THE EFFECT OF INSULIN ON THE NITROGEN METABOLISM OF TELEOST FISHES

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SUMMARY

The work presented here has investigated certain aspects of the action of insulin in controlling the overall metabolism of nitrogen in the goldfish (Carassius auratus). Morphological investigations of the Islets of Langerhans have shown them to be closely associated with the ducts of the diffuse exocrine pancreas and thus are present throughout the pleuroperitoneal cavity. The islets are discrete rounded richly vascularized bodies occurring in greater numbers around the cystic duct, common bile duct and the gall bladder.

Two distinct cell types, alpha and beta cells, are morphologically identifiable in all goldfish islets.

Ammonia production by goldfish was increased markedly after the administration of insulin and tolbutamide to the ambient water at 23°C. In addition, the amino-acid nitrogen content of muscle treated with insulin and tolbutamide was elevated, while alloxan lowered the content. The total nitrogen concentration of goldfish muscle was increased after injection of insulin and tolbutamide, but alloxan treatment reduced the total nitrogen content of goldfish muscle.

Leucine-^{14}C- incorporation used as an index of protein metabolism was significantly increased in muscle after insulin and tolbutamide treatment.

The effect of insulin, tolbutamide and alloxan on the total RNA and the total DNA content of goldfish muscle has also been studied.

Insulin and tolbutamide markedly increased the total RNA concentration of goldfish muscle, while alloxan significantly lowered the total RNA content of the same.

An attempt was made to study the secretion of insulin in response to high and low protein ingestion by Rainbow trout. The ingestion of high protein diet resulted in a significant increase in the plasma insulin level and an increase in the mean body weight of the trout. From this it is concluded that insulin plays a definite role in controlling the overall metabolism of nitrogen in goldfish and Rainbow trout.

GENERAL INTRODUCTION

Pathological and clinical studies in man as well as experimental investigations on animals have not yet been able to elucidate the aetiology and pathogenesis of diabetes mellitus. This is one reason why there has been a growing interest during the last few years in using other experimental animals for diabetes research rather than the conventional laboratory mammal. This situation may be improved by investigations on bony fishes (Doerr, 1950; Næe, 1955, 1960).

The bony fishes are of particular value in experimental diabetes research as their endocrine pancreatic tissue is concentrated into one or a few grossly visible islet-like structures in the abdominal cavity - the so-called "principal islet" (Rennie, 1905). This anatomical feature made it possible in the 1920's to show that insulin was produced by islet tissue of the pancreas and not by acinar tissue (Macleod, 1922). Bony fishes offer unequalled possibilities for chemical and metabolic studies on pure islet tissue (Lazarow, Makinen & Cooperstein, 1959; Davidson, 1959). Moreover, it is possible to extirpate the islet tissue selectively and completely without damaging the exocrine pancreatic tissue (McCormick & Macleod, 1925).

While the structures of the invertebrate "insulins" still require elucidation, the available data on their biological and immunological activities indicate that they differ greatly from vertebrate insulins (Falkmer & Wilson, 1967). In fish, an enormous discrepancy has

often been observed between the biological and immunological activity of insulin (Falkmer & Wilson 1967; Falkmer, 1969). Most authors suggest that exogenous insulin has a hypoglycemic action in fish and that excessive doses of insulin lead to hypoglycaemic convulsions and/or death (Seshadri, 1967; Young & Chavin, 1967). Falkmer & Wilson (1967) observed a weak hypoglycemic response even to species-specific insulin in Cottus scorpius. Tashima & Cahill (1964) failed to obtain hypoglycemia in Opsanus tau with a very high dose of bovine insulin. Young & Chavin (1967) on the other hand observed a moderate hyperglycemia after 1000 U/Kg and 5000 U/Kg of bonito insulin in the goldfish, while a dose of 1 U/Kg produced a significant hypoglycemia. Therefore, the duration and degree of hypoglycemia and the onset of hypoglycemic convulsions varies greatly. As regards the unpredictable action of insulin on the blood sugar of fish, it would seem more valuable to investigate the effects of insulin on protein metabolism in fish rather than in the mammal. The rapid fall in blood sugar which follows administration of insulin to animals and man, and the striking action of insulin in relieving the symptoms of spontaneous and experimental diabetes mellitus, naturally and quickly focuses attention on the mechanism of action of insulin in the regulation of carbohydrate metabolism. However, the role of insulin in increasing the rate of protein synthesis in mammals is well established (Sniper, 1968). Yet a review of the literature shows that relatively

few studies have investigated the effect of insulin on the rate of protein synthesis in fish.. However, in Ophicephalus striatus, insulin injections resulted in a reduced muscle free-amino-acids concentration and an increased protein-bound amino-acid fraction (Seshadri, 1959). Tashima & Cahill (1968) found that a protein meal, but not an oral glucose load, significantly increased the circulating insulin levels in toadfish and suggested that, in toadfish, which are essentially protein eating animals, amino-acids and not glucose may serve as the major stimulus for insulin secretion in this species. These workers used glycine 2-^{14}C incorporation as an index of protein synthesis and indicated that insulin accelerated glycine 2-^{14}C incorporation into skeletal muscle protein. They also suggested that one of the prime effects of insulin was the incorporation of amino-acids into toadfish skeletal muscle. Patent & Foa (1971) demonstrated that leucine (1 mg/ml) stimulated the release of insulin from incubated toadfish islets. Recently, Jackim & Laroche (1973) observed that leucine-^{14}C incorporation into Fundulus heteroclitus muscle was increased by insulin.

These present studies were carried out to examine further the effects of insulin, alloxan and tolbutamide on the rate of protein metabolism in goldfish and, in addition, to investigate insulin secretion in response to protein ingestion in Rainbow trout.

LITERATURE REVIEW

REVIEW OF STUDIES ON THE PANCREATIC ISLETS OF FISH

Comparatively few histological and histochemical investigations have been performed on the principal islets of fish. In 1846, Stannius and Brockmann described the Brockmann bodies in teleosts. An internal secretion of the pancreas, however, was not the subject of dispute in those days. About 20 years later, Langerhans discovered the islet bodies in rabbit pancreases (1869), and in the ammocoetes (1873), but considered them as "exocrine pancreas". After Diamare (1895) and Laguesse (1895) had shown that the Brockmann bodies contain the equivalent of the endocrine pancreas of other vertebrates, Massari (1898) demonstrated the existence of two different types of islet cells in teleosts. Again in teleosts, Bowie (1925) was the first to describe a third, granular type of islet cell.

McCormick (1924) and McCormick & Noble (1925) first considered the commercial production of insulin from Brockmann bodies, but in 1929, Jensen and co-workers first achieved a preparation of crystalline fish insulin. It was observed that the removal of Brockmann bodies was followed by a long-lasting hyperglycemia (McCormick & Macleod, 1925; Macleod, 1926; Simpson, 1926). Rennie and Fraser were the first to treat human patients with extracts from fish islets. Teleost insulin was used clinically in Germany (1957) and in Japan (1960).

In recent years, fish insulins have become important in research into the biological and immunological aspects of hormone specificity. Wilson (1966), Falkmer & Wilson (1967), and Yalow & Berson (1964) discussed the use of fish insulin in diabetics with immunological resistance to mammalian preparations.

Contrary to popular belief, the Brockmann bodies do not usually contain only pure islet tissue. Baron (1934) and Bargmann (1939) observed that, in many cases, they consisted of a giant islet which was often separated from a more or less complete rim of exocrine tissue by a connective tissue layer. The connective tissue capsule is not always complete (Bargmann, 1939- Falkmer, 1961, 1966) and exocrine tissue was observed to occur even within the capsule (Murrel & Nace, 1959; Lázarow, 1963; Bencosme et al, 1965).

In accordance with Siwe (1926), Baron (1934) considered the Brockmann bodies to be a direct derivation of the dorsal epithelium of the embryonic gut. To some extent, the Brockmann bodies might correspond to the splenic portion of the avian and reptilian pancreas (Clara, 1924; Miller, 1962) which have also been shown to be rich in islet tissue..

It is possible to free the exocrine components of the Brockmann bodies from other tissue (Falkmer, 1961; Hellman and Larsson, 1961; Lazarow, 1963). However, histological controls are prerequisite

when islet material from a few species is to be collected for biochemical studies, especially since cysts of nematodes (Macleod, 1922; Bergmann, 1939) or lymphatic tissue (Baron 1934) might be mistaken for pancreas nodules. The most rewarding species for the collection of islet material are Cottus scorpius (Falkmer, 1961), Cottus quadricornis (Hellman & Larsson, 1961), Opsanus tau (Humbel & Renold, 1963) and especially Lophius piscatorius (Macleod, 1922; Weitzel et al, 1953; Bauer et al, 1965). The tuna, Thunnus thynnus has two especially large Brockmann bodies with a different beta cell content and a correspondingly different amount of insulin (Planas & Garcia, 1964). Further information on the occurrence of pancreas tissues in teleosts may be obtained from the classic papers of Rennie (1905), McCormick (1925), and Siwe (1926) and from the critical studies of Baron (1934), Broman (1937) and Bergmann (1939).

It is clear from teleost pancreatic morphology that investigations on the effects of "isletectomy" (removal of the Brockmann bodies) must take into consideration the existence of islets at other visceral sites. It may be that a complete removal of islets and of all potentially insulinogenic tissue is only possible in species which have a "compact" pancreas (e g Anguilla vulgaris, Macleod, 1922), but then this would be pancreatectomy.

The histology of the teleost islets appears complex. Bowie (1925) has shown that there are at least three forms of granular cells.

Later studies revealed alpha, beta and delta cells in all species investigated (Falkmer & Hellman, 1961; Kenikar, 1963; Mosca & Solcia, 1964; Falkmer, 1965a, 1966; Epple, 1965, 1967; Honma & Tamur, 1968; Khana & Mehrotra, 1969). Electron microscopic studies have shown the existence of alpha and beta cells in all teleosts so far investigated. Falkmer & Olsson (1962) found five ultrastructurally different cell types in Cottus scorpius. Tiltback (1966) observed alpha, beta and delta cells, while Bencosme et al (1965) found a granules in the delta cells. Watanabe (1960) was able to identify delta granules only using the light microscopy. Like et al (1964) observed alpha,beta and agranular cells and considered the latter elements as progenitors of beta cells. The observation that Tilapia mossambica had only beta cells in its islets (Savadas 1964) could not be proved, (Savadas, 1964; Epple, 1965; Falkmer, 1965a). In some species, e.g. Cottus scorpius (Falkmer et al, 1964a) and Scorpaena scorfa (Mosca & Salcia, 1964) large numbers of agranular (c) cells were found, but were apparently absent in Salvelinus leucomaenis pluvius (Honma & Tamura, 1968). Amphiphils have been described in Tilapia mossambica (Epple, 1965), Salmo-trutta (Epple, 1967) and Salvelinus leucomaenis pluvius (Honma & Tamura, 1968).

Histochemically, the alpha cells show a positive reaction to tryptophan (Falkmer, 1966; Mosca & Solcia, 1964). They also contain glycogen (Bencosme et al, 1965). The beta cells of Opsanus tau

(Lazarow, 1963), Cyprinus carpio (Watanabe, 1960), and Ictalurus nebulosus (Bencosme et al, 1965) also contain glycogen. Further data on the histochemistry of the teleost islets has been provided by Pallot et al (1957) and Schatzle (1958). The islets of the teleosts were rich in zinc (Weitzel et al, 1953; Maske et al, 1956; J. Davidson, 1958). In Cottus scorpius the material was restricted to the central islet region which contained beta and delta cells (Falkmer et al 1964a). In Cottus quadricornis, Pihl (1967) was able to localize heavy metals electron microscopically within beta granules. Various authors have observed intracellular colloids droplets in alpha and/or beta cells. Although these structures were studied very thoroughly by histochemical techniques, their function remained obscure (Ermisch 1967).

Very recently, Chavin and Young (1970) found in the goldfish Carassius auratus that three distinct cell types were present in all the islets with no specific intra-islet localization. The beta cells were fusiform to round in shape. A centrally located nucleus contained chromatin and a small nucleolus. The alpha cells were irregular both in size and shape and were characterized by extremely fine, densely packed cytoplasmic granules. In many cells, a Golgi-like apparatus was present as a clear zone adjacent to the nucleus. The latter was round/oval with numerous minute chromatin granules prominent. The delta cells varied from oval to round in shape, with light green stained cytoplasm.

Hess (1935) showed that the islets of the Rainbow trout, Salmo irideus, were distributed around most of the organs, although the most anterior islet was somewhat larger than most. The pancreas of the Rainbow trout was diffuse, and found lying between the numerous pyloric caeca. In most other morphological respects it resembled the pancreas of higher animals.

Since the pioneer study of Macleod (1922) more than sufficient evidence has been accumulated for insulin to be present within teleost islets. The occurrence of glucagon was mainly concluded from physiological effects of islet extract; immunological evidence for the presence of this hormone was presented by Falkmer (1965a, 1966).

EFFECT OF EXOGENOUS ISLET HORMONE IN FISH INSULIN

(A) BLOOD SUGAR

Most authors have observed that insulin has a hypoglycaemic action in fishes and that excessive doses of insulin lead to hypoglycemic convulsions and/or death. Insulin hypoglycaemia has been observed in cyclostomes (Bentley & Follet, 1965; Falkmer & Matty, 1966a; Plisetskaya, 1967; Leibson & Plisetskaya, 1967) in chondrichthyans (W.C Grant, Jr , 1964; Leibson & Plisetskaya, 1967), and actinopterygians (Gray, 1928; Gray & Hall, 1930; Root, Hall and Gray 1931; Alghaubari, 1958; Falkmer, 1961; Yanni, 1964; Seshadri, 1967; Young & Chavin, 1967; Khana & Mehrotra, 1969). Data on the duration and degree of hypoglycaemia and on the onset of hypoglycaemic convulsions varies greatly. Most investigations have shown a slow response, the peak of hypoglycaemia or maximum intensity of convulsions occurring one or two days after injection. These observations are consonant with the effects of insulin in other poikilotherms (Bern & Nandi, 1964. Seshadri (1967) reported a mammalian-like hypoglycaemia response in Ophicephalus striatus, with a return to normal blood sugar levels within three hours. Falkmer & Wilson (1967) observed a weak hypoglycaemia response even to species-specific insulin in Cottus scopius. Tashima & Cahill (1964) failed to obtain any degree of hypoglycaemia in Opsanus tau with a very high dose of bovine insulin. On the other hand, Young & Chavin (1967) observed a moderate hyperglycaemia after 1000 U/Kg and 5000 U/kg

of bonito insulin in the goldfish, while only 1 U/Kg produced a significant hypoglycaemia.

(B) GLYCOGEN

In teleosts, the effects of insulin on the glycogen content of liver and muscle vary greatly. Root, Hall and Gray (1931) found in Stenotomus chrysops a transient increase in the liver glycogen, followed by hypoglycaemia and an increased glycogen deposition in muscles. Tashima & Cahill (1964) observed no effect on glucose ^{14}C- incorporation into toadfish tissues in vitro. While in Ophicephalus striatus, Seshadri (1967) observed a peak of both liver and muscle glycogen deposition 90 minutes after injection, returning to normal after 3 hours. On the other hand, blood sugar level showed an inverse response. A high dose of insulin caused an increase in both liver and muscle glycogen. In Clarias lazera, Yanni (1964) observed a slight increase in muscle glycogen and a slight decrease in liver glycogen, while insulin in the presence of glucose increased both muscle and liver glycogen. However, a double dose of insulin with glucose had an effect similar to insulin alone, i.e. a marked increase in muscle glycogen and a slight decrease in liver glycogen. Thus insulin might cause either an increase or a decrease of liver glycogen, while muscle glycogen is either unaffected or increased.

EFFECT OF ALLOXAN ON FISH

Ever since the observation that alloxan selectively destroyed the pancreatic beta cells in the rabbit (Dunn, J.S., Sheehan, H.L., and McLetchie, N.G.M. , 1943), this compound has been widely used to induce experimental diabetes or to facilitate the histological identification of the beta cells. In Lampreys, (Petromyzon planeri and Petromyzon fluviatilis), alloxan injection has occasionally been shown to cause beta cell destruction; however, very high doses were required and other organs (liver, kidney and intestine) might also have been damaged (Ermisch, 1966; Winbladh, 1967). Reports on beta cell destruction by alloxan in chondrichthyans are rather contradictory. After intraperitonial injections, Saviano 1946, 1947a) found an increased blood sugar but no islet damage in sharks. Even with high intramuscular doses of alloxan, Kern (1966) was unable to produce any alterations in the blood sugar level or the islet organ of Scyliorhinus canicula, Raja asterias, and Torpedo marmorata. However, he did observe lesions in the exocrine pancreas, the internal organs, adrenal bodies and kidney tubules. Clausen (1953) reported beta cell destruction in two specimens of Scyliorhinus canicula after subcutaneous injection of a relatively low dose of alloxan. The alpha cells of those animals appeared hypertrophied. Many studies have shown the diabetogenic action of alloxan in teleosts (Saviano, 1947b; Lazarow & Berman, 1947; Doerr, 1950; La Grutt, 1950; Grosso, 1950; Mosca, 1959; Clausen, 1953; Schatzle, 1954; Nace, 1955, 1960; Murell & Nace, 1959; Nace et al, 1958, 1959; Falkmer, 1961; Falkmer & Olsson, 1962; Moule & Nace, 1963; Young & Chavin, 1963, 1966; Khana & Mebratra, 1969).

