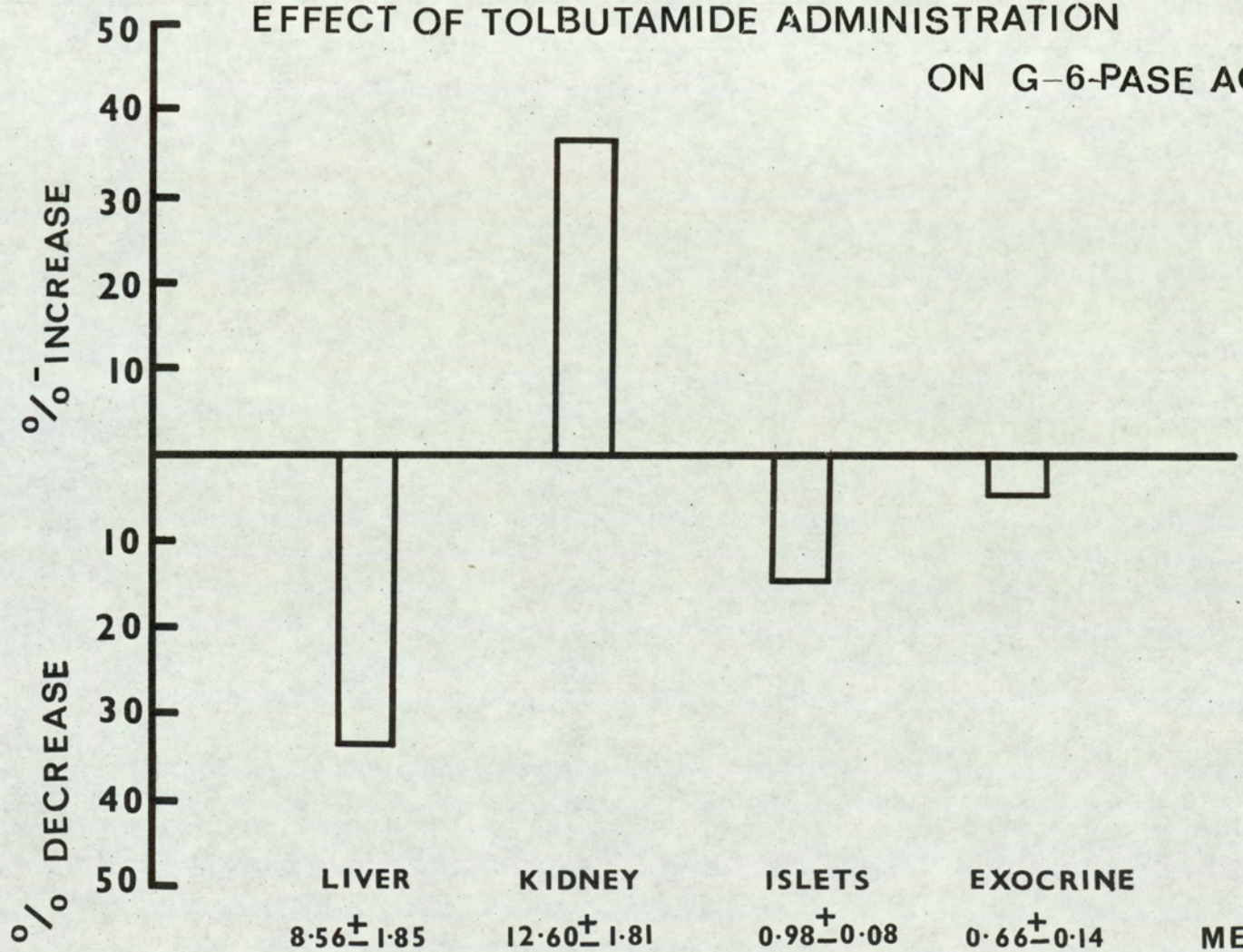
Total number of determinations given within parentheses.

It also appears from Table 27 that G-6-pase activity in liver and kidney are markedly affected by tolbutamide treatment. Liver showed a low G-6-pase activity in these animals as compared to untreated ones. On the other hand, kidney showed comparatively higher G-6-pase activity in treated animals than in untreated animals. In one treated animal, liver showed G-6-pase activity as low as 5.40 (normal average value 12.68) whereas kidney in some treated animals displayed the enzyme activity as high as 14.50 (normal average value 9.24).

The increase or decrease calculated as percentage of the normal values determined in untreated animals in pancreatic islets and other tissues after tolbutamide treatment is shown in Fig.19. It appears from Fig.19 that after tolbutamide treatment there occurred about 14% decrease of G-6-pase activity in the pancreatic islets which was not significant ($P < 0.1$). On the other hand significant ($P < 0.001$) decrease (about 32%) and significant ($P < 0.01$) increase (36%) of G-6-pase activity was found in liver and kidney respectively. The exocrine pancreas showed an insignifcant 4% decrease of G-6-pase activity.

Fig. 19

EFFECT OF TOLBUTAMIDE ADMINISTRATION ON G-6-PASE ACTIVITY



Chapter 4 DISCUSSIONS

In view of the controversial presence of G-6-pase (176, 184, 206) in the mammalian pancreatic islets and of its postulated role in the mechanism of regulation of insulin secretion (170) its properties were analysed and the activity measured in the isolated pancreatic islets of normal and obese-hyperglycemic mice, using a quantitative microchemical method. Histochemical demonstration of the enzyme in tissue sections could provide qualitative information, as obtained in mammalian pancreatic islets but the metabolic pattern and even the marked metabolic changes in the enzyme pattern of the tissue under study remain unrecorded. Also, only homogenized tissue can provide material for proper study of the enzyme characterization. The presence of G-6-pase enzyme in the islet beta cells by histochemical demonstration, therefore, can not be accepted particularly when reports of negative results have also appeared (23, 206). Low specificity of histochemical staining of this type of enzyme strongly recommends its analysis by quantitative microchemical methods.

During this study the properties studied for G-6-pase in the islet tissue have been compared with the corresponding characteristics in tissues which are known (liver and kidney) and not usually considered (exocrine pancreas) to display G-6-pase enzymatic activity, in order to establish the identity of this enzyme. In the principal islets of teleostean species, the PH characteristics of the activities have also been compared with those in gills and heart tissues.