Several investigations described beta cell lesions following alloxan injection, but the degree of these lesions varied greatly. Doerr (1950) concluded that a specific alloxan diabetes could not be produced in Cyprinidae. On the other hand, Falkmer (1961) found hyperglycemia and beta cell destruction for as long as three weeks after alloxan treatment in Cottus scorpius. The morphology of beta cell destruction in teleosts has been well documented in reports by Murrel & Nace (1959) and Falkmer (1961). Several investigators found destruction of other tissues such as liver, kidney, exocrine pancreas (Saviano, 1947b; Doerr, 1950; Grosso, 1950; Mosca, 1959; Schatzle, 1954; Murell & Nace, 1959; Falkmer, 1961; Young & Chavin, 1966). Matty & Qurashi (1967) have shown a decline in the RNA content of the islet of Cottus scorpius after alloxan treatment. La Grutta (1950) suggested that the alloxan diabetes in teleosts was similar to that shown by mammals, despite some differences in the physiology and biochemistry of mammalian and fish insulins.

EFFECT OF TOLBUTAMIDE ON ISLET TISSUE

The prolonged administration of sulfonylurea derivatives have been shown to produce degranulation of the pancreatic beta cells of rabbits (Volk, B.W. and Lazarous, S.S., 1958), rats (Williamson, J.R., Lacy, P.E. and Grisham, 1961) and guinea pigs (Theret, C. and Tamboise, E. 1963). The time of onset of degranulation in different species varied considerably. Histological changes in the pancreas after administration of tolbutamide closely resembled those after glucose stimulation (Lacy, P.E. 1963). Electron microscopic studies demonstrated that the beta cells of rats treated for several weeks with sulfonylurea derivatives showed increased activity reflected by hyperplasia of the Golgi apparatus and enlarged mitochondria (Volk & Lazarous, 1958; Williamson, Lacy & Grisham, 1961). Electron microscope observations in rats following the administration of tolbutamide indicated that the physical mechanism of beta cell secretion was not altered from that shown by normal beta cells and those stimulated by hyperglycaemia (Williamson et al 1961). It was generally agreed that the morphological effects of chromic administration of sulfonylurea derivatives was a reversible process and, despite degranulation, no degenerative changes occurred in the beta cells (Lacy, 1963; Williamson et al, 1961; Volk & Lazarous, 1958; Theret & Tamboise, 1963). It has also been shown by electron microscopic studies that degranulation of the beta cells after sulfonylurea administration was a primary effect of these drugs and not secondary to any hypoglycaemia which might have been induced (Volk & Lazarous, 1958).

THE EFFECT OF INSULIN ON AMINO ACID INCORPORATION INTO PROTEIN

The use of radioactivity labelled amino-acids, and the study of their movement and their incorporation in protein has provided a useful tool for the investigation of the effect of insulin on protein metabolism, and the determination of the mechanism of action of insulin in affecting this process. Forker et al (1951) by their observation that insulin stimulated the incorporation of ^{35}S from injected ^{35}S-methionine into the muscle protein of the eviscerated-diabetic dog, showed that the fall in the blood amino-acid level induced by insulin was more likely to be the result of a greater removal of amino-acid from the blood than of the smaller release of amino-acid by the tissue. In a later paper, Forker & Chiakoff (1952) found that rate of degradation of ^{35}S- labelled serum protein in the dog was not appreciably changed in diabetes. From this finding it was concluded that insulin did not affect the rate of proteolysis. More recently, however, Green & Miller (1960) showed that the rate of protein catabolism in perfused liver from alloxan-diabetic rats was markedly greater than that found in the normal rat liver. Using the isolated rat diaphragm preparation (Krahl, 1952, 1953, and Sinex et al, 1952) showed that insulin in vitro stimulated the incorporation of labelled amino-acids into muscle protein. This observation has been confirmed for a larger number of different amino-acids and their mixtures (Manchester & Young 1958a; Wool & Krahl, 1959a). In the experiments of Sined et al (1952), the labelled amino-acid studied was ^{14}C- alanine. These workers found that the addition to the medium of glucose or pyruvate depressed the incorporation of alanine into protein in the absence of insulin and

subsequently suppressed the effect of insulin on this process. In the experiments of Krahl (1952, 1953) in which the incorporation of glycine ^{14}-C into protein was studied, the addition of glucose to the medium markedly reduced the stimulatory effect of insulin on this process. Ketterer et al,(1957) .in the light of these findings, suggested that "an interference with the action of insulin on carbohydrate uptake might be expected to release the stimulating action of insulin on protein metabolism". Although this view may in fact be correct, the results of Sinex et al (1952) and of Krahl (1952-1953) were probably not relevant to the problem. Manchester & Young (1958a) using uniformly labelled ^{14}-C L-alanine, and DL - L - ^{14}C- alanine obtained results similar to those of Sinex et al (1952) but the reduced incorporation and loss of insulin effect brought about by the addition of glucose of pyruvate to the medium resulted from a substantial endogenous production of unlabelled alanine. When ^{14}C- labelled glucose or ^{14}C- pyruvate was added to the medium, a much greater incorporation of ^{14}C resulted, and under these conditions an effect of insulin on this process was again observed. Like Krahl (1952-1953), Manchester & Young (1958a) found that insulin stimulated the incorporation of ^{14}C- glycine into the diaphragm protein, but unlike Krahl, they observed no effect after the addition of glucose or pyruvate to the medium. In addition, they did not find that the addition of either of these substrates depressed the effect of added insulin. The discrepancy was partly resolved by the observations of Wool & Krahl (1959a, b) that addition of glucose to the medium had no effect on the incorporation of amino-acid into rat diaphragm protein.

Insulin stimulated incorporation of amino-acid into diaphragm protein during incubation in a medium containing no added glucose was brought about under conditions where the effect of insulin on carbohydrate metabolism was minimal. This observation strongly suggested (Manchester & Young, 1959a; Wool & Krahl, 1959a) that insulin could increase the incorporation of amino-acid into the diaphragm protein by a mechanism which did not involve a simultaneous stimulation by insulin of glucose uptake into the tissue /the primary point of action of insulin in stimulating carbohydrate metabolism was almost certainly on the entry of sugar into the muscle cell (Levine et al, 1950; Levine & Goldstein, 1955)/. The effect of insulin can be observed even after a lengthy preincubation of the diaphragm (Wool & Krahl, 1959a) and, during this time, any glucose carried over in the extra cellular fluid will have been used up (Krahl, 1957) and the glycogen content of the tissue will have declined almost to zero.

Results from various sources have shown that insulin stimulated amino-acid incorporation into protein was shown by the perfused rat heart and rat mammary gland slices (Wool & Manchester, 1962) as well as with isolated epididymal fat pad (Krahl, 1959). Insulin was able to stimulate the entry into diaphragm of the non-utilizable amino-acid a-aminoisobutyric acid (Kepnis & Noall, 1958). There is some uncertainty as to whether insulin could stimulate the uptake of utilizable amino-acids as well as of a-aminoisobutyric acid and, if it could, was this a secondary consequence of its ability to stimulate protein synthesis or was the stimulated rate of protein synthesis a secondary

consequence of the increased availability of the amino-acid for protein synthesis?

It was shown that only a few of the amino-acids normally present in protein were taken up to any greater extent by diaphragm muscle when insulin was present (Wool, 1964). It is suggested that the failure to demonstrate insulin stimulated entry of other amino-acids was a consequence of their rapid incorporation into protein. Castles & Wool, (1964) found that, when diaphragms were incubated with puromycin to inhibit protein synthesis, insulin could be shown to stimulate the entry of many of amino-acids into muscle cells. Furthermore, this showed that enhanced protein synthesis was not a requirement for the insulin stimulated entry of amino-acids. These studies were extended by Wool, Castles & Mayer (1965) and the conclusion that insulin stimulated amino-acid uptake and protein synthesis were independent of each other was confirmed. Similar results have been obtained with the perfused rat heart (Sharff & Wool, 1965).

Other work has shown that the stimulation of protein synthesis by insulin was not exerted by a stimulation of amino-acid entry into cells. Wool & Krahl (1959) for example, found that insulin incubated with diaphragm in vitro stimulated the incorporation of previously administered labelled amino-acid into rat diaphragm muscle protein when precursors, such as bicarbonate, were used which would have to enter the cell and be converted to amino-acids before being incorporated into protein. It appears from these studies that insulin acts on

protein synthesis at some point more intimately concerned with the mechanism of protein synthesis than the entry of amino-acid into the cell.

INSULIN AND NUCLEIC ACID METABOLISM

Nucleic acids play a decisive role in the regulation of protein synthesis. The various molecular species of nucleic acid impinge at several stages in the intracellular synthesis of protein. For that reason it was logical to explore the effect of insulin on nucleic acid synthesis, having in mind the possibility that nucleic acid synthesis might be the intracellular locus at which insulin acts to promote protein synthesis. In an early experiment, Wool, I.G (1960) found that insulin did increase the radioactivity in the nucleic acid fraction when diaphragm was incubated with labelled glucose, adenine, but not when the substrate was ^{14}C-thymine. Wool, I.G. (1963), in a second series of experiments found that insulin still increased the incorporation into nucleic acid of radioactivity from ^{14}C-adenine and from ^{14}C-glucose. The effect was solely on the RNA fraction; incorporation into DNA was not affected. Moreover, a new increase in RNA was obtained in diaphragms incubated with insulin. Insulin then enhances RNA synthesis. This was reflected both in an increase in incorporation of ^{14}C from several substrates (adenine and glucose) into the muscle RNA, and by an actual increase in the amount of RNA that can be extracted from insulin stimulated muscle. The action of insulin in increasing RNA synthesis not only parallels the effect of the hormone in stimulating incorporation of amino-acids and amino-acid precursors into muscle protein, but also shares the distinguishing characteristics of the latter. Thus it was independent of the effect of the hormone of glucose transport since it occurs in the

absence ofextra-cellular glucose and addition, even of large amounts of glucose, does not reproduce the insulin effect (Wool, I.G., 1960, 1963). Leslie (1952) has reported that insulin stimulates the synthesis of RNA and DNA by chick heart explants from 13-days old embryos. Leslie, Fulton and Sinclair (1957) found insulin to increase the synthesis of RNA by human skin fibroblasts and kidney cells in tissue culture. Carruthers & Winegard (1962) reported that insulin markedly potentiates the incorporation of radioactivity from ^{14}C-glucose into adipose tissue RNA, and Necheles (1962) has observed a similar effect of the hormone on incorporation of ^{14}C-adenosine into nucleic acid rabbit bone marrow.

The next step was to identify the fraction of RNA, the synthesis of which was increased (Wool and Munro, 1963). Analysis of RNA from insulin treated diaphragms revealed that the hormone had increased the specific activity of RNA of which the sedimentation coefficient was 19S; this fraction was of ribosomal origin. In some experiments insulin seemed to be responsible for the appearance of new peaks of radioactivity. The sedimentation constant of the new material was between 4 and 18S, suggesting that it was messenger RNA.

Realizing that the increase in messenger- and ribonucleoprotein, RNA might have resulted from a stimulation by insulin of the transport of RNA precursors into muscle cell. Wool (1965) sought to determine whether insulin altered the uptake of labelled adenine by diaphragm. The result was negative. Moreover, insulin failed to change the specific activity of the intracellular adenine pool. These facts affirmed the conclusion that insulin exerts a stimulatory effect on RNA synthesis

and led Wool (1965) to propose the theory that RNA synthesis is the molecular site of action of hormone. Accordingly, insulin would co-ordinate protein synthesis by initiating the transcription of messages, either by directly combining with a repressor molecule or by causing the synthesis of a molecule that combines with a repressor. The theory proved to be short-lived. It was tested by examining the effects of actinomycin on the response of diaphragm to insulin(Wool & Moyer, 1964; Eboue-Birnis, Chambout & Volfin, 1963). Despite complete inhibition, by actinomycin, of the labelling of RNA, protein synthesis remained unchanged (Eboue et al 1963) or was decreased, at the most, by 47% (Wool and Moyer, 1964). Actinomycin treatment of the muscle did not prevent the insulin effects on adenine incorporation into nucleic acids or glucose uptake (Wool & Moyer, 1964). It seems, therefore, that the stimulating of RNA synthesis is not the primary action of the hormone. The stimulation of nucleic acid synthesis is thus but one of the many unexplained anabolic effects of the hormone.

THE SYNTHESIS, STORAGE AND RELEASE OF INSULIN BY FISH

Studies on the mechanism of protein biosynthesis require tissues of great viability, synthesizing preferably a protein of known structure. Moreover, the tissue must be available in considerable amounts. These requirements are met by the Brockmann bodies. After Lazarow and co-workers had explained this possibility (Lazarow et al, 1964a), studies on the islet tissue of teleosts yielded important general information on protein synthesis, and particularly the biosynthesis of insulin.

When islet tissue of the Goosefish (Lophius piscatorius) was incubated with radioactive amino-acid, Bauer & Lazarow (1961) found that the specific radioactivity of the insulin fraction of the islet tissue was three times greater than that of other tissue proteins. Likewise, Goosefish islets incorporated radioactivity from glucose-U-^{14}C into many constituent amino-acids of the highly purified insulin fraction (Bauer et al, 1965). Similarly, Hellman & Larsson (1961) observed the incorporation of radioactivity from labelled glucose into amino-acids of Cottus quadricornis islet tissue. In addition, data on the uptake of glucose, carbon and leucine into insulin fractions of toadfish islets, Opsanus tau, were obtained by Humbel and co-workers (Humbel & Renall, 1963). Using labelled isoleucine for in vitro studies on toadfish islets, Humbel (1963) obtained preferential labelling of the A chain of insulin, suggesting the absence of this amino-acid from the B chain.

In extensive in vivo and in vitro studies by Lazarow and co-workers, the relationship between insulin synthesis and insulin storage was investigated in goosefish islets. Their findings suggested the microsomes as primary sites of insulin synthesis in the B cell and a subsequent transfer of the hormone to the secretion granules (Lazarow, 1963, 1965; Lazarow et al, 1964a; Bauer et al, 1965). During these experiments, Bauer et al (1966) suggested that the secretion granules of their preparation contained not only insulin and glucagon but other proteins as well. All insulins characterised consist of two polypeptide chains linked by two disulphide bridges (Eck & Dayhoff, 1966). Insulin can be split into its A and B chains and resynthesized. It has even been possible to prepare Cod and Ox "hybrid" insulin by mutual exhange of the isolated chains (S. Wilson et al, 1962). Two different pathways have been suggested for the final step of insulin biosynthesis. Lazarow and co-workers (Lazarow, 1965 and Humbel, 1965) concluded that insulin is formed from separate chains which are joined together via oxidizing sulfhydryl groups by disulphide bonds. For this process, Lazarow (1965) postulated the existence of a specific enzyme - "insulin zipase". However, recent evidence from studies on the biosynthesis of mammalian insulin suggests that insulin is derived from a large, single chain protein containing intrachain disulphide bonds. Division of this proinsulin into two chains is accomplished by cleavage of peptide bonds with resulting loss of the linking fraction of the original single chain molecule (Steiner & Oyer, 1967; Steiner et al, 1967; Chance & Ellis, 1968; Clarke & Steiner, 1968). While the structuresof invertebrate "insulins" are not fully

understood, the available data on their biological and immunological properties indicate that they differ greatly from vertebrate insulins (Falkmer & Wilson, 1967). In fish, there is often an enormous discrepancy between the biological and immunological properties of their insulin (Wilson, 1966; Falkmer & Wilson, 1967; Falkmer, 1969). As regards their evolution, this is not surprising. Davidson et al (1968a) demonstrated that guinea pig and nutria (coypu), although both are rodents, have insulins structurally very different from each other and from other known mammalian rodent species. In cyclostomes (Falkmer & Matty, 1966a; Falkmer & Wilson, 1967) ox and codfish insulin were much less effective in producing hypoglycemia than crude hagfish preparations. In mouse diaphragm assay, hagfish insulin showed lower potency than teleost insulins (Falkmer & Wilson, 1967). Studies on the structure revealed that N-terminal amino-acids are glycine in the A chain and probably a basic amino-acid in the B chain. The A chain contains no phenylalamine, and the histidine of the B chain shown by other known insulins is replaced by a different amino-acid. The intact molecule, and especially the A chain, is more basic than other known insulins (Weitzel et al, 1967). Macleod (1922) showed that dogfish pancreata contain an acid ethanol extractable substance, capable of causing hypoglycemia in rabbits. Falkmer & Wilson (1967) showed that insulin from the holostean Amia has very low potency in U-point mouse diaphragm assay. However, it was readily neutralized by both ox and codfish anti-insulin sera,

suggesting that it has an antibody-combining site in common with both of them.

The hypoglycaemic action of teleost insulins in mammals is well known (Macormick & Noble, 1925 and Vincent et al, 1925). However, recent investigations (Wilson, 1966; Falkmer & Wilson, 1967) made it clear that there are considerable differences in both biological and immunological properties, not only between mammalian and teleost insulins, but also among teleost insulins themselves. The blood glucose of teleost appears markedly resistant to both teleost and bovine insulin preparations (Falkmer, 1961; Tashima & Cahill, 1964; Falkmer & Wilson, 1967). Also, antisera to ox insulin failed to produce hyperglycaemia in Cottus scorpius. Even homologous insulin of this species was surprisingly ineffective (Falkmer & Wilson, 1967). On the other hand, Young & Chavin (1967) reported a hypoglycaemic effect by low doses of bovine, bonito, tuna, hydrologus and squalus insulins in the goldfish, Carassius auratus.

Insulin from Opsanus tau influenced glucose uptake into rat adipose tissue but not into muscle (Cahill et al, 1964). But mammalian insulin had no effect on the incorporation of glucose U-^{14}C into lipids, fatty acids, glycogen or CO_2 of tissue preparations of heart, liver, and skeletal muscle. These differing biological and immunological properties of insulin result from differences in its molecular structure (Wilson, 1966; Smith, 1966; Eck & Dayhoff, 1966; Grant & Reid, 1968).

CHAPTER I

HISTOLOGICAL INVESTIGATIONS ON THE PANCREATIC ISLETS OF FISH

INTRODUCTION

The islets of Langerhans form an important endocrine part of the pancreas in vertebrates. Although important contributions have been made to the study of mammalian islets, our histophysiological knowledge of this structure in fishes is still limited. Earlier workers (Rennie, 1903, 1905; Bowie, 1925; McCormick, 1924; Thomas, 1940) have provided brief information on the structure and distribution of islet tissue in fishes. More recently, a few investigations have studies the histophysiology of the teleostean islet with the help of modern staining techniques (Nace, 1955; Lazarow, 1957; Mosca, 1957, 1958; Falkmer, 1961; Khana, 1962 and Falkmer & E.V. Gerharl(1964).