The present microchemical study clearly shows that the rate of hydrolysis of G-6-pase in the pancreatic islets, as in liver and kidney, of both normal and obese-hyperglycemic mice, was higher at PH 6.5 than at either PH 5.0 or PH 9.0 which are optimal for acid and alkaline phosphatases respectively (358) and it is also known that G-6-pase has an optimal PH of 6.5 (358). Similarly, G-6-pase activity in the principal islets of teleostean species was found to be much higher at its optimal PH (6.5) when

compared with that at either PH 5.0 or PH 9.0. On the other hand, in other tissues, the activities towards G-6-P at differing PH levels was not found to be significantly different from one another.

G-6-P has not yet been purified either from the very small amounts of pancreatic islets available from mammals or from principal islets of fish. The islet cells have been shown to display a distinct activity of non-specific acid phosphatase (35). It was assumed, therefore, that both non-specific acid phosphatase and G-6-pase, if present, may hydrolyse G-6-P, whereas cleavage of β-glycerophosphate was considered to reflect non specific activity. The determination of G-6-pase and glycerophosphate-splitting activities in islet tissue demonstrated that it has been possible to distinguish G-6-pase activity from that of acid phosphatase.

The results that the islet tissue, like liver and kidney, of all the species under study displayed much higher activity towards the specific substrate (G-6-P) than towards the non-specific substrate (β-glycerophosphate) as expressed by comparing the calculated ratios between the respective activities towards the two substrates, demonstrated strongly the substrate specificity in the pancreatic islets for G-6-P, which is the specific substrate for G-6-pase enzyme (358). Response of the islet tissue towards other non specific substrates was similar to that towards β-glycerophosphate. Furthermore, the findings that G-6-pase hydrolysing activity was much higher in the pancreatic islets when compared with that in exocrine, gill or heart tissues suggests the presence of an activity which is specific. The enzyme activity in the exocrine, gill or heart tissues, as a matter of fact, was of the same order.

It is known that specific G-6-pase is rapidly inactivated at 37°C (359) whereas the non-specific acid phosphatase in the islet homogenates of obese hyperglycemic mice is characterised by a greater stability (35). The enzyme activity towards G-6-P in the present study was shown to be thermally unstable, as preincubation of the pancreatic islet homogenates at 37°C for 15 minutes resulted in a marked inactivation of the enzymatic activity.

On the other hand, the activity towards glycero-phosphate remained apparently unaffected in such experiments. Also, such inactivation of the G-6-P hydrolysing activity was observed in liver homogenates but could not be established in the homogenate of exocrine tissue. Similar results have been reported for human jejunal mucosa (360) and were considered as indicating the presence of specific G-6-pase. Furthermore, addition of ammonium molybdate into the reaction mixture resulted in drastic inhibition of the enzyme activity towards G-6-P in the pancreatic islet homogenates and it is known (358) that this compound acts as inhibitor of specific G-6-Pase.

Thus the present study indicates that pancreatic islets of normal as well as of obese-hyperglycemic mice, like liver and kidney but unlike the exocrine pancreas and other tissues, display an activity with most of the characteristics and well known properties of specific G-6-pase enzyme previously confirmed in mammalian liver and kidney (353, 354, 358, 359).

G-6-Pase enzyme activity in the present study is clearly distinguished from acid phosphatase and from non specific activity acting on glycerophosphate. A similar suggestion is evident from the studies performed on the principal islets and other tissues of Cottus scorpius and Tilapia mossambica. It seems, therefore, reasonable to conclude from the present investigation that the islet tissue of all the four species under study contain a specific G-6-pase and that probably the presence of this enzyme is a characteristic enzymatic feature of the islet tissue in both mammalian and non-mammalian species. As the reaction products for this enzyme, whenever reported to be present in the pancreatic islets by histochemical staining, has been demonstrated in the beta cells (35, 170, 178), it is also concluded that probably this enzyme is a characteristic of enzyme pattern in the pancreatic beta cells.

It must be mentioned as well, that as G-6-pase enzyme is known to be extremely labile, some if its activity in the tissue homogenates might have been lost due to inactivation, although extreme care was taken to

perform all the tissue handling prior to incubation at about +2°C to +4°C. The high degree of sensitivity used in the present study, however, suggests that such studies are possible even if micrograms only of islet tissue are available.

Previously, G-6-pase enzyme was demonstrated in the pancreatic islets of some mammals, by histochemical staining (170, 177-179, 181, 182). This enzyme was shown to be present in pancreatic islets of dog, guinea pig (170) as well as of rabbits (170, 179, 181, 182), where it was confined to the beta cell cytoplasm and appeared as a fine granular precipitate. It was found to be present even in the surviving beta cells of alloxan-diabetic rabbits (361). The present study confirmed the earlier histochemical findings of the presence of this enzyme in pancreatic islets of normal and obese-hyperglycemic mice (35, 177), which showed that islet cells display a cytoplasmic reaction whereas the activity of exocrine pancreas was weak. In the present study no specific G-6-pase enzyme was found to be present in the exocrine pancreas. On the other hand, in an earlier extensive study of the distribution of G-6-pase in different tissues of mouse, evidence was found for the existence of G-6-pase enzymatic activity only in liver, kidney and intestinal epithelium, but not in endocrine or exocrine pancreas (206).

During the course of this investigation, a few histochemical studies regarding G-6-pase enzyme have also appeared (175, 183, 362-364). But, histochemical studies on a KK strain of mouse (diabetic) showed (178, 362) an apparently higher G-6-pase activity in contrast to the control mouse (C 57 BL/6 strain), and very recently intense cytoplasmic reaction in the beta cells from the adult obese-hyperglycemic mice has also been observed (364). Similarly, application of histochemical studies to isolated beta cells of microdissected islets from guinea pig (183) also demonstrated the presence of this enzyme which was seen to be localized on the nuclear membrane and as irregular aggregations dispersed in the cytoplasm. G-6-pase is known to be a microsomal enzyme (358) and observations of its presence

on nuclear membrane is probably due to technical faults.

In the pancreatic islets of rat, on the other hand, G-6-pase activity was found to be absent (179, 365) even with an incubation time of 60 minutes (179), although the same authors reported the enzymatic activity in rabbit pancreatic islets (179). It appeared difficult to explain whether the difference in result was due to variations in optimal conditions of the enzyme activity in different species or whether it represents more biochemical differences. Species difference has been shown to exist in the enzyme activity of the islets between rabbit and rat (179). Histochemical techniques are liable to produce different results as well. Recently, histochemical investigations on human pancreas failed to demonstrate the presence of this enzyme in the pancreatic islets (175).

No study has yet been reported confirming the presence of G-6-pase enzyme in the pancreatic islets of teleost or any other non-mammalian species. The only study carried out on the principal islets of a teleost fish denied the presence of this enzyme in the islet tissue (23, 162, 193). Determining the enzyme activity by quantitative method, the only quantitative report prior to the present investigation, the authors found (23, 162, 193) that slightly more phosphate was split from glycero-phosphate than from G-6-P, and no evidence was obtained for increased activity at its optimal PH 6.5 when compared at PH 5.0 and PH 9.0. They instead, attributed the liberation of inorganic phosphorus by toadfish islet tissue to be due to a non specific phosphatase rather than a specific G-6-pase (23, 162, 193). The present study, on the other hand, clearly distinguished between the activities due to specific and non-specific substrates as well as between specific G-6-pase activity and that of non-specific acid phosphatase. G-6-pase is an extremely labile enzyme (358) and there is every possibility that most of the enzyme activity was lost during the handling of the tissue homogenates prior to incubation. Probably, the low phosphate splitting activity of the islet tissue at PH 6.5 as determined by them was in fact due to to the presence of a decreased specific G-6-pase and not due to non

specific phosphatase. It may also be pointed out that the buffer used as incubation medium in the present determinations was different from that used by them (193) and it is possible that composition of the incubation media may produce moderate, if not intense, degree of variability in the results. The present findings, however, were confirmed (personal communication from I. Taljedal, of Histology Department, Umea University, Umea, Sweden at Sixth Conference of International Diabetic Federation 1967 in Stockholm) by the demonstration of this enzyme, using a quantitative microchemical method, in the microdissected islets obtained from obese-hyperglycemic mice as well as from cortisone treated guinea pigs, a brief report of which appeared (366) later on.

The present study confirms the previous findings of the presence of specific G-6-pase enzyme in mammalian liver (353-356, 358, 367, 368) and kidney (354, 356-358, 367, 368). G-6-pase, besides being present in liver and kidney has also been reported to be present in small intestine (360, 369, 370) using a biochemical procedure, and in rectum, cartilage and skin by non-specific hostochemical methods (371). The suggestion that specific G-6-pase is present in blood (372) was shown to be due to non-specific phosphatase (373). Histochemical studies indicated the presence of this enzyme in epididymis (374). Also, reaction products for this enzyme were demonstrated in endoplasmic reticulum and nuclear membrane of hepatic cells (375).

G-6-pase has been considered to be important in releasing glucose into the circulation by hydrolysing G-6-P (phosphorylated form of sugar in the cells). It was shown that presence of this enzyme enables the liver to supply the necessary glucose for the maintenance of blood glucose, either from glycogen by gluconeogenesis or by removal of glycolysis (376). Later on it was confirmed by extensive studies on liver homogenates that release of glucose for the liver is mediated by this enzyme. It was also indicated (357) that kidney may also contribute blood sugar but in amounts which are relatively small as compared with liver. In contrast to non-specific

acid and alkaline phosphatases, the presence of histochemically demonstrable G-6-pase in kidney was assigned to a particular function i.e. production of glucose (368). A greatly increased G-6-pase activity has thus been reported in connection with an increased glycogen breakdown in the liver cells (355) while enzyme activity in the kidney appears to be found mainly in those tubular cells which absorb glucose from glomerula filtrate and transport it to the blood (206). A report about the absence of this enzyme (376) in liver and kidney of human cases of glycogen storage disease is consistent with this view. Though a number of enzymes have been detected with histochemical staining methods (170-180, 205) as well as measured by quantitative microchemical procedures (23, 35-39, 122, 162, 184-186, 189-192) in the pancreatic islets of many mammalian species, their functional significance particular in relation to beta cell metabolism is poorly understood. Among such enzymes particular attention has been paid to phosphatases, pronounced activities of which were observed in the pancreatic cells of many species (35-37, 173, 174, 176, 177, 185, 186, 203). These studies have provided evidence to suggest that some of these enzymatic activities are related to the beta cell function, although biological significance of these phosphatases remains obscure (179). A more obvious relationship to the metabolism of beta cell was suggested especially for specific G-6-pase when histochemical observations revealed its strikingly high concentration in the beta cells (170). The presence of this enzyme in the pancreatic islets has clearly been established in the present study. It can reasonably be assumed that this enzyme function as it does for mammalian liver and kidney i.e. hydrolysing G-6-P, to release necessary glucose in relation to the insulin secretion from the beta cell. As it has been shown that increase in glucose concentration in the beta cells increases insulin secretion whereas the increase in glucose concentration in the brain or liver does not (377). The pancreatic islets of fish and mice display relatively low G-6-pase activity which is about 9-11 times less than that of liver and about 7-8 times less than that of kidney as determined in the

present study. If there is to be an important function of G-6-pase enzyme as far as regulation of insulin release is concerned, it might be supposed that the enzymatic activity should have been of the same order as in the liver and kidney in order to release sufficient glucose to influence insulin secretion from the beta cells. It must, however, be pointed out that the enzymatic determination was carried out on homogenates of whole islets and expressed as per unit weight and, therefore, it cannot be attributed to beta cells only as pancreatic islets are a mixture of islet cells as well as of vascular tissue. The contribution made by the beta cells towards the total weight of the whole islet is not known but as rough estimations show beta cells to compose about one third or one fourth of the total islet mass, the enzymatic activity of the beta cells can presumed to be probably three or four orders of magnitude of the activity determined. It is evident that the data obtained on pure beta cells would be desirable. As said earlier, the possibility of some loss of the enzymatic activity during the experimental handling particularly with such a small amount of islet tissue cannot be ruled out, suggesting the enzymatic activity in fact is greater than the determined values.