The pancreatic islet tissue of most strains of teleosts is concentrated into one or a limited number of grossly visible structures in the abdominal cavity, the so-called "principal islet" (Rennie, 1905). In some strains of teleosts, e g Cottus scorpius and Lophius piscatorius (angler fish, goose fish) the principal islets are exclusively composed of endocrine parenchyma. They provide a favourable object for experimental diabetes research (Falkmer, 1961).

There is evidence for the presence of alpha and beta cells in these principal islets. Falkmer (1961) has shown that in the two principal islets of Cottus scorpius, there is a dark central region containing cells analogous to the mammalian beta cell and alpha cell.

A light peripheral region has been shown to contain both granular cells, which apparently correspond to mammalian alpha cells, and an agranular unclassified cell type. These agranular cells are considered to be young, immature forms of the granular parenchymal cells of the islet tissue (Falkmer, 1962b). In the principal islet of the Bullhead fish (Amierus nebulosus), Bencosme, S.A., Meyer, J., and Bergman, B.J. (1962) found a morphological difference between the secretory granules of the alpha and beta cells by using alternate light and electron microscope sections of the islet. While both types of granules had a dense core and a light peripheral zone with a surrounding membrane, the cores of the alpha granules were smaller and homogenous. The cores of the beta granules were larger and irregular. Falkmer & Olsson (1962) concluded that the structure of the principal islet cells in Cottus scorpius resembled those found in mammals. The central feature of the islets, which contain granular cells with fine structure resembling that of mammalian beta cells, in that they become severely damaged after alloxan treatment.

As regards the teleostean principal islet in general, there is some controversy regarding the cell types which occur in islets with purely endocrine tissue. There is a general agreement of the occurrence of a central region with dark cells and a peripheral region with mainly light cells. The disagreement concerns the identification of the various cell types. Most authors maintain that the insulin producing beta cells are found in the central region (Falkmer, 1961), but some claim that the beta cells are in the light peripheral region (Saviano, 1947a; Weitzel et al, 1953).

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 been proposed (Bowie, 1925; Clausen, D.M., 1953).

Recently Chavin & Young (1970) demonstrated that goldfish islets contained alpha and beta cells, together with a third kind - the delta cells, like most other teleosts (Bowie, 1925; Grosso, 1950; Clausen, 1953; Algaubari, 1958; Falkmer, 1961; Kenikar, 1963; Pasha, 1964 and Bencosme et al, 1965). It has been suggested that teleostean delta cells might represent degranulated alpha cells (Mosca, 1957; Planas & Garcia, 1964) or beta cells (Pallot & Schatzle, 1953; Savadas, 1964), but the differences in staining reaction, size and nuclear morphology of the goldfish delta cells tend to preclude these suggestions.

Hess (1935) only gave a rough picture of the trout pancreas (Salmo irideus), but, more recently, O.H. Robertson; Marcus A. Krupp; Sydney F. Thomas, Cotting, B; Favour, Satoshi Hane and B.C. Wexler (1961) reported alpha and beta cells in the trout islet (Salmo gairdneri). Also, Epple (1967) demonstrated alpha, beta and D cells in the trout islet (Salmo trutta).

However, the present study describes the normal morphology of the goldfish (Carassius auratus) and Rainbow trout (Salmo gairdneri) pancreas.

MATERIALS AND METHODS

ANIMALS

FISH - (Plates 1, 2)

The fish used were goldfish (Carassius auratus) about 50-60 g in weight (5-6 in. length), obtained from WOLVERHAMPTON AQUATICS, WOLVERHAMPTON, STAFFS. Trout (Salmo gairdneri) were obtained from BIBURY TROUT FARM, GLOUCESTERSHIRE. They were about 250 - 300 g in weight and 10 -11 in length.

The goldfish (Carassius auratus) is a fresh water teleost. Experimental fish were kept at 25°C in a constant temperature room, with constant illumination and aeration. Every two days the tanks were thoroughly washed out with clean tap water at the appropriate temperature to remove all excreta and uneaten food. Fish were fed daily with commercial baby fish food (TETRA WERKE MELLE, WESTERN GERMANY) and also with beta floating trout pellets (COOPER NUTRITION PRODUCTS LTD., WITHAM, ESSEX).

The Rainbow trout (Salmo gairdneri) were kept at 10°- 12°C in large tanks with continuously running well-aerated water. Sometimes carbon filters were incorporated into the inflow system of the water supply to check the variations in chlorine content of the tap water. Commercially available beta floating trout pellets were given daily to the animals. (COOPER NUTRITION PRODUCTS LTD., WITHAM, ESSEX) Every four or five days the tanks were thoroughly cleaned to remove all excreta and uneaten food.

PLATE 1

PLATE 2

Prior to experimental work, fish were brought to the laboratory and maintained in experimental aquaria containing aerated tap water.

PREPARATION OF TISSUE SECTIONS FOR LIGHT MICROSCOPY

PREPARATION OF PARAFFIN SECTIONS

Fish were killed by a blow on the head, and the cystic duct, common bile duct and the base of the gall-bladder were removed, because the larger islets were observed around these organs.

FIXATION

Tissues were fixed in Bouin's fluid for about 24 hours.

DEHYDRATION

Routine dehydration was facilitated by bringing the tissue through a succession of alcohols of increasing strength, usually ranging from 70% to absolute, until all the water has been removed from the specimen. The tissue remained in each strength of alcohol as mentioned below:-

70% alcohol	2 washes
70% alcohol	1 change for 6 - 12 hours each
90% alcohol	2 changes for 6 - 12 hours or overnight
Absolute alcohol	2 changes for 6 - 12 hours each.

CLEARING

Clearing is not the making of the tissue transparent as the title implies, although this may occur in some instances, but is the replacement of the dehydrating fluid with a substance that is miscible with the embedding medium to be employed. Xylene was the clearing reagent used, with two changes for 1 - 2 hours each.

EMBEDDING

Paraffin wax - 2 changes for 1 - 2 hours each.

The tissue pieces were then placed in a third paraffin bath in the oven for 2 - 4 hours before the final block was made. Paraffin wax, melting point 56^{o}C was used and was filtered prior to use to make it free from grit, dust, water droplets and other foreign matter. On certain occassions, overnight impregnation was carried out and it appeared not to injure the tissue.

SECTIONING

Serial sections were prepared to determine the position of the islet of Langerhans. 4 - 6$\mu$of the tissues were cut using a Cambridge rocking microtome (CAMBRIDGE INSTRUMENT CO. LTD., CAMBRIDGE).

STAINING

Sections were dewaxed with Xylene and hydrated in alcohol of decreasing strength, usually ranging from absolute to 70%, and then in water. This was the exact reverse of the procedure that was used to impregnate the tissue. Hematoxylin and eosin was used as routine stain.

After staining, sections were dehydrated, cleaned and finally mounted.

PHOTOMICROGRAPHY

Islets for photography were selected from different sections. Photographs of trout and goldfish islets were taken on a Zeiss Mk. I Photomicroscope (CARL ZEISS, DEGENHARDT, AND CO. LTD., LONDON) using EKTACHROME EHB 135-20, (KODAK , LONDON) bulb temperature 3200°C.

OBSERVATIONS AND RESULTS

Normal Pancreatic Morphology of the Goldfish (Carassius auratus)

The exocrine pancreas of goldfish (Carassius auratus) was diffuse with the cells arranged in bands. The greater part of these were found in the mesentric area around the anterior part of the intestine and spleen. Often they extended posteriorly into the abdominal cavity. Interlobular septa and interlobular ducts were clearly seen. (Plate 3).

The islets were closely associated with the pancreatic ducts of the diffuse exocrine pancreas and thus were present throughout the pleuroperitoneal cavity. The islets were rounded bodies, varying insize. However, a number of larger islets occurred around the cystic duct, common bile duct and gall-bladder and may be considered as Brockmann bodies. (Plate 4 &5).The islets were surrounded by a thin fibrous connective tissue capsule. Also connective tissue trabeculae were frequently present, especially in the larger islets. The islets were

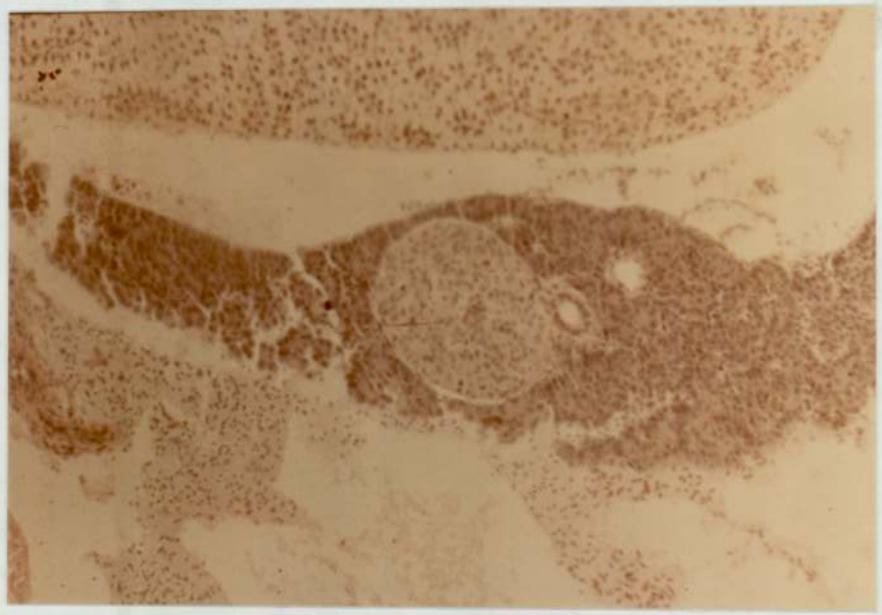


PLATE 3 x50

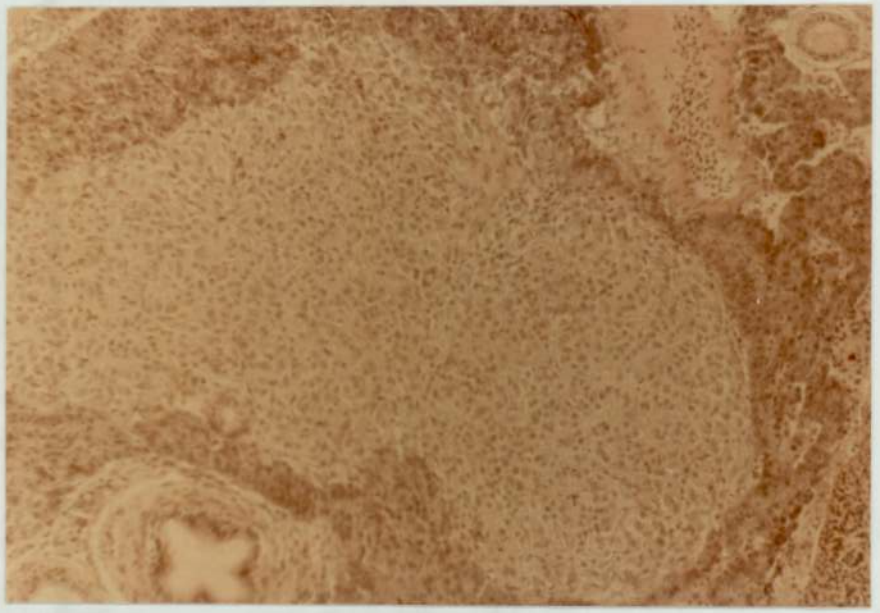


PLATE 4 x50

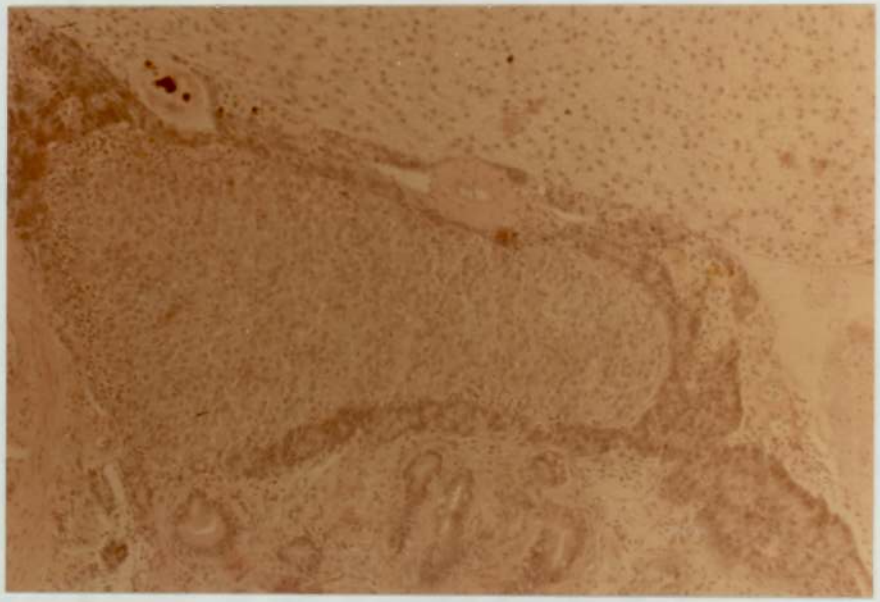


PLATE 5 x50

richly vascularized.

Two types of cells were found in the islets designated as alpha and beta cells. Many of the beta cells were longer than broad and somewhat triangular in shape. A large circular nucleus occupied the centre of the cell. Most of them were singly placed but sometimes more than one were visible together. (Plate 6).

The alpha cells were fairly large in size and were evenly distributed within the islets. More than two alpha cells were often found together so that the cell outline was indistinct. (Plate 6).

Morphology of the Pancreas of Salmo gairdneri

The exocrine pancreas of rainbow trout was again diffuse in type, like other teleosts. Although the pancreas was situated between the numerous pyloric caeca, in structure it resembled the pancreas of higher animals. The pancreas was found throughout the entire extent of the body cavity. The exocrine cells were arranged in bands. The greater part of the pancreas was found in the mesentric area near the anterior part of the intestine and liver. It often extended posteriorly to the abdominal cavity. Interlobular septa were observed together with interlobular ducts.

The endocrine pancreas of the rainbow trout proved amenable for study of the islets. Unlike the Cottus scorpia (Falkmer, 1961) which had two principal islets, the islets of the rainbow trout were

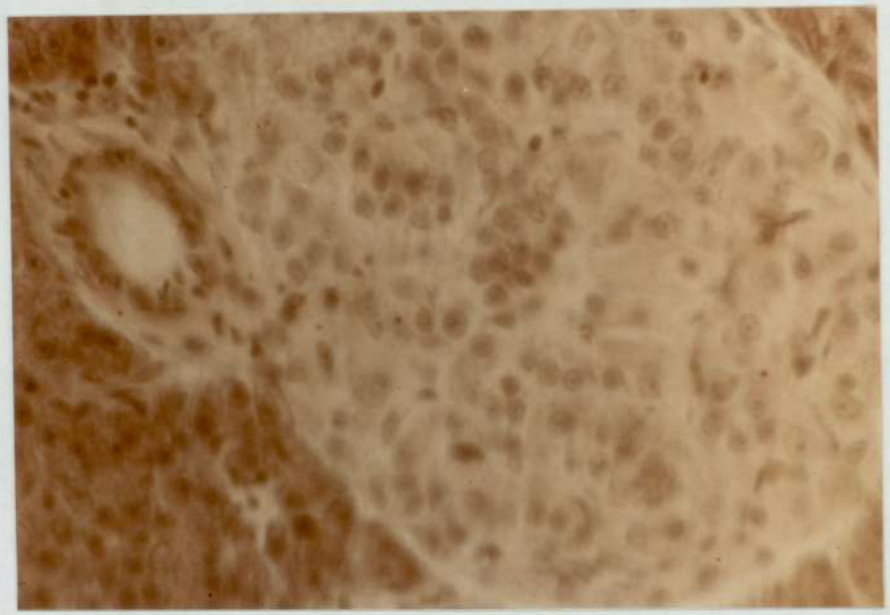


PLATE 6 x120

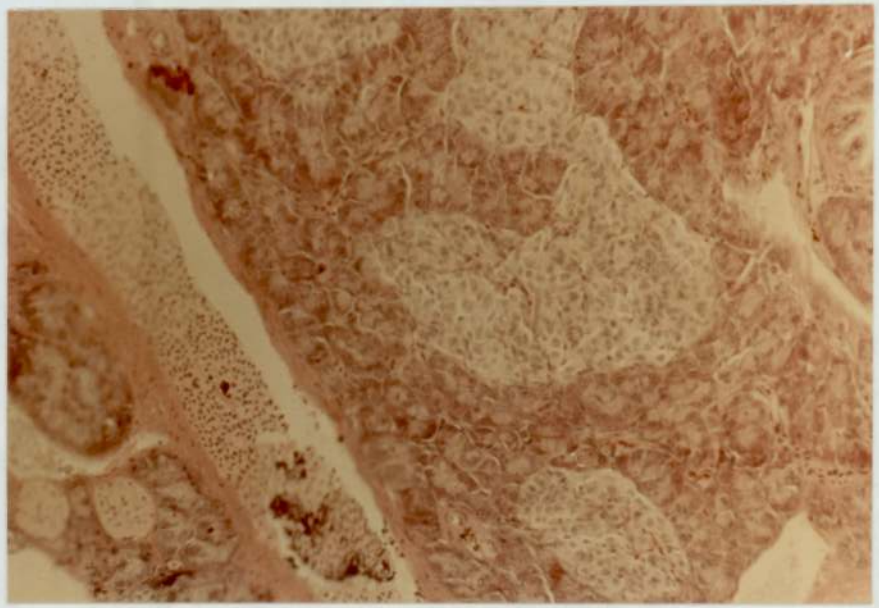


PLATE 7 x50

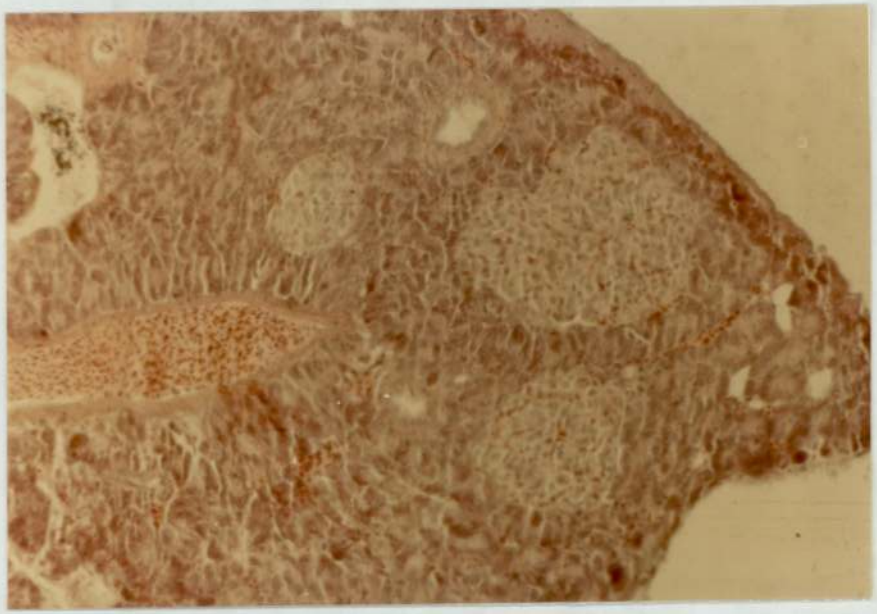


PLATE 7 x50

found throughout the entire extent of the organs, although the most anterior islets were somewhat larger than the others. The islets were rounded or oval in shape and surrounded by a delicate fibrous connective tissue capsule. Occasionally, a partial encirclement by the exocrine pancreatic cells was noted. Very delicate supporting connective tissue was visible in some sections. No large blood vessels were seen to penetrate the islets, although an abundant blood supply was received via arterioles from the adjacent larger blood vessels. Large conspicuous capillaries were abundant, but were not equally distributed throughout the organ.