If an enzyme, involved in the glucose metabolism of the pancreatic islets controls in some way or another the regulation of insulin secretion, then it should be expected that the increased or decreased insulin secretion is related to the alterations in the level of that enzymatic activity. Therefore, it becomes essential to study the change in the concentration of such intermediate metabolites in the pancreatic beta cells induced by various experimental conditions and to correlate these changes with their different functional states of insulin secretion. Since glucose is phosphorylated to G-6-P upon entering the cell, the amount of G-6-P available for beta cell metabolism may be the limiting factor which governs the rate of insulin output. Presumably an increase in G-6-P would increase insulin output. The net rate of formation of G-6-P would depend upon relative velocities of hexokinase and G-6-pase activities, and the velocity of

glucose phosphrylation by hexokinase would depend upon the blood glucose concentration. Thus a decrease in the activity of this enzyme or an increase in blood glucose concentration would probably increase the amount of G-6-P available for beta cell metabolism and hence increase insulin out-put. In other words, lowering of blood sugar concentration or a raised G-6-pase activity, by breaking down more G-6-P would decrease available G-6-P and thus would diminish insulin production (170). It was, therefore, concluded that G-6-pase might be working as a limiting enzyme in the control of insulin out-put (170). To test this hypothesis it becomes extremely important first to establish the presence of specific G-6-pase. Though its presence has been denied in the pancreatic islets of certain species, as mentioned earlier, but it is known that G-6-pase is an extremely labile enzyme and failure to demonstrate it histochemically certainly is no evidence for its absence. Hence the fact that G-6-pase may be absent from the islet cells of some species, as species difference has also been noted to occur in enzymatic pattern (179), in no way should modify our thinking about its possible significance in the islet cells of species in which it is present. In this concept, after clearly demonstrating the presence of the specific G-6-pase activity in the pancreatic islets of normal mice as well as of teleostean species, the change in the level of G-6-pase activity was studied in the pancreatic islets of obese-hyperglycemic mice, as well as in those of starved, alloxan-treated and tolbutamide treated mice.

According to the hypothesis of Lazarus (170) it would mean that a decreased G-6-pase activity in the pancreatic islets of obese-hyperglycemic mice should be expected, as it is known that these animals have islet beta cells in considerably raised functional state of insulin secretion (40). However, the present study shows that the pancreatic islets from this strain of mice do not contain a low G-6-pase activity compared with normal mouse pancreatic islets. The enzyme activity was in fact slightly higher than that in the pancreatic islets of normal mouse, though not as high as

might have been assumed if a comparison were to be made between the present results and previously published histochemical observations (35, 177) which indicated an intense enzyme activity in the islet beta cells of obese-hyperglycemic mice, and a weak reaction in the islet beta cells of the lean littermates. It is not known whether this discrepency depends on limitations experienced during the present biochemical approach or on the artifacts introduced by histochemical staining techniques or on the assumption that visual quantitative assessment of intensity of a reaction product may not necessarily correspond accurately to the concentration of enzyme activity. It is also probable that this difference is due to the difference of age of the obese-mice used for histochemical (177) study and for biochemical (present) investigation. The later concept finds support by the observations in an old obese-hyperglycemic mice describing the pancreatic beta cells to be characterized by a considerably weaker reaction, the staining intensity being approximately the same as in islet cells of lean mice. This observation, therefore, is in agreement with the results obtained in the present study. Also, Westman did not find any difference in the islet G-6-P splitting activity between lean mice of different ages (364). On the other hand apparently higher G-6-pase activity was observed in the beta cell cytoplasm of KK(diabetic) strain of mouse as compared to the faint reaction in the control group of animals (178, 362). This high G-6-pase activity, inconsistent with the suggestion of Lazarus (170) was plausibly explained as an adaptive phenomenon to the presumably increased amount of the substrate of the enzyme (178, 362). The average G-6-pase enzyme activity of liver of obese-hyperglycemic mice is slightly higher than that of normal mouse liver. This overall increase in the G-6-pase activity between two strains of mice may probably be considered as the result of the adaptive increase in liver size of obese-hyperglycemic mice (378).

Fasting for 48 hours was not found to have any significant effect on the G-6-pase activity of mouse pancreatic islets. According to the Lazarus

hypothesis (170) lowering of blood sugar resulting in diminished output of insulin is associated with a raised G-6-pase activity. As it is known that fasting results in slight decrease in blood sugar (379) and hence has an effect on pancreatic beta cell function, a raised level of G-6-pase activity would have resulted, if G-6-pase activity is directly controlling the regulation of insulin release. Up till now no study, biochemical or histochemical has been attempted to observe the effect of fasting on G-6-pase activity in the pancreatic islets. On the contrary, the present study revealed a marked increase in G-6-pase activity of both liver and kidney and this is in agreement with previous findings on liver and kidney of rat (380-384). It was suggested that the low liver glycogen found in fasting rats is the result of the increased phosphatase activity observed, and the rise in hepatic G-6-pase was explained as an adaptive change (380, 381). It is also possible, as indicated when the results were expressed in terms of total activity/unit original body weight (384), that increase is not significant since due to fasting there is a decrease in body weight and liver sizes which may result in high concentration of the enzyme activity rather than increase in its activity.

After alloxan treatment the average G-6-pase activity of pancreatic islets was found to be approximately less than half of the corresponding values of the normal mouse islets. A slight reduction in the enzyme activity in the pancreatic beta cells has been observed histochemically at 15 and 30 minutes after alloxan injection but it was also found that the enzyme activity remained unchanged at 1, 2 and 4 hours following alloxan administration (181, 182). But a complete loss of the activity in the necrotic portion was observed at 24 hours after the injection. On the other hand, histochemical investigations on rabbit pancreatic islets (184, 185) indicated that the enzymatic activity continued to show the reaction in the beta cell evan at 18 hours and disappeared completely at 24 hours. But the later studies (181, 182) failed to demonstrate such activities at 8 hours. Considering their intimate relation of this enzyme with G-6-P, it may have a

function at a moderately long distance from the direct energy-producing metabolism in the mitochondria; probably some role in cellular specific functions (181). It cannot be said positively whether there was a complete loss of G-6-pase activity in the pancreatic beta cells of alloxan-treated mice in the present study, but occurence of significant decrease in its activity probably supports the earlier findings (181). It is quite probable that the decrease in the G-6-pase activity has occured secondarily as a result of decrease in ATPase activity as after alloxan administration the first and most significant reduction was observed in the ATPase activity in the islet cells, especially in the beta cells, followed by a slight reduction in G-6-pase activity (181). Dithiozone and 8-hydroxyquinaldine compounds have also been shown to decrease the activity of G-6-pase in rabbit pancreatic islets (181) the former resulting in marked inhibition of the enzyme activity immediately after the administration while the latter produced an abrupt and marked reduction in the enzyme activity of the beta cells at 6 hours after the injection. The difference in response of G-6-pase activity to these chemical agents is probably due to the fact that each presents a different process by which it leads to the beta cell destruction after reaching the islet, although all the three agents produce a similar end result. And it has been indicated that dithiozone and hydroxyquinaldine have different mechanism of action from that of alloxan. In the liver and kidney of alloxan treated mice, by contrast, the enzyme was elevated by 84% and 44% respectively. The increase in hepatic G-6-pase activity is in agreement with previous findings on rat liver homogenates (380, 382, 385) and it was concluded that the increased G-y-pase activity in the liver is the result of an increased gluconeogenesis (382) and that there is no correlation between the blood sugar concentration and hepatic G-6-pase activity. Rise of G-6-pase activity in kidney of alloxan treated mice as well confirms the earlier findings (384). There is evidence that glucose ~~in~~ G-6-pase response in kidney may represent a physiological response of the organ to the conditions in which rise of

blood sugar imposes a greater metabolic load on the reabsorption mechanism. Comparing the hepatic G-6-pase activity of obese and alloxan-treated mice, it was obvious that the activity in the latter case was approximately 1.5 times that in the former. It is interesting to note that G-6-pase as reported in liver of diabetic rats (382) in comparison with the activity reported in liver of obese-hyperglycemic mice (378) was seen to have a similar ratio. It is thus reasonable to assume that hyperglycemia of obese-hyperglycemic mice is distinguished biochemically from that of alloxan-diabetic animals even by the difference in the activity of G-6-pase in liver.

A decrease in the activity of G-6-pase enzyme in the pancreatic beta cells would be expected after the administration of sulfonyluerea derivative, should the hypothesis put forward by Lazarus (170) that G-6-pase might be the limiting enzyme in the control of insulin output, be accepted. However, the present study does not seem to support this view. Though the average enzyme activity in the pancreatic islets was found to be slightly lower in the tolbutamide treated mice than in the normal mice, it was apparent that the decrease is not significant. On the other hand, it has been observed by Lazarus using histochemical staining, that after chloropropamide treatment to rabbits, intense staining of G-6-pase activity was found to be considerably less than in the normal animals (184, 186), thus obtaining evidence for his hypothesis. It must be mentioned, however, that, as he pointed out himself, the difference in the enzyme activity became less apparent after prolonged incubation of pancreatic sections in the substrate reaction mixture (186). It seems quite possible, therefore, that the results obtained by histochemical staining may not be relevant to the true enzymatic activity in the pancreatic islets. Besides Lazarus' own investigations by histochemical staining, no study to my knowledge has appeared to indicate the change in G-6-pase activity of the pancreatic islets after sulfonylurea derivatives. On the other hand, a slight but definite increase in G-6-pase activity has been regularly observed in the pancreatic

islets of cortisone-treated rabbits (179), and this is inconsistent with the hypothesis put forward by Lazarus (170), as in steroid treated animals one would expect a decreased activity in view of the increased insulin output. Lazarus, however, did not observe any change in cortisone-treated rabbits (185, 186). The insignificant depression in the G-6-pase activity in the tolbutamide treated mice as observed in the present study does not seem to support the view that G-6-pase activity is the limiting factor controlling the insulin release mechanism. G-6-pase activity of liver, however, was significantly decreased after tolbutamide treatment. Similar results have been reported on rat liver homogenates (382, 383). On the other hand, in another study, hepatic G-6-pase activity did not change as a result of an acute effect of sulfonylureas both in vivo and in vitro experiments carried out on rat (386). Similarly, an in vitro effect of 1-butyl-3-P-tolylsulfonylurea on the G-6-pase activity of rat liver homogenates could not be found (387). Whether or not an inhibition of G-6-pase activity can account for hypoglycemia observed in some of these studies is questionable, as no dimunition in G-6-pase activity was observed during the occurence of hypoglycemia within three hours of high dose of tolbutamide (382, 383). It, therefore, seems unlikely that the decrease in hepatic G-6-pase activity in tolbutamide treated mice is due to hypoglycemia caused by sulfonylurea treatment, thus confirming the previous suggestion (387). It can probably be explained as an adaptive response similar to that observed in rat liver homogenates after insulin administration (388). The same explanation probably can be suggested for the insignificant diminution of G-6-pase activity in the pancreatic islets of tolbutamide treated mice. On the contrary to the decrease observed in hepatic G-6-pase, a considerable increase was found in the G-6-pase activity of kidney after tolbutamide treatment. This is surprising because it has been suggested (356) the G-6-pase enzyme of both liver and kidney seem to possess similar properties and therefore, presumably perform similar functions. The present results indicate that experimental condi-

tions of hypoglycemia in this case, may result in certain metabolic changes in kidney which are not similar to those induced in the liver. The significance of this increase is, however, not clear.

Numerous findings have established the fact that increase in glucose concentration in the pancreatic beta cells increases insulin secretion (146-151, 377). Presumably the insulin release is a secondary consequence of changes in the intracellular glucose concentration. Moreover, it has also been consistently confirmed that insulin release mechanism is presumably activated by one of the metabolic products of glucose metabolism occuring in the beta cells (147, 149, 151, 162, 163) as insulin release is stimulated mostly by sugars known to be metabolized by the beta cells, and by the fact that glucose stimulation of insulin release is blocked by inhibitors of its metabolism (163-166). A strong suggestion, therefore, is present that further utilization of G-6-P rather than its formation in the beta cells which accelerates insulin secretion, is presumed. (149, 160, 166). Since glucogen can appear in the beta cells especially in diabetes, the pathways of synthesis and breakdown of polysaccharides are presumably present(23, 162), and enzymes involved in a number of pathways of glucose metabolism have been studied in pancreatic islets of various species. The metabolic pattern in islet tissue as it appears from various studies and summarised by Lazarow (23, 162) can be briefly discussed here. After passing through the cell membrane glucose is phosphorylated to G-6-P by hexokinase. The G-6-P may then be converted to G-1-P (and stored as glycogen) oxidised to 6-phosphogluconate (via the pentose shunt) or further phosphorylated to glucose-1-6-diphosphate (and metabolized by the glycolytic Embden-Meyerhof pathway). The pyruvate formed via glycolytic pathway is converted to acetyl.CoA and oxidized to CO_2 (and water) via the tricarboxylic acid (Kreb's) cycle. The reduced pyridine nucleotides, formed in the course of these reactions are oxidized by the electron transport (cytochrome) system with the consequent production of high energy phosphate. Inhibition of glucose stimulation by