In routine Hematoxylin-eosin preparations, the pancreatic acinar cells stained very deeply. The spherical nucleus with a centrally placed nucleolus was situated at the base of the cell. The islet cells were smaller and stained more lightly than exocrine pancreatic cells. (Plate 7).

Rainbow trout islets contain both alpha and beta cells. The beta cells made up almost exclusively the median border of the islet, but were not restricted to this area. A few were found scattered in other regions. The alpha cells were lighter-stained elements. These cells were fairly large and evenly distributed in the islets. (Plate 8).

DISCUSSION

The existence of "principal" islets in some species of fishes has

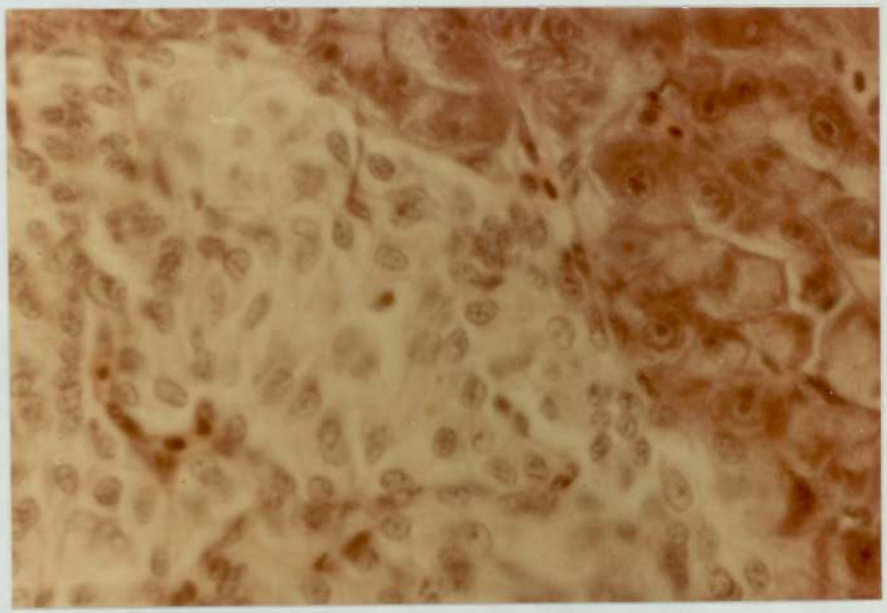


PLATE 8 X120

been reported by Rennie (1903, 1905), McCarmick (1924), Mosca (1958) and Falkmer (1961). In goldfish (Carassius auratus) and in rainbow trout (Salmo gairdneri) however, the pancreas was in the form of a more or less compact structure and containing a large number of islets of different sizes scattered in it. There appeared to be no "principal" islet independent of the exocrine tissue in these fish. These observations were similar to those reported previously by Chavin & Yount (1970) in Carassius auratus and by Hess (1935), and Epple (1967) in Salmo irideus and Salmo trutta respectively. Histologically, cell types comparable with those found in mammals have been identified in some teleosts (Bowie, 1924; Lepori, 1952; Lazarow, 1957; Mosca, 1955, 1958; Leiner & Schmidt, 1957; Falkmer, 1961 and Khana, 1962). Fish islets were generally believed to consist of two kinds of cells, the darker one in the centre of the islet, and the lighter one at the periphery. Mosca (1955, 1957) considered the lighter cells to be alpha cells and the darker ones as beta cells. It was suggested that the reverse might be true (Savainno, 1947). Three types of cells have been reported by Bowie (1925), Falkmer (1961), Falkmer & Hellman (1961), Falkmer & Olsson (1962) and Khana (1962). Leiner & Schmidt (1957), on the other hand, have reported the existence of four types of cells in protopterous. Chavin & Young (1970) found that the islets of Carassius auratus contained alpha, beta and delta cells, but the present study has only shown the presence of alpha and beta cells in Carassius auratus. The beta cells

probably secrete insulin, as suggested by the presence of aldehyde-fuschinophilic granules in them (Lazarow, 1957; Falkmer, 1961). The beta cells were reported to be present in the centre of the islet while the alpha cells were towards the periphery (Lazarow, 1957; Mosca, 1955, 1957; Falkmer, 1961). But the present study has shown that in Carassius auratus, however, alpha and beta cells were more or less uniformly distributed in the islets.

Epple (1967) found three types of cells - alpha, beta and delta in Salmo trutta, but the present study has shown that only alpha and beta cells were discernible under the light microscope with haematoxylin-eosin stain, similar to those reported previously by Robertson et al (1961).

Using the silver impregnation procedure in Cottus scorpius, Falkmer & Hellman (1961) have shown that the argyrophilic A_1 cells were present in the central region and the non-argyrophilic A_2 cells towards the periphery of the islet. Hellerstrom, C., B Hellman, B Petterson and G. Alm (1964) reported that cobalt chloride treatment damaged A_2 cells only, while the A_1 cells remained intact. On the other hand, Mosca (1957) was of the opinion that the islets of Scorpaena scorfa were only composed of alpha and beta cells; the third type (gamma or D cells) were only a functional stage in the differentiation of one of the two cell types. In Carassius auratus and Salmo gairdneri the two types of cells were dispersed throughout the islets. Falkmer et al (1964) have reported the presence of a few agranular cells along the periphery of the principal islet of Cottus scorpius. Such agranular cells could not be observed in the goldfish and rainbow trout.

SUMMARY

In the goldfish (Carassius auratus) and in the rainbow trout (Salmo gairdneri), the pancreas is a more or less compact structure, containing a large number of islets of different sizes dispersed throughout. There appeared to be no "principal" islet independent of the exocrine tissue either in the goldfish or in the rainbow trout.

The numerous islets of Langerhans were discrete, encapsulated nodules located in the pleuroperitoneal cavity. The larger islets occurred around the cystic and hepatic ducts and might be considered as Brockmann bodies.

The islet contained alpha and beta cells, uniformly distributed throughout the islet. No "D" cell or agranular cell types appeared to be present.

CHAPTER II

THE EFFECT OF INSULIN ON AMMONIA PRODUCTION BY THE GOLDFISH (CARASSIUS AURATUS)

INTRODUCTION

No systematic examination has been made of the pathways of nitrogen metabolism in fish. The meagre literature available suggests that they are similar to those occurring in higher vertebrates. What makes fish unique and of particular interest from a comparative standpoint is the wide variety of nitrogenous end-products found in their species and their adaptive significance with respect to varying degrees of water availability and embryonic habitat.

Ammonia is the chief nitrogenous end-product in aquatic animals (Delaunay, 1931). This generalization extends from the simplest protozoa to the highest vertebrates. The formation and elimination of ammonia as a nitrogenous end-product has many biological advantages. There is no expenditure of energy involved in a conversion of the nitrogen in proteins to ammonia. In fact, many of the reactions, such as deamination of glutamate, ultimately lead to the production and capture of free energy. The small size and highly lipid soluble nature of the free base provides for ease of elimination of the compound without the obligatory loss of water from the aquatic organism. A further advantage is the ability of NH_4^+ to exchange with Na^+ across the gills (Maetz & Romeu, 1964) in goldfish (Carassius auratus) and in the fresh water eel (Anguilla anguilla) (Romeu & Motais, 1966). Mechanisms of excretion of nitrogenous compounds in various fish range from simple diffusion to active transport. The chief route of nitrogen excretion is via the gills (Smith, 1929; Wood, 1958;

Fromm, 1963). Approximately 80% of the nitrogen eliminated is as ammonia. Smith (1929) originally proposed that this ammonia was derived from that in the blood which diffused across the gills. Smith later (1930) suggested the peripheral site of formation to be the gill rather than a central site such as the liver and kidney, as in most vertebrates. It has recently been shown, however that excreted ammonia is derived mainly from that circulating through the gills and delivered there as preformed ammonia (Goldstein, Foster and Fanelli, 1964). The mechanism of passage across the gills is not certain. The simplest hypothesis is that the free-base diffuses down a concentration gradient from blood to water. However, Maetz & Fomeu (1964) have shown that NH_4^+ in the blood of goldfish can be excreted in exchange for Na^+ in the water. Thus there may be two mechanisms in existence for the excretion of ammonia - simple diffusion as well as an exchange component at the gills. The quantity of ammonia excreted in the urine of fishes is relatively small compared to gill excretion (Smith, 1929) even though the urine of these species may be highly acidic.

The physiological role of the teleostean pancreas is not completely understood. The purpose of the present study, therefore, was to determine the effects of insulin on the production of ammonia by fish in their ambient water. To evaluate its role, the effect of alloxan and tolbutamide were also evaluated on the production of ammonia by goldfish. Alloxan studies have aided in the clarification of mammalian islet function by presumably destroying beta cells and thereby producing

a persistent hyperglycaemia (Lazarus & Volk, 1962). The actions of alloxan upon teleostean islet tissue are variable. No apparent effects are produced in some species (Doerr, 1950; Pallot & Schatzle, 1953), while beta cell degeneration may occur in other species (Lazarow & Berman, 1948a; Grosso, 1950; Nace et al, 1958; Murrell & Nace, 1959; Falkmer, 1961). Alloxan, in addition, may induce necrotic changes in the liver (Lazarow & Berman, 1947; Doerr, 1950; Grosso, 1950; Falkmer, 1961) and kidney (Grosso, 1950; Falkmer, 1961). Alloxan has been shown to produce hyperglycaemia in most teleosts, but the time, onset, duration and severity of the hyperglycaemic reaction was variable (Saviano, 1947a; Lazarow & Berman, 1947, 1948a; Murrell & Nace, 1959; Nace, 1955; Falkmer, 1961). Recently, Chavin & Young (1970) observed the destruction of beta cells in goldfish (Carassius auratus) after alloxan administration.

Lacy, (1963), Williamson et al (1961), using the electron microscope, have described the sequence of events occurring during the release of insulin secretory granules from the pancreatic beta cells following stimulation by tolbutamide. The process, termed emiocytosis, is thought to be the margination of beta granules, followed by fusion of the sac enclosing the granule with the plasma membrane of the beta cell, resulting in the rupturing of the sac and the release of the granule contents directly into the extra-cellular space (Williamson et al, 1961; Lacy, 1963). The cytoplasm between the two granules liberated close to each other appears as a "bulge" at the surface of the cell, forming a microvillus (Williamson et al, 1961; Lacy, 1963).

It is generally accepted that sulfonylurea drugs exert their pancreatic stimulatory action by enhancing the islet beta cell secretion of insulin (Levine, Mahler, 1964).

MATERIALS AND METHODS

Goldfish (Carassius auratus) about 10 - 15 g in weight (3 - 4 in. length) were obtained from WOLVERHAMPTON AQUATICS, WOLVERHAMPTON, STAFFS.

For determination of the ammonia concentration in the ambient water, fish were maintained at 25°C in a constant temperature room, with constant illumination and aeration. Every two days the tanks were thoroughly washed out with clean tap water at the appropriate temperature. Fish were fed daily with commercial baby fish food (TETRA WERKE MELLE, WESTERN GERMANY).

Prior to experimenta work, fish were brought to the laboratory and maintained in experimental aquaria at approximately 23°C. Each individual goldfish was kept in 5 litres of water in a glass aquarium. Each day, at the same time, a sample of water was removed for analysis. After about 50 min. the aquarium was thoroughly washed out with clean tap water at the appropriate temperature, so as to remove all the excreta. One hour after taking the sample, washing was stopped and the volume adjusted to 5 litres. Thus, samples were the product of 23 hours of metabolism by the fish.

After about 7 days, during which time the normal resting level of ammonia production was established, groups of fish were treated in separate experiments with insulin, alloxan and tolbutamide. The dose of insulin used was 0.16 1.U/fish, injected intramuscularly (INSULIN NOVO ACTRAPID, NOVO INDUSTRIALS; COPENHAGEN, DENMARK).

Alloxan monohydrate (SIGMA CHEMICAL COMPANY) was dissolved 0 9% saline, prepared rapidly and injected within 2 minutes to avoid deterioration. The administrated dose was 600 mg/kg at the volume of 0.3 ml.

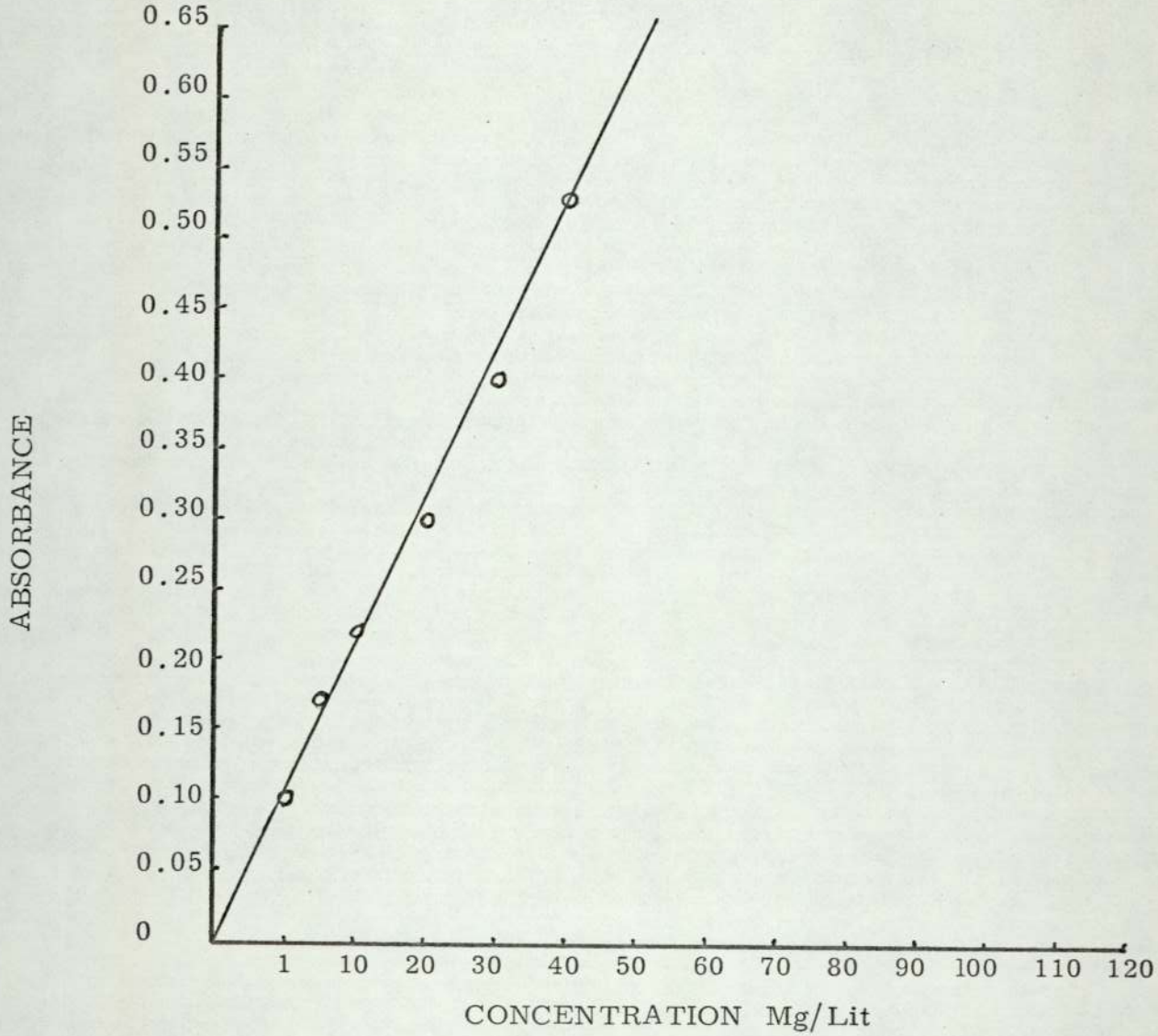
The dose of the tolbutamide (as the sodium salt) was 10 mg/Kg prepared in double distilled water.

Control fish were injected with 0.9% saline.

ANALYTICAL TECHNIQUES

The ammonia concentration in the ambient water was determined using Nessler's reagent. 1 ml of a freshly-prepared mixture of equal volumes of Nessler's reagent and 50% sodium potassium tartrate was added to a 25 ml sample, and the absorbance measured on BECKMAN D.B. SPECTROPHOTOMETER (BECKMAN INSTRUMENTS LTD.) at 500 nm. To the tartrate had previously been added 1% of Nessler's reagent, and after standing for a few days, the clear solution was decanted off and used. Standard solutions of ammonium chloride were treated in exactly the same manner as described above for the

FIG. 1 STANDARD CURVE OF AMMONIUM CHLORIDE



samples and a standard curve was prepared by diluting the stock ammonium chloride solution as shown in Fig. 1.

Total nitrogen in the ambient water was determined by the micro-Kjeldahl method. 10 ml of sample was heated in a pyrex boiling tube with 1 ml of concentrated sulphuric acid, potassium sulphate and a crystal of copper sulphate until clear. The ammonia was released with 50% sodium hydroxide and aerated into approximately N/50 sulphuric acid, and estimated with Nessler's reagent.

OBSERVATIONS AND RESULTS

Ammonia Excretion after Insulin Administration

The administration of insulin raised the ammonia production of goldfish at 23°C from a mean resting level of 11.5 ± 1.3 mg/100 g fish/24 hours to a mean level of 18.6 ± 1.8 mg/100 g fish/24 hours (Fig. 2). On the second day the ammonia production was increased with a peak at the value of 24.6 ± 1.3 mg/100 g fish/24 hours two days after insulin administration. The ammonia production fell after that, and a resting level of 15.5 ± 1.3 mg/100 g fish/24 hours was recorded on the third day. But the normal mean resting level of 11.5 ± 1.3 mg/100 g fish/24 hours was not regained until 6 days later, when the experiment was terminated, though it appeared to be levelling off at about 12.1 ± 1.3 mg/100 g fish/24 hours.

Total Nitrogen Excretion in the Goldfish after Insulin Administration

The result appears in Fig. 3.

The effect of insulin administration on the total nitrogen excretion

FIG. 2 EFFECT OF INSULIN (0.016 I.U/100 G) ON AMMONIA EXCRETION OF GOLDFISH

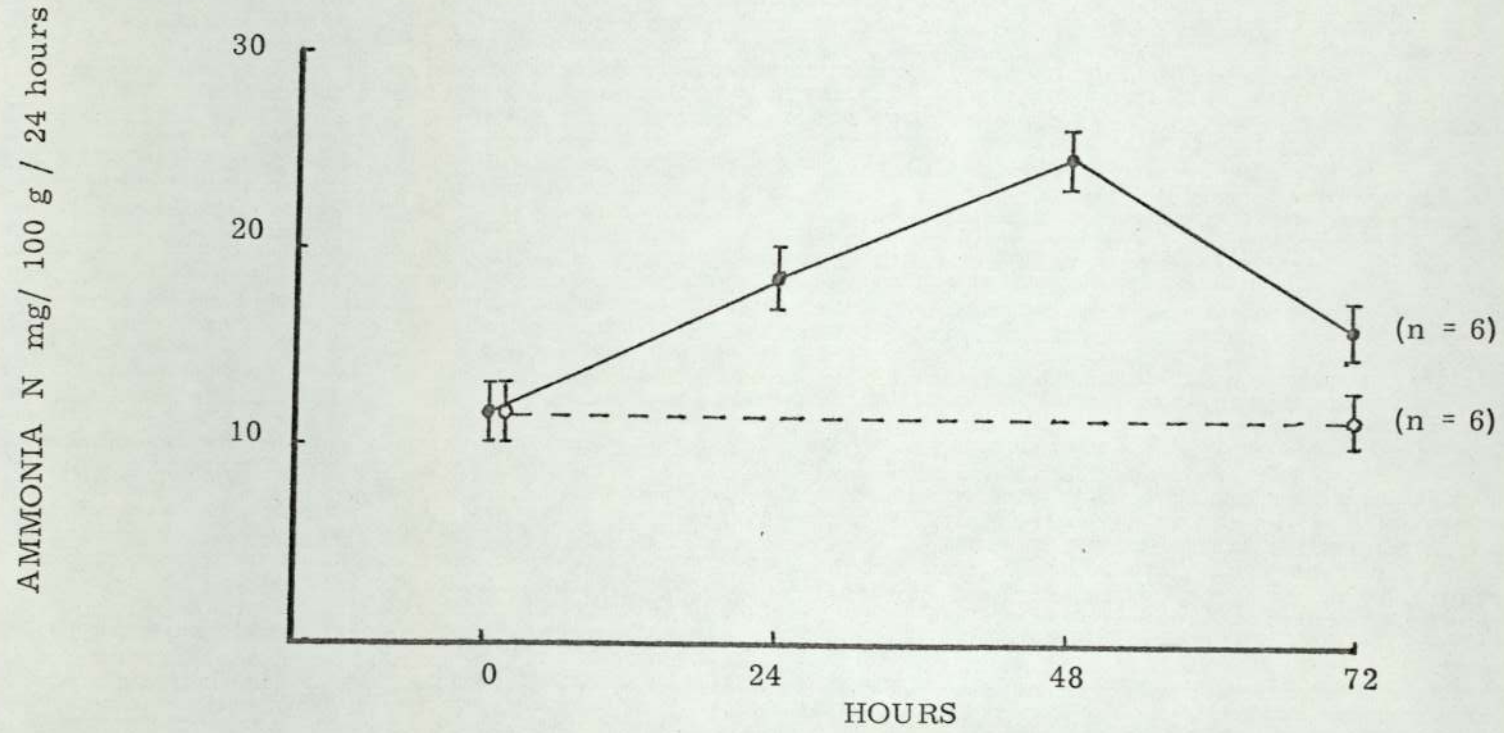
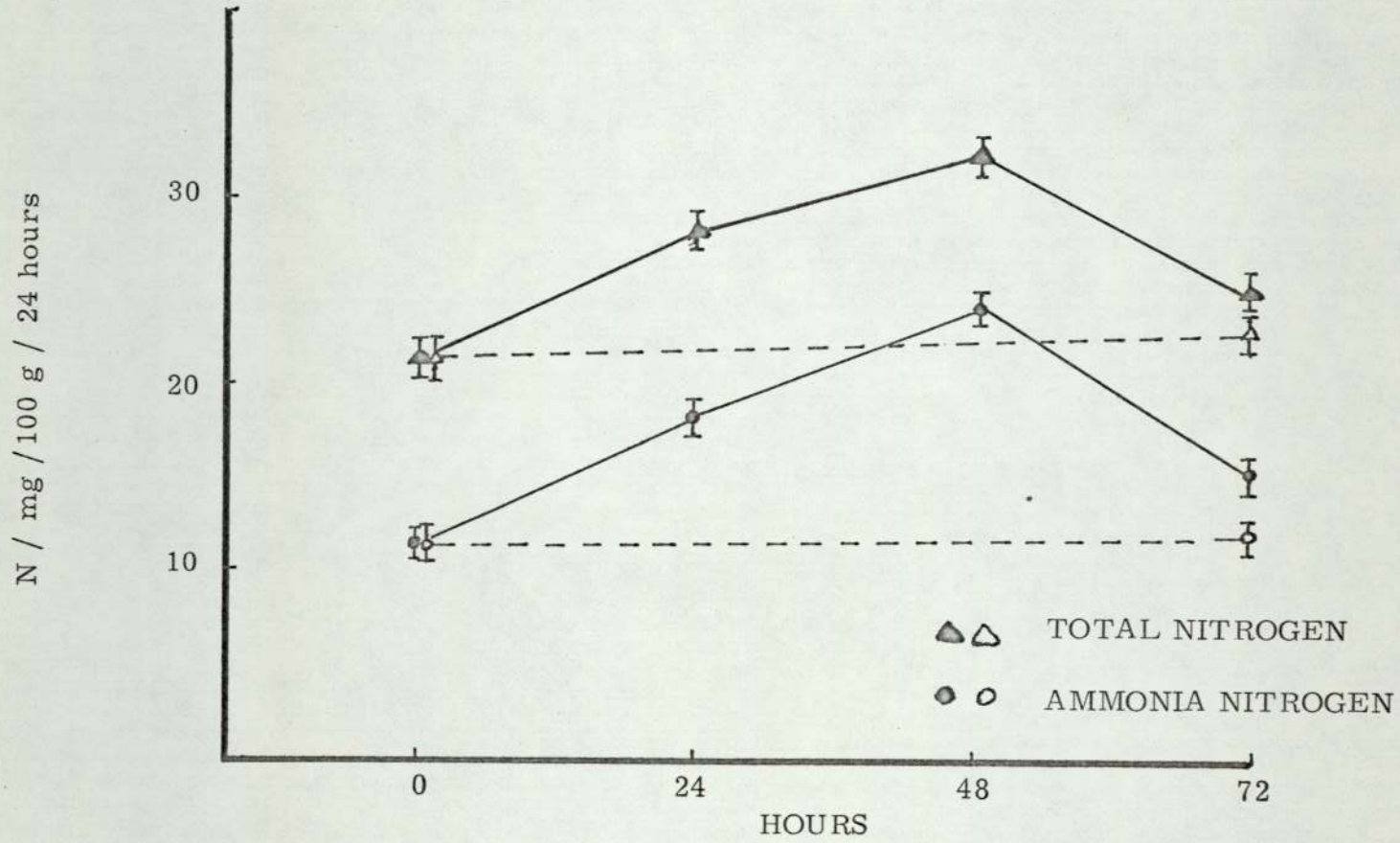


FIG. 3 EFFECT OF INSULIN (0.016 I.U/100 G) ON NITROGEN EXCRETION OF GOLDFISH



by fish was most marked. From an average resting level of 21.5 ± 1.3 mg/100 g fish/24 hours, the total nitrogen production was increased to a mean level of 28.1 ± 1.2 mg/100 g fish/24 hours, with a peak at 32.5 ± 1.7 mg/100 g fish/24 hours 48 hours after administration. On the third day it fell, and a resting level of 25.0 ± 0.8 mg/100 g fish/24 hours was found. But the normal average level of 21.5 ± 1.3 mg/100 g fish/24 hours was not reached for a further 6 days, when the experiment was terminated, though it appeared to be levelling off at 23.0 ± 0.6 mg/100 g fish/24 hours.

The total nitrogen content of ambient water followed the same pattern as the ammonia concentration of ambient water.

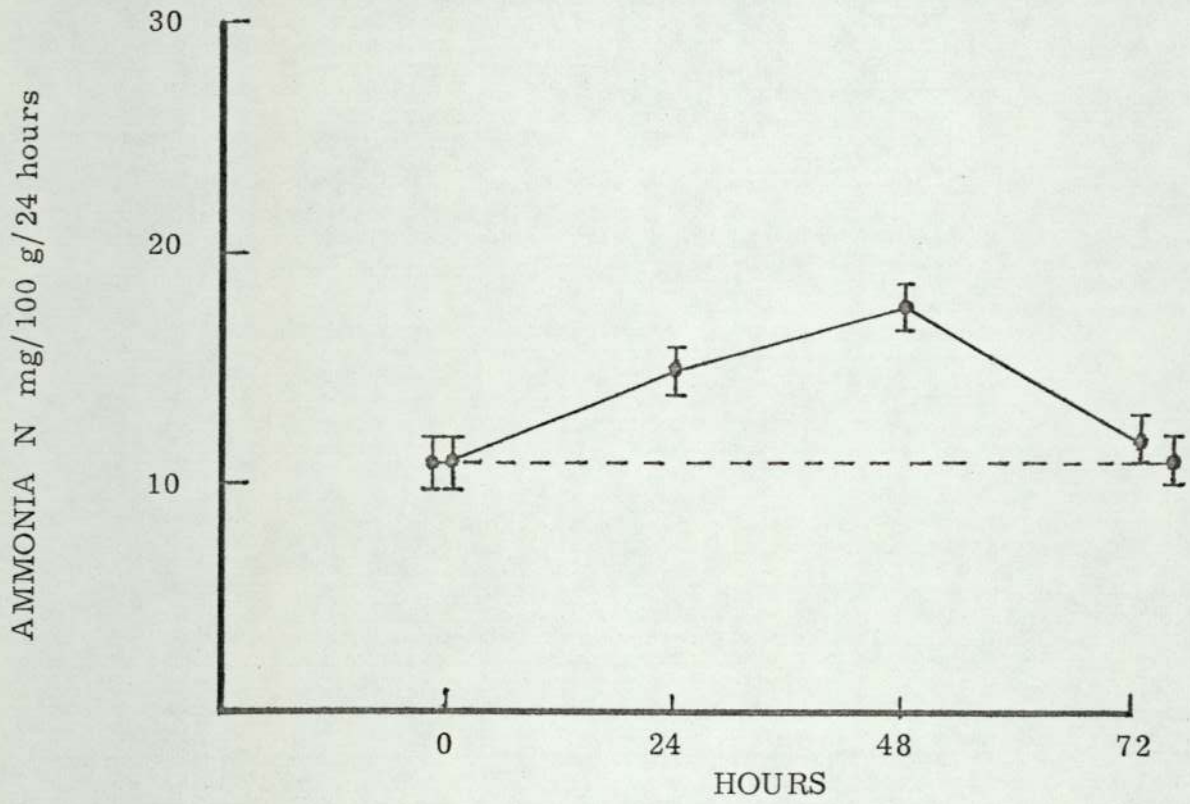
Ammonia Excretion after Alloxan Administration

No change was observed in the production of ammonia after alloxan administration. The mean resting level recorded was 11.6 ± 0.8 mg/100 g fish/24 hours, and was not altered by alloxan treatment.

Ammonia Excretion after Tolbutamide Administration

Results presented in Fig. 4 show that the injection of tolbutamide increased the ammonia production of goldfish at 23°C, from a mean resting level of 11.0 ± 1.0 mg/100g fish/24 hours to a mean level of 15.6 ± 1.0 mg/100 g fish/24 hours with a peak at 18.5 ± 1.0 mg/100 g fish/24 hours. The peak was reached two days after the tolbutamide administration. On the third day it fell from the peak value and gradually it appeared to be levelling off at the normal resting level.

FIG. 4 EFFECT OF TOLBUTAMIDE ON AMMONIA EXCRETION OF GOLDFISH



DISCUSSION

It appears from the present study that the marked increase in the production of ammonia, produced by the insulin and tolbutamide, is due to the hormone stimulating some aspect of tissue metabolism and possibly that protein metabolism is concerned. It is possible that there may be some aspects common to both the mammal and the goldfish in this respect.

Among fish, two major pathways of nitrogen elimination are utilized, one leading to the formation of ammonia and the other to the synthesis of urea. The main source of nitrogen in both pathways can be assumed to be the amino-acids. The mechanisms of excretion of nitrogenous compounds in fish range from simple diffusion to active transport. The chief route of nitrogen excretion in fish is the gill (Smith, 1929; Wood, 1958; Fromm, 1963). Smith (1929) originally proposed that this ammonia was derived from that already present in the blood which diffused across the gills. Goldstein & Forster (1965) proposed that the production of ammonia was likely due to the enzymatic deamination of glutamine and other amino-acids in the gill. It has recently been shown, however that excreted ammonia was derived mainly from that circulating through the gills and delivered there as preformed ammonia (Goldstein et al, 1964). The simplest explanation for this was that the free base diffuses down a concentration gradient from blood to water via the gills.

In the present study it has been shown that the administration of insulin increased the production of ammonia. From this it might be concluded that insulin exerts its effect via the enzymatic deamination of amino-acid in the gill. As far as the action of insulin is concerned, there seems to be two possible mechanisms of action. Insulin might facilitate the entry of amino-acid into the cell, in which case the increased intracellular substrate would result in increased ammonia production. It might enable ammonia to leave the cell more quickly, thus allowing the deamination reaction to proceed faster.

It is generally accepted that the sulphonylurea drugs exert their pancreatic stimulatory action by enhancing the beta cell secretion of insulin (Levine, R. and Mahler, R, 1964). These drugs, exemplified by tolbutamide, produce an increase in plasma insulin concentration as determined by immunoassay in normal subjects and in maturity-onset diabetic subjects, whether given by mouth or intravenously (Yalow, Black & Berson, 1960). This situation might also prevail in fish. The administration of tolbutamide might have increased the secretion of insulin from beta cells. The raised levels of insulin might then have been responsible for the elevated production of ammonia. It would seem possible that insulin exerts its effect via the deamination of amino-acids.

SUMMARY

(1) Ammonia production by goldfish increased markedly after the administration of insulin at 23°C.

(2) Total nitrogen production was increased after the administration of insulin in the goldfish to the ambient water at 23°C.

(3) Tolbutamide injection of goldfish increased the concentration of ambient water ammonia.

(4) Alloxan had no effect on the production of ammonia by goldfish.

CHAPTER III

EFFECT OF INSULIN ON THE AMINO-ACID NITROGEN CONTENT OF GOLDFISH (CARASSIUS AURATUS) MUSCLE AND LIVER

INTRODUCTION

Insulin influences many biological processes, but its role in the control of protein metabolism is not as widely appreciated as its effects on glucose and fat metabolism. The rapid fall in blood sugar that follows the administration of insulin to animals and man, and the striking action of insulin in relieving the symptoms of spontaneous and experimental diabetes mellitus, naturally focused attention on the mechanism of insulin action on carbohydrate metabolism. Free fatty acids have served as an index of the effect of insulin on fat metabolism. Until recently no such simple, easily measured, index of protein metabolism was available. Although changes in free amino-acid concentration as an index of protein metabolism have been used for a number of years (Lotspeich, W.D., 1947; Seshadri, 1959). Efforts in this field have been hampered by difficulties in methodology and complexity of the system.