anoxia (which stimulates glycolysis) is inconsistent with a significant involvement of the glycolytic pathways. Also, inhibition of insulin secretion by phenazine methosulphate (which stimulates oxidation of HMP shunt) suggests that these pathways do not provide the signal for insulin secretion. Pyruvate itself does not stimulate insulin secretion (though it may enhance the action of glucose) indicating probably that components of the citrate cycle are not connected with insulin secretion (149, 147). The proposal for mechanism of insulin secretion that a general increase of cytoplasmic viscosity within the cell may act as a trigger for insulin secretion does not hold good since other sugars that also increase viscosity proved inactive to stimulate insulin release (23). The role of zinc in the storage and secretion of insulin has also been postulated (141). The fact that most of the compounds like histidine, cystein and oxalacetate citrate (showing greater affinity for zinc) can be formed from glucose led Mask (141) to suggest that binding of zinc by these substances may be responsible for the release of insulin during hyperglycemia. The evidence for this view was obtained when zinc level and beta cell granulation were observed to show parallel changes as a result of glucose or insulin administration (389). A reduction in the level of zinc before the reduction in aldehyde fuchsin positive granules was also reported in pancreatic islets stimulated by tolbutamide (141), although later on the membranous sac and not the electron dense content of the granule was shown to be aldehyde fuchsin positive (201, 203), but this suggested a relationship between zinc and electron dense content of the granule. The theory of insulin secretion involving zinc removal is not supported by electron microscopic demonstration of secretion by granule extrusion (288). Though insulin itself does not stimulate insulin secretion, it has variably been reported as causing zinc depletion in the pancreatic islets (359) or having no such effects. On the other hand, injections of zinc does not affect insulin release and furthermore it is not even present in the pancreatic islets of all species (389). The observations that phlorrhizin

a known inhibitor of membrane transport system for sugars in the mammalian cells, failed to influence insulin release at high or low glucose concentrations (149) suggests that any transport system for the sugars in the beta cell membrane which regulates the insulin secretion, seems unlikely to exist. But it was found that N-acetyl glucosamine and 2-deoxyglucose (may inhibit glucose phosphorylation in other tissues) do not inhibit glucose stimulated insulin secretion in the beta cells (149) and this indirect evidence may not rule out some unique membrane transport system insensitive to phlorrhizin.

Integrity of the membranes surrounding the beta cell granule and beta cell wall is probably the requirement for insulin secretion. It is possible that non specific agents required for the maintenance of membranes could influence insulin secretion without being involved in the direct quantitative control by glucose. Thus calcium has been shown to be necessary for the secretion of insulin in response to glucose from the perfused rat pancreas (390). Calcium deficiency completely blocked the insulin secretion despite high glucose concentration (390). The established role of calcium in membrane viability suggests that this calcium dependency is a membrane phenomenon. Similarly it was demonstrated that insulin secretion was inhibited by a high extracellular magnesium concentration (391). Recently, evidence was presented that a sodium pump plays a part in insulin secretion, that extracellular sodium is a prerequisite for secretion in response to a variety of stimuli and that secretion may be associated with a rise in sodium concentration in the beta cells (392). Such studies serve to emphasize that many factors shown to influence insulin release may not be involved in the intimate control of this hormone directly but may act on the general mechanism required for the release of any secreted protein.

During the present investigations though the G-6-pase enzyme did not correlate directly with the amount of insulin, produced due to different functional states of the pancreatic islets, as one should expect if it is

to play the role as a limiting enzyme in the control of regulation of insulin secretion, its increase or decrease, nevertheless, signifies its importance in the beta cell metabolism. It, however, supports the suggestions previously put forward that glucose metabolism in pancreatic beta cells may provide a signal for release of insulin and it further confirms strongly that phosphorylation of glucose probably is the key step with regard to control of insulin release. Recently a report involving the determination of ATP-D-glucose 6-phosphotransferase in mouse islet homogenates has appeared (393). It was assumed in this study that phosphorylation of glucose is perhaps catalysed by an ATP-D-glucose 6-phosphotransferase with a high km for glucose (glucokinase) may be, therefore, a rate-determining step in the process (393). Mouse islets showed no evidence for the presence of glucokinase under conditions where glucokinase was readily detected in liver homogenates (393). It was concluded that glucokinase is unlikely to be responsible for the increased rates of glucose oxidation and insulin release (393). It may as well be possible that stimulation of insulin release by glucose may occur by a mechanism which does not primarily involve glucose metabolism but which leads secondarily through the release process to acceleration of glucose oxidation.

It appears, therefore, from the present investigation that no conclusive evidence was obtained suggesting role of G-6-pase enzyme as a limiting factor controlling insulin release from the beta cells, although its specific presence was clearly demonstrated in the islet tissue of both mammalian and teleostean species. It could also be concluded from the present investigation that the specificity of this enzyme appears to be identical to the G-6-pase enzyme present in liver.

The precise role of this enzyme in relation to the pancreatic islet metabolism remains largely unknown, although its importance in the beta cell function is signified. There is still a possibility that the oxidation of phosphorylated sugar by glucose oxidase as well as by glucose dehydrogluc ase may occur. Large amounts of glucose may as well be meta-

bolized by a pathway which probably does not include the formation of G-6-P (394). Moreover, liberation of inorganic phosphate may suggest that the beta cell G-6-pase is mainly responsible for producing inorganic phosphate required for different processes functioning in the beta cells. Whether phosphate concentration itself may be acting as a limiting factor in the beta cell metabolism, as indicated in other cells (395), is not known. Although the present attempt (Appendix II) to localize G-6-pase enzyme in the pancreatic islets at the ultrastructural level was not successful, another investigation on rabbit pancreatic islet demonstrated its activity in the endoplasmic reticulum and nuclear membrane in the islet beta cells (396), and there is a possibility that glucose released during the hydrolysis of G-6-P by this enzyme may be taking part in some way or another in the process of insulin synthesis by an as yet unknown process.

Chapter 5. SUMMARY

The isolated pancreatic islets microdissected from the fresh pancreas from normal and obese-hyperglycemic mice and the principal islets of Cottus scorpius and Tilapia mossambica have been subjected to a quantitative micro-enzymatic method and study of enzymatic activities towards G-6-P and glycerophosphate has been made. Analysis of optimal assay conditions as well as of enzyme properties (PH optimum, substrate specificity, inhibitors, activators and thermal stability) were made on tissue homogenates in comparison to those determined in liver, kidney and exocrine pancreas.

A specific G-6-pase activity as observed in liver and kidney has been demonstrated in the islet tissue of both mammalian and teleost species. It was absent in exocrine pancreas. The comparative distribution of this enzyme in various tissues of these species has been presented. The enzymatic activity of islet tissue of different species fell within a certain range; teleost species showing higher activity than both strains of mice. The islet tissue displayed 10 - 15 times less activity than liver and 6 - 8 times less than kidney.

G-6-pase activity of islets and other tissues of mouse was determined in different functional states of pancreatic islets in relation to insulin secretion. It was found that the enzymatic activity in the islets remained apparently unchanged after fasting for three days, did not decrease significantly after tolbutamide treatment and was markedly reduced after alloxan administration. Moreover, the enzymatic activity of islets of obese mouse was fractionally higher, though insignificantly, and not lower than that of normal mouse islets. Fasting and alloxan treatment elevated the enzyme activity markedly in liver as well as in kidney whereas slight decrease and significant increase in enzymatic activity was found in liver and kidney respectively after tolbutamide treatment.

G-6-pase activity probably plays an important role in the beta cell function. Significance of the findings in the present study in relation to beta cell function particularly to its role as the controlling factor for insulin synthesis/release has been discussed.

Appendix I

MECHANISM OF ACTION OF ALLOXAN STUDIED BY ELECTRON MICROSCOPE-AUTO RADIOGRAPHY

Since the observation of necrosis of pancreatic beta cell (see 346) by alloxan, this compound has widely been used for the induction of experimental diabetes. Though extensively studied, the precise mechanism underlying the destruction of beta cells by alloxan, however, remained obscure (162, 346). Two theories have been considered to understand the diabetogenic action of alloxan. The first explanation is that alloxan may become selectively concentrated in the islet beta cells in excess of other tissues and thus lead to their damage (397). Greater sensitivity of the islet beta cells as compared to other tissue cells was considered the second possibility for islet damage even without selective accumulation, and such a sensitivity might be the result of a specific reaction of alloxan with zinc in the islet (398) or by alloxan inactivation of a sulfhydryl containing enzyme system at the cell membrane (23, 346) or by another as yet unknown mechanism. Experiments failed to produce support for the first theory and also no positive evidence is present even for the second suggestion. It seemed essential, therefore, to demonstrate whether or not alloxan enters the beta cells and selectively accumulates there. For this purpose, an attempt to follow the course of ^{14}C alloxan in the islet tissue was made using electron-microscope-autoradiographic technique. Although these techniques e.g. stripping film or liquid emulsion, for non-water soluble compounds have become more or less well established, but attempts to apply such procedures to water soluble compounds have not been as successful (399). Methods involving liquid fixation, solvent dehydration and embedding, and wet emulsion application have a strong threat of translocation of developed photographic grains. Diffusion and loss of isotope or both, during tissue preparation has been recognised as the major hinderance for localization of water soluble compounds. There have been numerous approaches made in various ~~tissues~~

tissues to solve such problems. These include vacuum drying, freeze substitution, dry stripping film techniques and very recently described dry mounted freeze-dried frozen section technique, but all have severe short comings of one sort or another, and have been briefly outlined (400, 401). In the present attempt freeze-drying and vapour fixation instead of coventional liquid fixation and dehydration steps, were employed during tissue preparation. Preliminary experiments with OsO_4 or formaldehyde fixation in vapour state for 16 hours did not produce good fixation at E/M level, though comparatively better fixation resulted with OsO_4 vapour fixation for 36 hours. Decomposition of alloxan has been shown to decrease markedly by lowering the temperature (+ 2°C) at which the half life of ^{14}C alloxan is over three hours, and half life of alloxan at body temperature is about one minute (see 346). Alloxan 2-^{14}C monohydrate (specific activity 6.2 mc/mM, obtained from Volk Radio-chemical Co., U.S.A.) was used in the present study. Ideally the experiment must be done using tritium labelled alloxan to obtain maximum resolution; however, all of the hydrogen atoms on alloxan are readily exchangeable with water so that a related compound e.g. methyl alloxan will have to be used.

Three to four mouse islets separated quickly from exocrine tissue by microdissection were incubated in a small flask containing one ml of alloxan 2-^{14}C solution (0.1 mc alloxan 2-^{14}C dissolved in 1.0 ml of chilled saline) and kept in the cold water bath (+ 2°C). After 5 minutes the islets were rapidly frozen in isopentane chilled by liquid N_2 and transferred quickly to a small aluminium foil placed in a pre-set freeze-drying apparatus (temp. -40°C; pressure - 0.1 mm.Hg). After 48 hours, the apparatus was brought to room temperature, the vacuum broken by nitrogen-air. OsO_4 crystals were placed inside the chamber of the apparatus and the tissue specimen were left inside for 40 hours. After the fixation time the tissue samples were infiltrated with Araldite as in normal embedding for E/M study, but in vacuo, at room temperature, and finally polymerised for 24 hours at 60°C. Sections (0.5 - 1 μ) were cut on an ultratome

and unfortunately had to be floated on distilled water before picking up on the grids, as attempts to cut and pick a dry section were not successful. The sections after drying quickly in the freeze-drying apparatus were subjected to nuclear emulsion (L 4-10) following strictly Budd's membrane method for E/M autoradiography (402). After appropriate time of exposure (2-6 months), the sections were examined with electron microscope. The results were not satisfactory and it was apparently obvious that either partial diffusion or complete loss had occured during the course of tissue preparation or the resolution of the grains was not achieved at the ultrastructural level. Previous studies as well failed to demonstrate any accumulation of this compound in the pancreatic islets. Concentration of alloxan in islet tissue was not found (397) to be greater than in other tissues e.g. liver or lung, but this failure may possibly be due to dilution of the alloxan compound by exocrine pancreas. Moreover, the islets constitute a small fraction of total weight of whole pancreas, thus the results cannot be attributed to the islets. Overcoming the first difficulty, autoradiographic approach was made in a later study (402) but it was found that alloxan concentration in islets was no greater than in other tissues, although it was higher than in the surrounding exocrine pancreas, and a great susceptibility of the beta cells, rather than the unusual accumulation of the drug, was considered as the factor underlying alloxan's toxic action. These results are not surprising as ^{14}C alloxan is known to have a half life of about one minute at body temperature (see 346), and it is also possible that diffusion of alloxan during tissue preparation might have occured. Furthermore, the fact that high doses of alloxan are so destructive so as to render the beta cells incapable of retaining any alloxan which they may initially have taken up, may explain previous failure to demonstrate alloxan concentration. Recent studies (346) involving determination ^{14}C alloxan distribution in toad fish tissues suggested that alloxan did not penetrate the cell membrane but remain in the extracellular compartment and it was concluded that although

a selectivity of alloxan for beta cell is demonstrated, it is not due to selective concentration of injected alloxan by islet tissue, but due to its early action on the beta cell membrane (346). Their studies lacked the autoradiographic approach. Very recently it has been reported (347) that in fact alloxan is concentrated in the pancreatic islets as shown by autoradiographic studies and this supported the first theory. Their study did not reveal localization of alloxan concentration within the beta cell, the aspect which was aimed for in the present investigation.