Free amino acids play a key role in the overall picture of nitrogen metabolism, since it is in this form that most of the metabolically important nitrogen is absorbed from the intestinal tract and the form in which it is transported to and from the various organs. Numerous studies have been performed to show the distribution of free amino-acids in the tissues and body fluids of various fishes. As regards blood and plasma, the major difference between fish and higher vertebrates

is the relatively low concentration of glutamine (Goldstein & Forster, 1970). This low concentration has been correlated with the lack of glutamine synthesis in all tissues of fish except for brain (Wu, 1963). Indirect evidence suggesting the presence of glutamine synthesis in fish was obtained by Pequin & Serfaty (1966), who showed that an injection of glutamate into the carp lowered the excretion of ammonia. The study of free amino-acid pattern of fish muscle has been a subject of considerable interest in recent years because of the growing importance of fish as a source of animal protein in human nutrition (Cowey, 1965). The concentrations of individual free amino-acids in blood and in tissues vary widely, and the individual concentrations can vary independently. The variations in free amino-acid patterns occur during different stages in the life cycle of fishes. These variations may be due to the diet in marine or fresh water environment, varying osmotic stress, development and difference in temperature. A recent detailed study of the free amino-acid pattern in the muscle of the Atlantic Salmon, Salmo salar, was conducted using migrating marine and fresh water adults and young fresh water parr and older smolt (Cowey, Daisley, Parry, 1962; Cowey & Parry, 1963). This study showed that there were gradual changes in the concentrations of free amino-acids in the muscle of maturing salmon, which contrasted with the sudden changes in fatty acid composition during this period. Since most cell membranes contain an active transport system for amino-acids, the concentration of individual amino-acid in the intracellular water is somewhat higher than the concentration in plasma. However, all amino-acids are not

concentrated within the cell to the same degree. These widely and relatively independent variations in amino-acid levels in part account for the difficulty in assessing the effects of insulin on amino-acids.

However, the knowledge that insulin can stimulate protein synthesis in muscle by a mechanism not involving the utilization of glucose has naturally focused attention on the possibility that the site of stimulation might be the process whereby amino-acids are transferred in the cell interior (Levine, 1965; Wool & Krahl, 1959). To be more specific, does insulin accelerate amino-acid transport in muscle and, if so, is the effect of insulin on protein synthesis the result of this acceleration? Obviously, an increased availability of amino-acids would favour their intracellular utilization and incorporation into protein.

Alloxan is thought to act directly and specifically on the beta cells of the islets of Langerhans, causing them to undergo degeneration. Alpha cells and acinar tissue have been shown to remain relatively uneffected in goldfish (Chavin & Young, 1970). On the other hand, it is now generally accepted that tolbutamide exerts its pancreatic stimulatory action by enhancing the secretion of insulin (Levine, Mahler, 1964). Maingay, Ruyter, Touber, Croughs, Schopman & Lequin (1967) have shown that the administration of tolbutamide and the rise in plasma insulin was consistent with the view that tolbutamide stimulated the beta cells of the islets of Langerhans to release their preformed insulin content immediately.

The present study described below was performed to evaluate the

effect of insulin, alloxan and tolbutamide on the amino-acid nitrogen content of goldfish muscle and liver.

MATERIALS AND METHODS

The fish used were GOLDFISH (Carassius auratus) about 10 - 12 g in weight (2 - 3 in. lenght), and were obtained from WOLVERHAMPTON AQUATICS, WOLVERHAMPTON, STAFFS.

Fish were kept at 25°C in a constant temperature room, with constant illumination and aeration. Every two days the tanks were thoroughly washed out with clean tap water at the appropriate temperature in order to remove all excreta and uneaten food. The fish were fed daily with commercial baby fish food (TETRA WERKE MELLE, WESTERN GERMANY).

Prior to experimental work, fish were brought to the laboratory and maintained in experiment aquaria containing aerated tap water, approximately at the temperature of 23°C. Fish were allowed to acclimatise for 3 days prior to experimentation. Food was always withheld for 24 hours prior to the experiment.

Experimental fish were treated with insulin, alloxan and tolbutamide separately as described below:-

0.16 I.U/fish Insulin (NOVO, ACTRAPID, NOVO, INDUSTRI A/S COPENHAGEN, DENMARK) was injected intramuscularly.

Alloxan monohydrate (SIGMA CHEMICAL COMPANY) was dissolved in 0.9% saline and injected within 2 - 3 minutes to avoid deterioration. The administered dose was 600 mg/Kg at the volume of 0.3 ml.

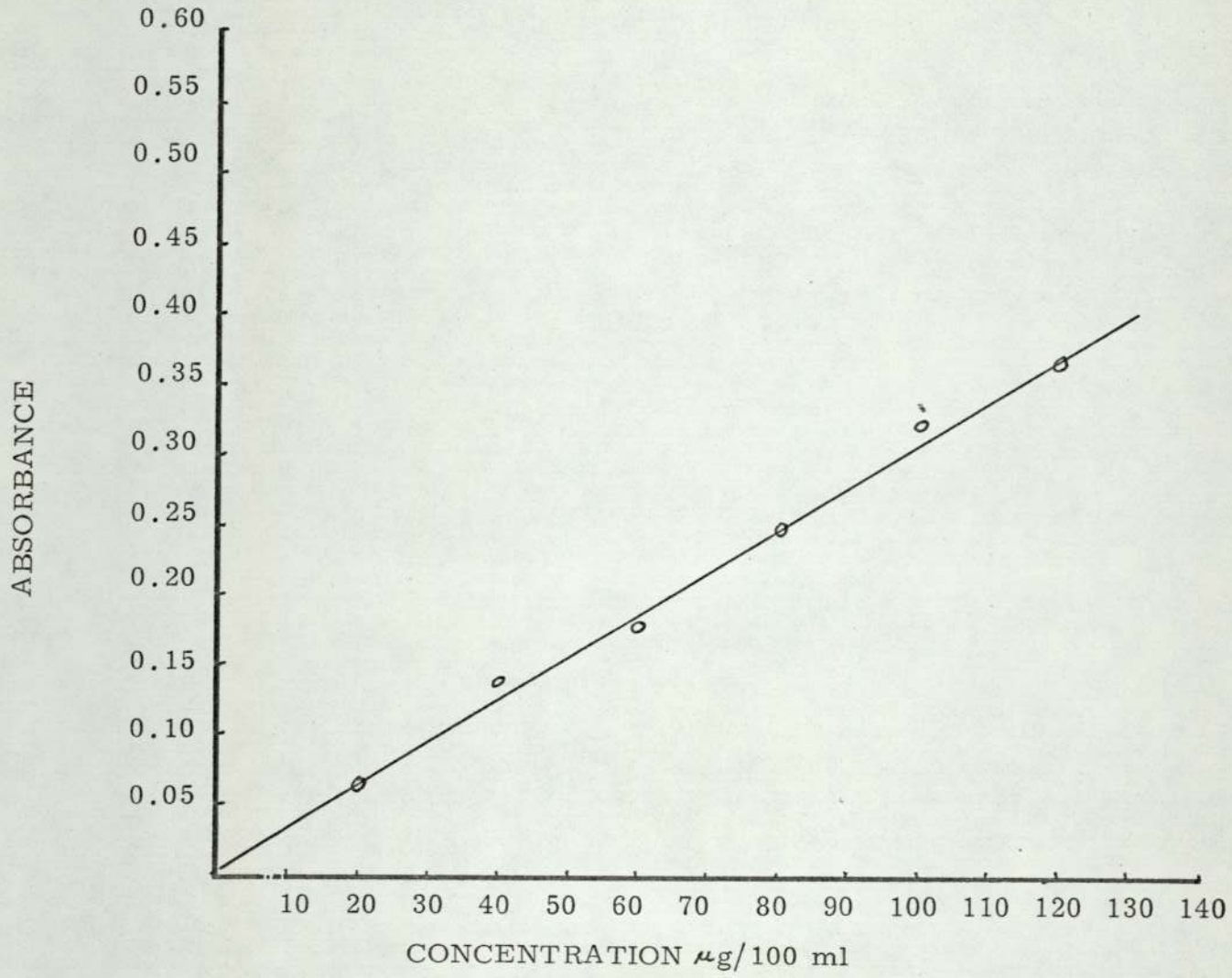
Tolbutamide (as sodium salt) (RASTIONON HOECHST) was prepared in double distilled water and injected intramuscularly. The dose was 10 mg/Kg body weight.

Control fish were injected with 0.9% saline. Anæsthetics were not used.

ANALYTICAL TECHNIQUE

Amino-acids in muscle and in liver were estimated using the method of Varley (1958). Fish were killed by a blow on the head 48 hours after administration had been started, and approximately 100 mg portions of tissue were quickly removed and homogenized with 5 ml of a mixture of equal volumes of 10% sodium tungstate and 0.66 N sulphuric acid. The homogenate was washed into a centrifuge tube with a further 5 ml, and after centrifugation free of debris, amino-acid nitrogen was determined on 3 ml of the clear supernatant and values were expressed in μg/mg of tissue. The absorbance was measured on a BECKMAN D.B. SPECTROPHOTOMETER (BECKMAN INSTRUMENTS LTD.) at 420 nm. Amino-acid standards were treated similarly and a suitable standard curve was prepared by diluting the stock solution (Frame, Russel & Wilhelmi, 1943). The amino-acid standard curve is shown in Fig. 5.

FIG. 5 STANDARD CURVE OF AMINO-ACID



OBSERVATIONS AND RESULTS

Tissue Analysis

(A) EFFECT OF INSULIN

Fig. 6 shows the results of determination of the amino-acid nitrogen concentrations in muscle and liver of the goldfish at 23°C, 48 hours after administration, compared with controls. The amino-acid nitrogen of insulin treated muscle was much higher than that of untreated muscle. The mean values were 104.5 μg/100 mg tissue and 69.2 μg/100 mg tissue respectively. In liver there was no change. The mean value of amino-acid nitrogen concentration in untreated tissue was 59.7 μg/100 mg tissue and it was 62.2 μg/100 mg tissue in treated goldfish liver.

(B) EFFECT OF ALLOXAN

The amino-acid nitrogen concentration in muscle and in the liver of goldfish at 23°C 48 hours after alloxan injection is shown in Fig. 8. The amino-acid nitrogen content of alloxan treated muscle was less than that of untreated muscle. The mean value of alloxan treated muscle was 45.3 μg/100 mg tissue, while it was 69.5 μg/100 mg tissue in the untreated one. There was no difference in the mean amino-acid nitrogen content of liver from treated and untreated fish, 56.8 μg/100 mg tissue and 58.7 μg/100 mg tissue respectively.

(C) EFFECT OF TOLBUTAMIDE

Fig. 7 shows amino-acid nitrogen content of muscle and liver of goldfish treated with tolbutamide at 23°C 48 hours after injection, compared with controls. The amino-acid nitrogen concentration of

FIG. 6 EFFECT OF INSULIN ON THE AMINO-ACID NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER (48 HOURS AFTER SINGLE INJECTION)

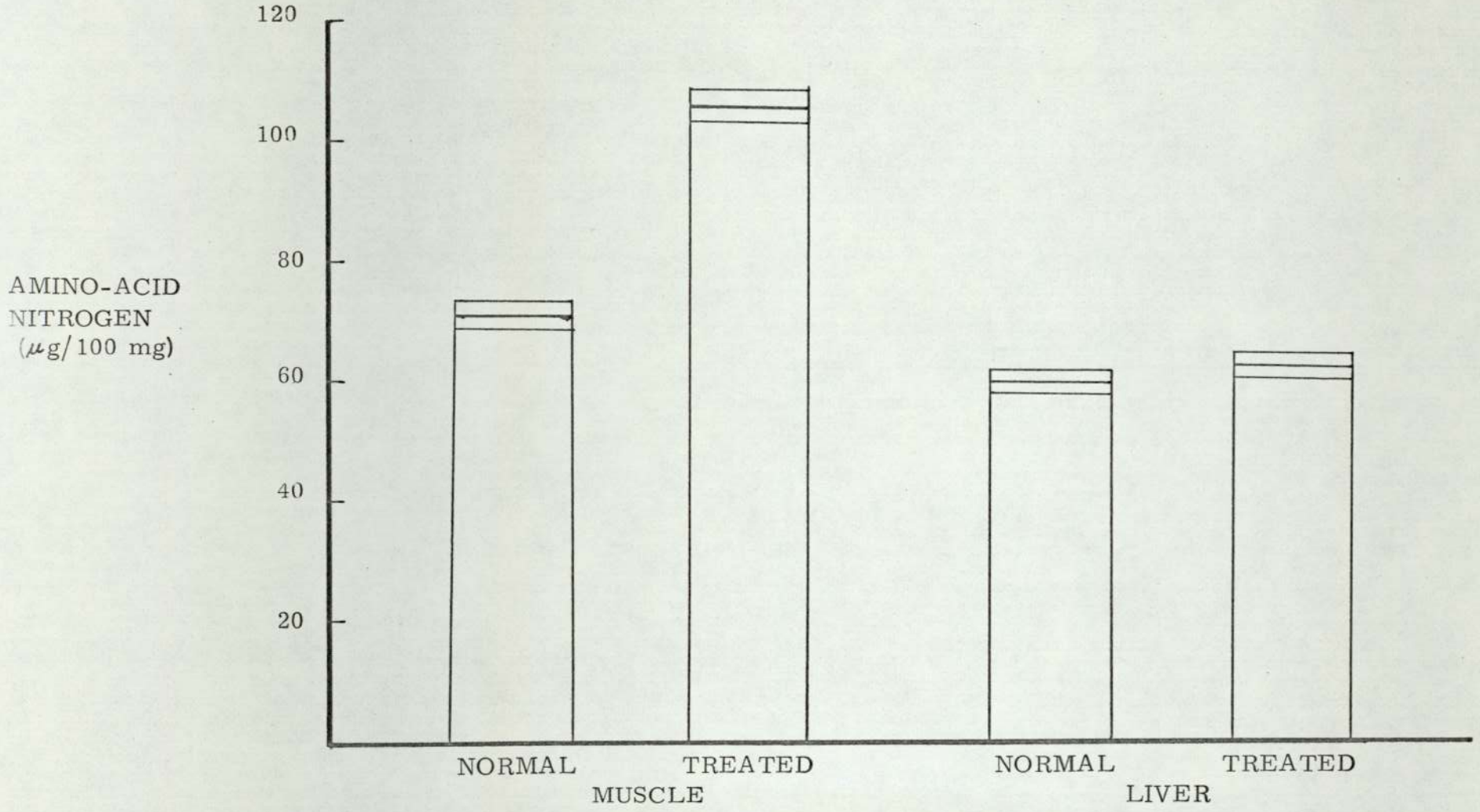


FIG. 8 EFFECT OF ALLOXAN ON AMINO-ACID-NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER (48 HOURS AFTER SINGLE INJECTION)

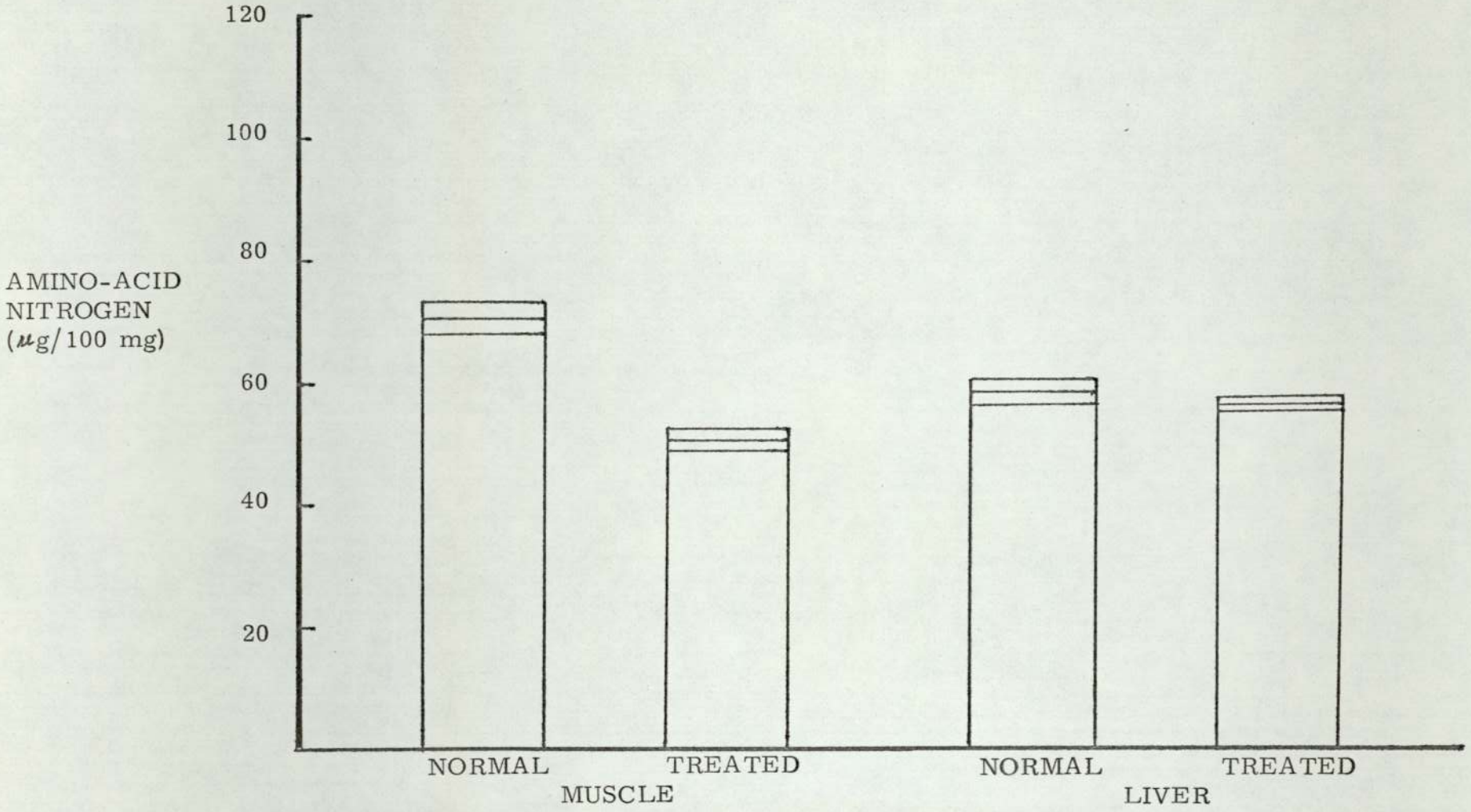
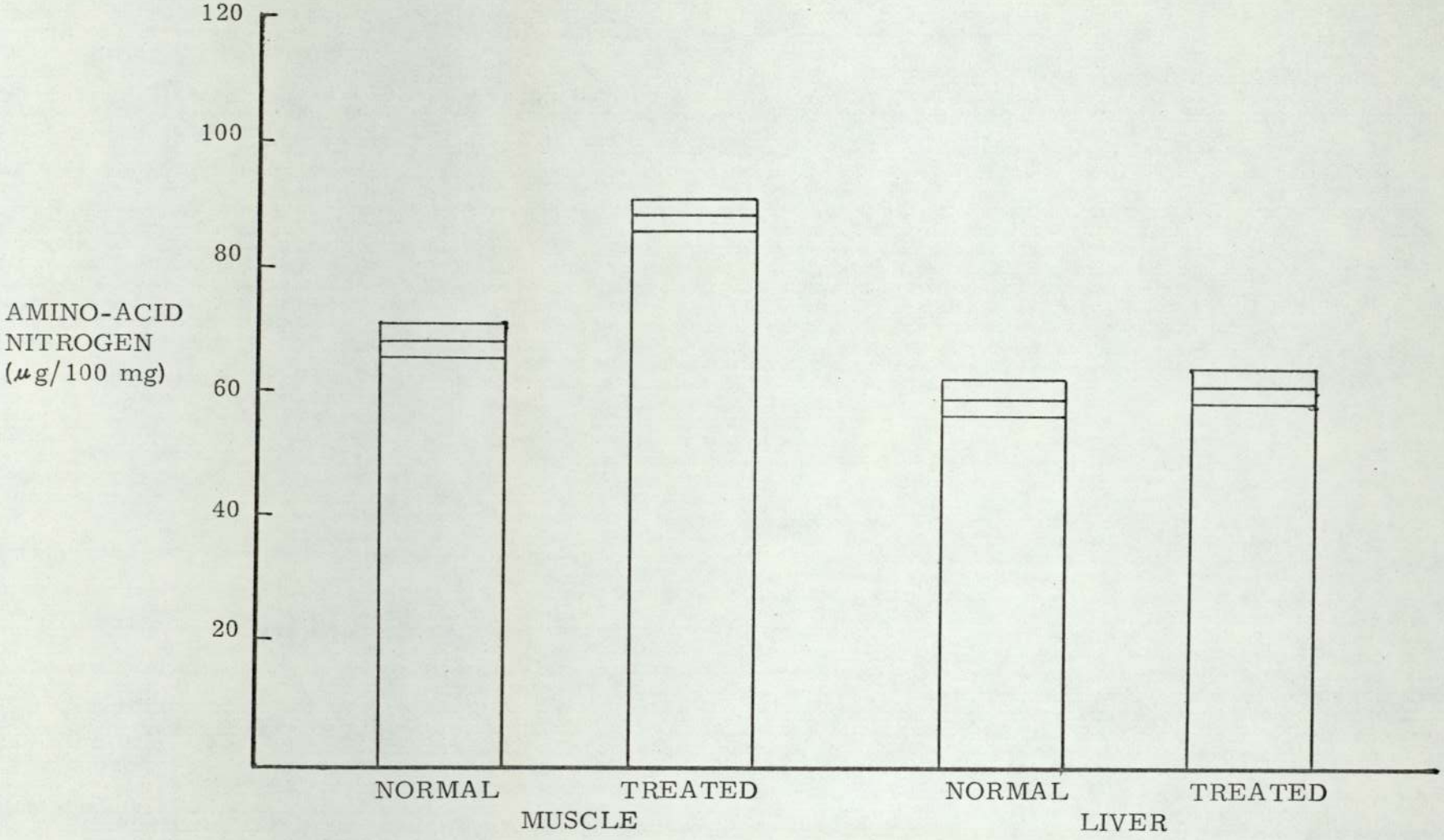


FIG. 7 EFFECT OF TOLBUTAMIDE ON THE AMINO-ACID NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER (48 HOURS AFTER SINGLE INJECTION)



treated muscle was higher than that of untreated muscle. The mean values were 88.4μg/100 mg tissue and 68.3μg/100 mg tissue respectively. No change was observed in liver amino-acid nitrogen content after tolbutamide treatment. The mean value of controls was 59.6μg/100 mg tissue and it was 61.3μg/100 mg tissue in treated fish.

DISCUSSION

Although little direct effect on protein anabolism over all can be obtained by treatment of the intact animal with insulin, its administration does rapidly lower the blood amino-acids level of numerous species of animal (Luck, Morrison & Wilbur, 1928). Kiech & Luck (1928 and Kerr & Krikorian (1929) found that insulin increased the rate of urea formation in normal rats, and concluded that a lowering of the blood amino-acid level produced by insulin was accounted for by the increased excretion of urea. They suggested that insulin increased the rate of amino-acid catabolism and inhibited protein hydrolysis. However, Mirsky (1938) found that the administration of insulin to eviscerated nephrectomized dogs depressed the rate of increase in blood urea and diminished the rate at which the amino-acids were liberated from the muscle. The rate of removal of exogenous amino-acid from the blood of eviscerated nephrectomized animals was raised by the administration of insulin. Mirsky suggested that insulin depressed the rate of oxidative deamination of amino-acids in liver and increased their rate of utilization by muscle for protein synthesis. Direct evidence for the promotion by insulin of the uptake of amino-acids by skeletal muscle was obtained by Forker and associates (1951), consonant with the results of Mirsky (1938). Frame Russel (1946) and Ingle & Nezamis (1947) found that insulin suppressed the rise in blood amino-acid concentration in the eviscerated rat, an effect which was observable within 15 minutes of its administration (Ingle, Torralbo & Flores, 1956).

Lotspeich (1949), who studied the effect of insulin injection on amino-acid concentration of the blood and muscle of dog, found that there was a proportional correlation between the fall of the free amino-acids concentration of the blood and the rise in protein bound amino-acids of muscle following insulin injection. From this he concluded that insulin promoted protein synthesis. In Ophicephalus striatus insulin injection resulted in a decrease of muscle free amino-acid concentration and an increase of protein bound amino-acid concentration (Seshadri, 1959).

The present study using the goldfish (Carassius auratus) has shown an increase in the amino-acid nitrogen of muscle after the administration of insulin. It is therefore concluded that insulin might facilitate the entry of amino-acids into cells and that the increase in amino-acid nitrogen content of muscle was due to the transport of amino-acid into the cell. However, there was no significant change in the amino-acid nitrogen content of liver after insulin treatment. This was perhaps due to the fact that liver is not specifically necessary for the action of insulin on amino-acids. This is suggested by the fact that insulin delays the accumulation of non-protein nitrogen in the nephrectomized eviscerated dog (Mirsky 1938) and of amino-acids in blood of the eviscerated rat (Frame & Russell, 1946). Thus, the effect of insulin on amino-acid must be expressed on tissue in general rather than in liver, or perhaps that insulin has no effect on the deamination of amino-acids in liver.

Many studies have described the diabetogenic action of alloxan on teleosts (Saviano, 1947b; Young & Chavin, 1963; Khana & Mehratra, 1969). Several investigations have described beta-cell lesions following alloxan injection, the degree of these lesions varying greatly. Recently, Chavin and Young (1970) described beta cell destruction after alloxan treatment in goldfish (Carassius auratus). In the present study a similar dose of alloxan was used to make the experimental fish diabetic. Alloxan treatment reduced the amino-acid nitrogen content of goldfish muscle. It is assumed that alloxan damaged the beta cells and due to this destruction of beta cells, a deficiency of insulin was assumed. Due to the reduced availability of insulin in the fish, the amino-acid nitrogen content of the muscle was lowered. Either the amount of circulating insulin was not sufficient to facilitate the entry of amino-acids into the muscle cells, or the transport of amino-acids did not take place due to the reduced availability of insulin.

In mammals, tolbutamide is thought to stimulate the beta cells of the islets of Langerhans to secrete more insulin. This might also be truein fish, since the present study has shown the amino-acid nitrogen content of tolbutamide-treated goldfish muscle to be elevated. It is assumed that tolbutamide induced release of insulin, and due to this the amino-acid nitrogen content of goldfish muscle was increased. Hence, here also insulin might be facilitating the membrane transport of amino-acids.

SUMMARY

(1) The amino-acid nitrogen content of muscle from goldfish treated with insulin was increased 48 hours after the injection. There was no change in liver amino-acid nitrogen content.

(2) Alloxan lowered the free amino-acid content of muscle from goldfish. There was no difference in the amino-acid nitrogen content of treated and untreated liver.

(3) Administration of tolbutamide produced an increase in free amino-acid content of muscle from goldfish. No change in amino-acid nitrogen content was observed between treated and untreated goldfish liver.

CHAPTER IV

EFFECT OF INSULIN ON TOTAL NITROGEN CONTENT OF GOLDFISH (CARASSIUS AURATUS) MUSCLE

INTRODUCTION

Under physiological circumstances, the first step in protein biosynthesis must of necessity be concerned with providing a supply of amino-acids for the manufacture of protein and transporting them to the site of synthesis. Since the intracellular pool of amino-acids appears to be large compared with the concentration of amino-acids in the extracellular fluid (I.G. Wool, 1965), it is conceivable that protein synthesis might proceed for some time without requiring additional amounts of amino-acids. This appears to be the case with isolated rat diaphragm where amino-acid incorporation was not appreciably increased over that observed with Krebs bicarbonate buffer by addition of a complete amino-acid mixture (Wool, M.E., Krahl, 1959). However, at some point, the intracellular amino-acid pool must be replenished, and a system of transport of amino-acids from the extracellular compartment into the cell interior is required. Insulin has been shown to decrease the concentration of amino-acids in the plasma of intact animals (Luck, Morrison & Wilbur, 1928). Insulin, at physiological concentration,has been shown to stimulate the incorporation of all 20 natural amino-acids (Wool, I.G. & P. Karlson, 1965). On the other hand, amino-acid incorporation into liver proteins does appear to require the presence of glucose (Wool, I.G., 1965). In adipose tissue, amino-acid incorporation into

protein does not require the presence of glucose, but some other substrate, possibly acetate or pyruvate (Krahl, M.E., 1964). The fact remains that, in muscle, which contains the bulk of the body protein, the synthesis of protein under the influence of insulin is probably independent of glucose transport. It is here that the effect of insulin on protein synthesis is being studied more intensively than in any other tissue. Lotspeich (1949) found a proportional correlation between the fall of the free amino-acids in the blood and the rise in protein bound amino-acids of the muscle following insulin injection. This observation supports the hypothesis that insulin might increase amino-acid transport into the cell with a resulting increase in protein synthesis. From this information alone it is uncertain whether the increase in amino-acid uptake is secondary to any increased intracellular utilization of amino-acid for protein synthesis, or increase in availability of amino-acids. This second possibility has been postulated as accounting for the insulin mediated stimulation of protein synthesis in muscle (Kipnis & Noall, 1958).

The finding that insulin could increase amino-acid transport has suggested that this might be the mechanism of action of insulin in stimulating protein synthesis. Wool & Krahl (1959) demonstrated amino-acid accumulation into diaphragms of rat before removal. Subsequent incubation of the tissue with insulin resulted in an increased amino-acid incorporation into protein, that in the hormone had acted after the muscle cells had accumulated the amino-acids. Manchester& Krahl (1959) were able to show an insulin induced amino-acid incorporation using labelled carboxylic acid precursors of amino-acids. In those experiments the amino-acids were formed intracellularly

and their transport was therefore not involved. Another experiment was performed just to trap any labelled amino-acids that might have leaked out, thus preventing their reaccumulation (Wool & Krahl, 1964). Under that condition insulin still stimulated incorporation of pyruvate carbon into muscle protein. This experimental evidence suggests that the enhanced incorporation of radioactivity into muscle protein in the presence of insulin is not the result of a stimulation of amino-acid transport. Thus the action of insulin on the membrane transport of amino-acids and on protein synthesis probably occur at separate sites in the muscle cell.

Insulin has been shown to enhance the transport of amino-acids into the muscle of goldfish at 23^{o}C (Page 56 This Thesis). The purpose of this present study, therefore, is to evaluate the effect of insulin on the total nitrogen content of goldfish muscle. Alloxan and tolbutamide were used to investigate their effect on the total nitrogen content of goldfish muscle.

METHODS AND MATERIALS

Goldfish (Carassius auratus) about 10 - 12 g in weight (2 - 3 in. length) were obtained from WOLVERHAMPTON AQUATICS, WOLVERHAMPTON, STAFFS.

Fish were maintained at 25°C in a controlled temperature room, with constant illumination and aeration. Every two days the tanks were thoroughly washed out with clean tap water at the appropriate temperature to remove all excreta and uneaten food. Fish were fed daily with commercial baby fish food.

Prior to experimental work, fish were brought to the laboratory and maintained in experimental aquaria containing aerated tap water at 23°C. Fish were allowed an acclimation period of at least 3 days prior to use in the experiment. Food was withdrawn 24 hours before experimental work.

Fish were separately treated with insulin, alloxan and tolbutamide as follows: -

INSULIN 0.16 I.U/Fish (NOVO, ACTRAPID, NOVO INDUSTRI A/S, COPENHAGEN, DENMARK) was injected intramuscularly.

ALLOXAN MONOHYDRATE (SIGMA CHEMICAL COMPANY) was dissolved in 0.9% saline and injected within 2 - 3 minutes to avoid deterioration. The administered dose was 600 mg/Kg at the volume of 0.3 ml.

TOLBUTAMIDE (as sodium salt) (RASTIONON HOECHST) was prepared in double distilled water and injected intramuscularly.

The dose was 10 mg/Kg body weight.

Control fish were injected with 0.9% saline. Anaesthetics were not used.

ANALYTICAL TECHNIQUE

Total nitrogen content of goldfish muscle was determined by the standard micro-Kjeldahl method. This is an established routine technique. No other analytical method has done so much to further metabolism investigation as Kjeldahl's method for the determination of total nitrogen. While applicable to all kinds of nitrogenous products of interest to the biochemist, it has proved serviceable. In its modern modification, it is one of the most rapid, convenient and accurate methods.

Fish were killed by a blow on the head 48 hours after injection, and approximately 100 mg protions of tissue were quickly removed and homogenized (all glass homogenizer) with 10% TCA. After centrifugation, the clear supernatant was removed and retained precipitate was dried in an oven at 80^{o}C, powdered and digested in a digestive mixture (Bock & Benedict, 1915) to determine the total nitrogen. The optical density was measured on the Beckman D.B. Spectrophotometer (BECKMAN INSTRUMENTS LTD.) at 500 nm.

OBSERVATIONS AND RESULTS

Tissue Analysis

EFFECT OF INSULIN

Fig. 9 shows the total nitrogen content of goldfish muscle at 23°C 48 hours after injection, and are compared with control values. The total nitrogen content of insulin treated muscle was higher than the untreated muscle. The mean values were 3.6 mg/100mg tissue and 2.4 mg/100 mg tissue respectively.

Fig.12 illustrates the dose response curve of insulin with the concentration of total nitrogen in muscle of goldfish at 23°C 48 hours after injection.

EFFECT OF ALLOXAN

The total nitrogen content of goldfish muscle at 23°C 48 hours after alloxan injection is shown in Fig. 11.

The total nitrogen content of alloxan treated muscle was less than untreated muscle; the mean values were 1.4 mg/100 mg tissue and 2.5 mg/100 mg tissue respectively.