Appendix II

ULTRASTRUCTURAL LOCALIZATION OF G-6-PASE ENZYME ACTIVITY IN ISLET TISSUE

Specific G-6-pase enzyme is present in the pancreatic islets as concluded from the present microchemical study. It was thought worthwhile to perform parallel histochemical study at electron microscopic level in order to get some useful information regarding its role in the beta cell function. Its presence in any particular organelle of the cell may indicate its precise involvement in the synthesis or secretion of insulin from the beta cell. The present study has indicated the suggestion that islet G-6-pase is probably identical to hepatic G-6-pase. If so, then the enzyme localization within the cell must correspond to that observed in liver (404). Though localization of a variety of enzymes has been extensively and successfully studied in various tissues but these specific staining reactions have not successfully been extended to include electron microscope histochemistry. However, electron microscopy has demonstrated localization of reaction products of a number of enzymes. The normal procedure involves incubation of thick frozen section or small pieces of tissue sample in the presence of the specific substrate, recommended in the conventional method for light microscopy, following by routine processing for electron microscopy. Problems of penetration of components of the incubation medium into tissue pieces or sections exist and may differ in severity with different fixatives. For instance OsO_4, a fixative of choice for fine structural preservation, leads to a very extensive loss of both ATPase and 5-Nucleotidase (405). Glutaraldehyde solutions have been recommended strongly for studies which require the combination of electron microscopy and histochemistry (406) but it has also been shown to be a powerful inhibitor for a number of enzyme activities (404). Similarly the fixatives better for histochemical study may result in poor preservations at ultrastructural level. As has been pointed out previously, there is such a variation in behaviour of different enzymes towards

different fixatives that no standard technique is useful to demonstrate a variety of enzymes. Although G-6-pase activity is said to be abolished by formaldehyde (374) and glutaraldehyde (375, 406) the enzyme activity has been shown to survive two hours fixation in 5% glutaraldehyde (404).

The localization of G-6-pase activity at ultrastructural level was attempted on the principal islets of Cottus scorpius. The dissected principal islets were either quickly frozen in chilled isopentane for frozen sectioning as described in section II, or were directly fixed in 5% buffered glutaraldehyde (404) for two hours before cutting the frozen sections. Thick frozen sections (20-40 μ) obtained from both specimens were incubated in the reaction mixture as described for this enzyme at light microscope level (407). After 30 minutes of incubation, the sections were rinsed in 7.5% sucrose solution, and were fixed and processed for electron microscopy as described previously (Chap.1, Section II). Examination of the thin sections cut by ultratome, revealed that proper localisation of the enzyme as well as preservation of fine structure did not occur. Severe vacuolization of the cytoplasm and extensive damage to the endoplasmic reticulum was observed in the present study and apparently it is due to incubation, as it is known that even brief period of incubation in the presence of reaction mixture may result in a considerable damage to fine structure (408). It was very difficult to interpret partly localization of this enzyme as the endoplasmic reticulum was severely damaged and hepatic G-6-pase enzyme activity has been observed in the endoplasmic reticulum. Whether partial or complete loss of the enzyme activity has resulted from fixation or not, is not known. It may, however, be pointed out that an attempt to localize the precise sites of enzyme activity of an extremely labile enzyme such as G-6-pase, at the ultrastructural level is at least a compromise between the conditions required for visualization of the reaction product in the staining procedure and those required for electron microscopy.

GENERAL SUMMARY

Cellular composition of the principal islets of two teleost fish (Cottus scorpius and Tilapia mossambica) have been studied using differential histological techniques. Principal islets of Tilapia mossambica have also been examined with an electron microscope, confirming the present findings in contrast to earlier observations, that more than one granular cell is present in the principal islets of this species and also demonstrating a fourth cell type - D cell. The principal islets of teleosts seem to be composed of at least three kinds of granular cells: alpha, beta and D cells analogous to mammalian alpha(a_2), beta and D (and a_1) cells respectively, in addition to 'agranular' cells. In vitro cytological changes in the islet cells of Cottus scorpius have been studied after incubating with alloxan, tolbutamide, growth hormone and hydrocortisone for different periods of time (up to 4 hours). Electrom microscopy of tolbutamide-incubated islets has also been performed and significant changes in the beta cells have been described. The involvement of endoplasmic reticulum in the mechanism of insulin synthesis and the release of secretory granules from the islet cells have been discussed in the light of present in vitro morphological findings.

Isolation of pancreatic islets from exocrine pancreas by free-hand micro-dissection has been accomplished in normal and obese strains of mice. Nucleic acids were quantitatively measured in the isolated pancreatic islets obtained from these animals as well as from both species of teleost fish. The effect of alloxan and tolbutamide on the RNA content of pancreatic islets in Cottus sp. and mice have been studied. The role of nucleic acids in the process of insulin

synthesis/secretion have been assessed and discussed in the light of present findings.

An attempt was made to study the mechanism of action of alloxan by tracing the course of ^{14}C alloxan in the islet beta cells applying electron microscope-autoradiography for water soluble compounds.

Specific glucose-6-phosphatase enzyme has been shown to be present in the pancreatic islets of all the four species studied. Assay properties and quantitative measurement of this enzyme have been carried out by ultramicrochemical procedure in the isolated pancreatic islets of fish as well as mice. The enzymatic activity has also been determined in differing functional states of pancreatic islets in mice (effect of tolbutamide, alloxan and fasting). The specific presence of this enzyme, in contrast to earlier investigations signifies its importance in being involved in glucose metabolism of the pancreatic islets. An attempt was also made to localize this enzyme in the beta cells of mouse pancreatic islets at the ultrastructural level.

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