EFFECT OF TOLBUTAMIDE

The total nitrogen content of goldfish muscle after tolbutamide treatment at 23°C is shown in Fig. 10. The total nitrogen content of treated muscle was higher than that of untreated muscle. The mean

FIG. 9 EFFECT OF INSULIN ON PROTEIN OF MUSCLE OF GOLDFISH
(48 HOURS AFTER SINGLE INJECTION)

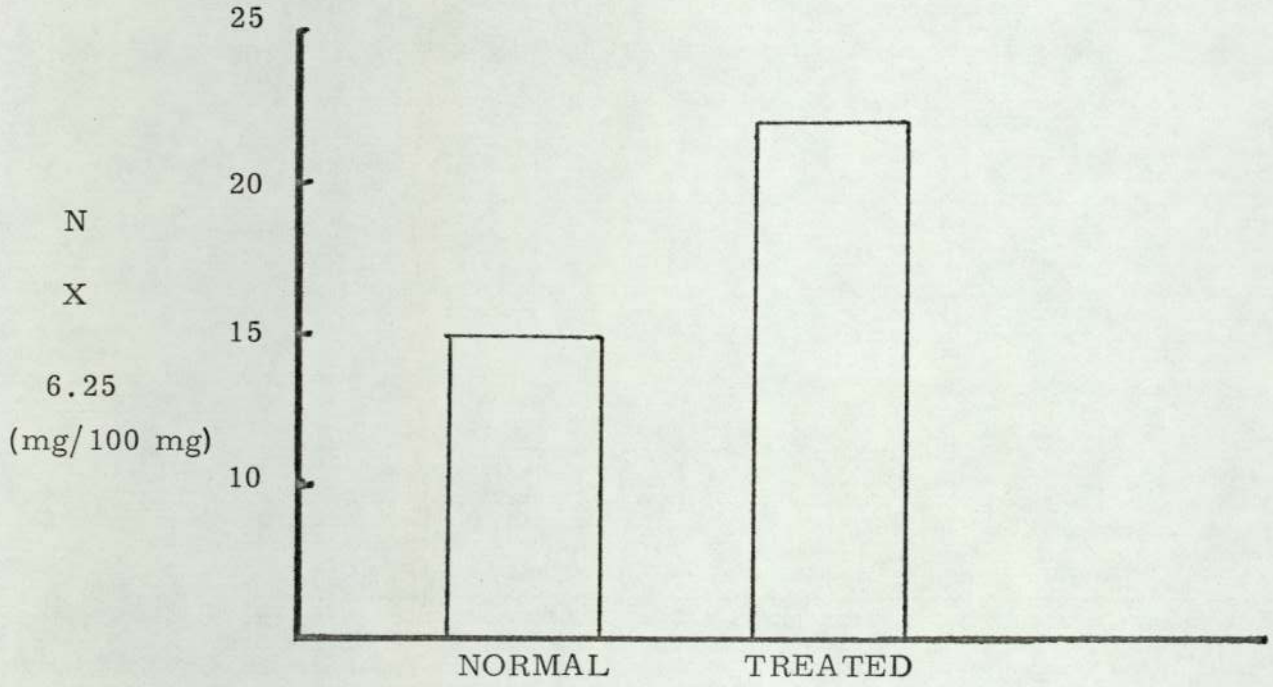


FIG. 12 EFFECT OF MAMMALIAN INSULIN ON PROTEIN OF MUSCLE OF GOLDFISH (48 HOURS AFTER SINGLE INJECTION)

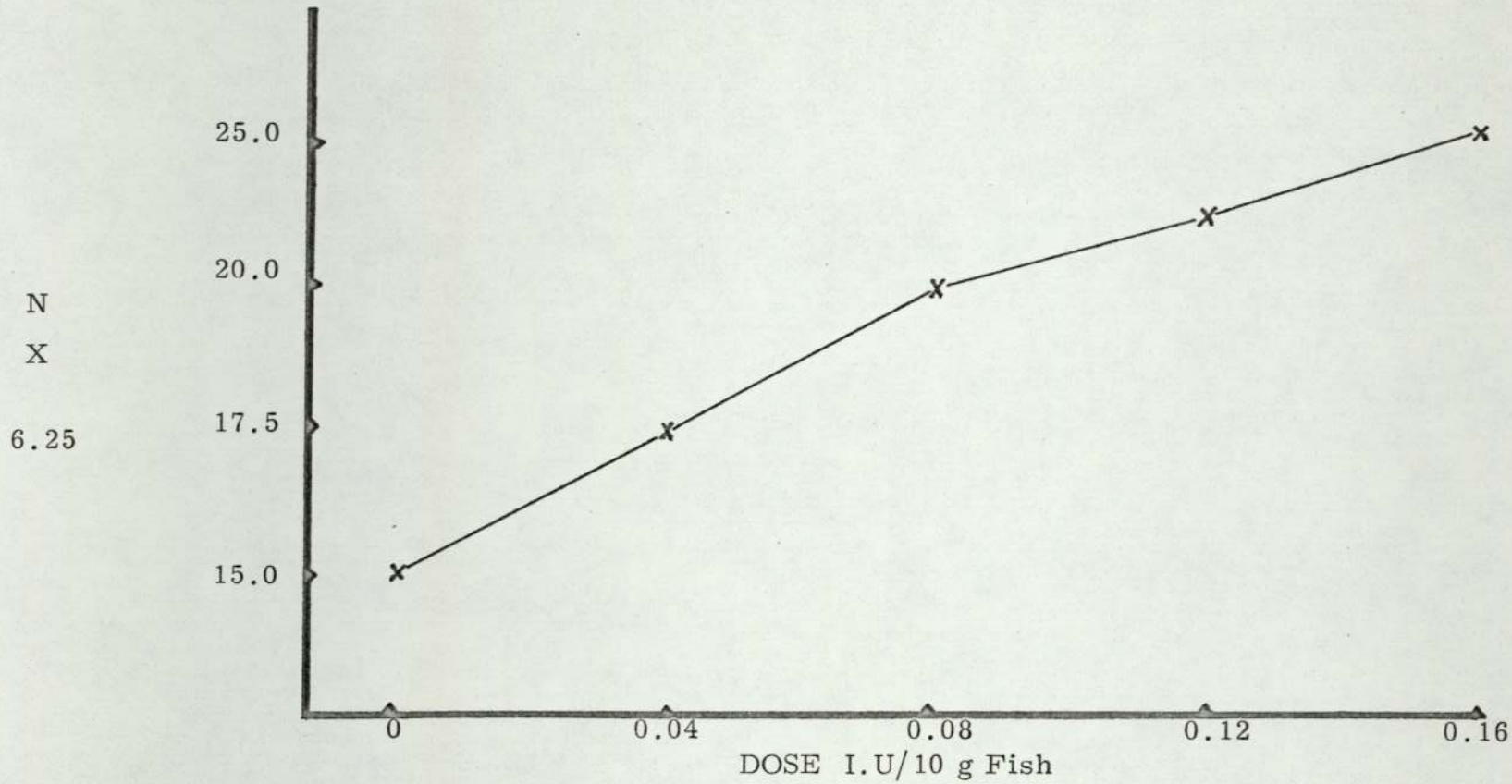


FIG. 11 EFFECT OF ALLOXAN ON PROTEIN OF MUSCLE OF GOLDFISH
(48 HOURS AFTER SINGLE INJECTION)

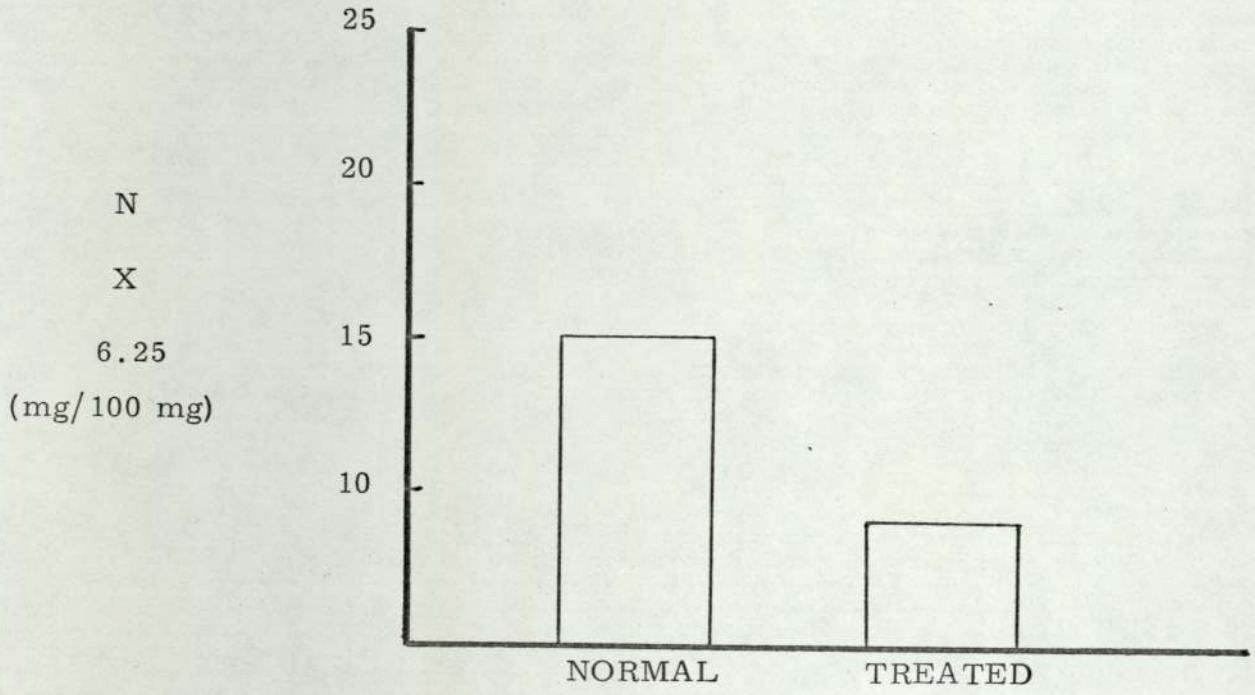
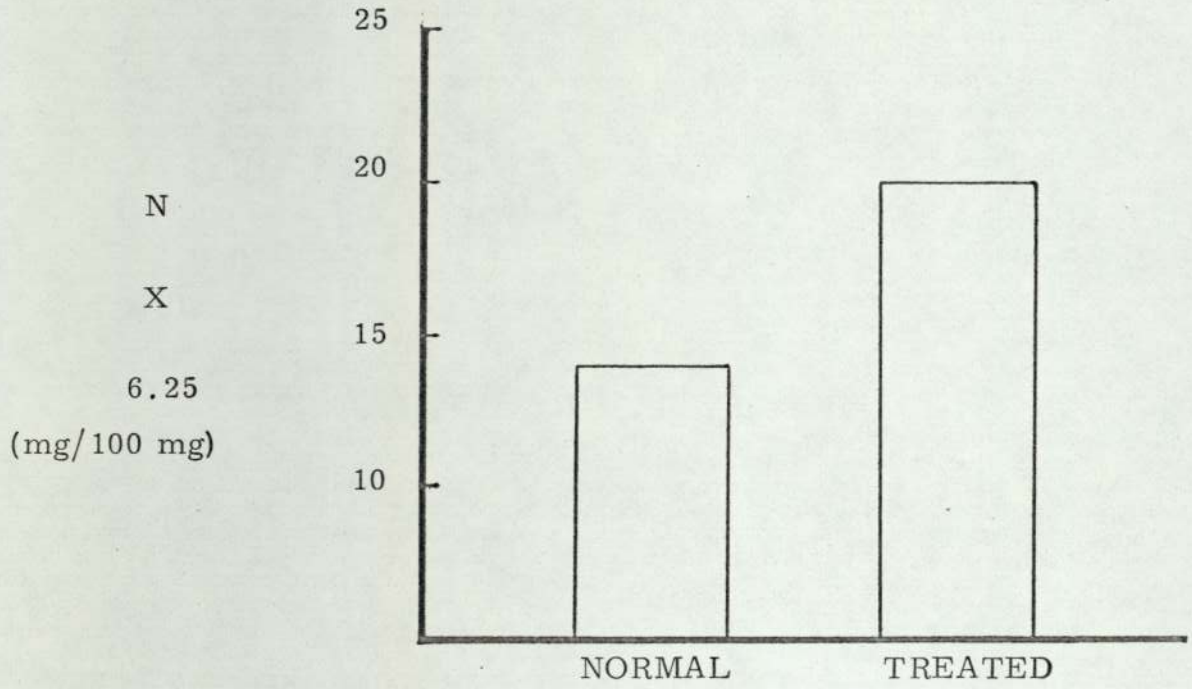


FIG. 10 EFFECT OF TOLBUTAMIDE ON PROTEIN OF MUSCLE OF GOLDFISH (48 HOURS AFTER SINGLE INJECTION)



values were 3.2 mg/100 mg tissue and 2.3 mg/100mg tissue respectively.

The most frequently used method of estimating the amount of protein in a tissue or in a foodstuff is by determining the nitrogen content. The procedure is to multiply the N_2 content of the tissue by 6.25 in order to give the quantity of protein present. This depends on certain criteria being established. Firstly, can one safely assume that all, or nearly all, the content of the tissue present is in the form of protein? If this can be accepted as a reasonable approximation, then one has to consider whether one is justified in using a single factor of 6.25 to convert from nitrogen to protein. This is for the purpose of the nutritional studies; the nitrogen content of the diet consistsessentially of protein. This is not strictly correct for animal tissue. If one assumes that by multiplying N content by the factor 6.25, it means that the most proteins do not deviate seriously from 16% N content. When such a factor is used, it is best to designate the result as crude protein.

DISCUSSION

The observation that uncontrolled diabetes, whether of natural origin or induced by pancreatectomy or administration of alloxan, is accompanied by loss of body-weight, depletion of tissue protein and a rise in the rate of nitrogen excretion has led to the acceptance of the idea that insulin plays a part in stimulating protein biosynthesis. The excessive nitrogen loss and tissue wastage of the diabetic animal can be prevented by treatment with insulin (Atchley, Loch, Richards, Benedict & Driscoll, 1933; Chiakoff & Forker, 1950).

Although little direct effect on total protein synthesis can be obtained by treatment of normal animals with insulin, its administration does rapidly lower the amount of amino-acids in the blood of several species of animal (Luck, Morrison & Wilbur, 1928), and promotes the uptake of amino-acids by skeletal muscle (Forker, Chiakoff, Entenman & Tarver, 1951). Furthermore, the proportions in which ten essential amino-acids disappear from the blood of the fasting dog after insulin administration correspond with the relative proportions of these amino-acids in the protein of muscle (Lotspeich, 1949). In Ophicephalus striatus, insulin administration resulted in a decreased muscle free amino-acid content and an increased proportion of protein bound amino-acids (Seshadri, 1959). Convincing evidence suggesting that insulin might have a direct effect upon protein biosynthesis, has come from in vitro experiments in which added insulin has been shown to stimulate the rate of incorporation of amino-acids into protein.

Insulin added in vitro has been shown to stimulate the incorporation of radioactive amino-acids into the proteins of isolated rat diaphragm (Sinex, MacMullen & Hastings, 1952; Manchester & Young, 1955a). The incorporation of radioactive amino-acid into the protein of alloxan diabetic rat diaphragm was depressed, but later restored to the normal level by treatment with insulin (Krahl, 1953; Manchester & Young, 1960a). The results of all experiments in which the incorporation of radioactive amino-acids into protein is used as a measure of protein biosynthesis must be viewed with some reservation, for it is by no means certain that the incorporation of amino-acids can be equated with the synthesis of specific, biologically active proteins.

In the present study the total nitrogen content of goldfish muscle was markedly increased after the administration of insulin. From this it is concluded that insulin increases the amount of protein in the muscle. In a similar way, tolbutamide, which is thought to stimulate the B-cells of pancrease to release the insulin content immediately after its administration has increased the tot al nitrogen content of goldfish muscle 48 hours after injection. It might be suggested that tolbutamide, by elevating the circulating levels of insulin, promoted increased muscle protein synthesis.

Alloxan, on the other hand, decreased the total nitrogen content of goldfish muscle. This was probably due to a reduced secretion and hence supply of insulin, by destroying B-cells. One might conclude that circulating insulin levels exercised control over protein anabolism

in the goldfish.

However, the rate of protein biosynthesis depends upon the rate at which protein synthesizing mechanism works and/or the rate at which amino-acids are transported across the cell membrane from the extracellular fluid into the muscle cell. Insulin might influence one or both of these mechanisms.

SUMMARY

(1) The total nitrogen content of goldfish muscle was increased at 23°C, 48 hours after insulin injection.

(2) Alloxan treatment lowered the total nitrogen content of goldfish muscle at 23°C, 48 hours after injection.

(3) The administration of tolbutamide increased the total nitrogen content of goldfish muscle.

CHAPTER V

EFFECT OF INSULIN ON THE INCORPORATION OF LEUCINE ^{14}-C INTO GOLDFISH MUSCLE PROTEIN

INTRODUCTION

The use of radioactivity labelled amino-acids, and the study of their transport and incorporation into protein, has provided a useful method for the investigation of the effect of insulin and the mechanism of action on protein metabolism. Insulin, added *in vitro*, has been shown to stimulate the incorporation of labelled amino-acids into protein (Sinex, MacMullen & Hastings, 1952). This highly significant observation has been confirmed for a large number of different individual amino-acids and for mixtures of amino-acids (Manchester & Young, 1958; Wool & Krahl, 1959). Insulin initiated amino-acid incorporation into the proteins of diaphragm incubated in a medium containing no added glucose was induced under conditions where the effect of insulin on carbohydrate metabolism was assumed to be minimal. Manchester & Young, 1958a; Wool & Krahl, 1959a, strongly suggested that insulin could increase amino-acid incorporation into diaphragm protein by a mechanism which did not involve a simultaneous stimulation by insulin of glucose uptake by the tissue./ The primary point of action of insulin in stimulating carbohydrate metabolism has been suggested to be almost certainly on the entry of the sugar into the muscle cell (Levine, Goldstein, Huddleston, Kein, 1950; Levine & Goldstein, 1955)/.

A significant rise in amino-acid incorporation into isolated rat diaphragm can be produced by as little as 0.05 milliunits (2.5μg) of insulin/ml of medium (Manchester & Young, 1959b), an amount well within the physiological range (Randle, 1957). Even at a low concentration of insulin, addition of glucose did not quantitatively alter the response of diaphragm muscle to the action of the hormone on amino-acid incorporation (Manchester & Young, 1958a). Under conditions where the concentration of insulin was rate limiting, there appeared to be no competition between the utilization of glucose and the incorporation of amino-acids induced by the available insulin (Ketterer, Randle & Young, 1957).

The incorporation of labelled amino-acid into protein of diaphragm from alloxan-diabetic rats was depressed, and subsequently restored to the normal level by treatment of the animal with insulin (Krahl, 1953; Manchester & Young, 1960a). So insulin seems to have a well-established role of increasing protein biosynthesis in mammals (Snipes, 1968). There is very little literature on protein biosynthesis in fish, but Tashima & Cahill (1968) demonstrated that insulin markedly stimulated incorporation of glycine ^{14}-C into skeletal muscle in toadfish. Recently, Jackim & G. Laroche (1973) showed that insulin increased leucine ^{14}-C incorporation into the muscle of Fundulus heteroclitus.

In an attempt to further evaluate the effect of insulin on protein biosynthesis in fish, the effects of insulin, alloxan and tolbutamide have been investigated on protein biosynthesis in the goldfish.

Protein synthesis in muscle was chosen rather than liver, since, in muscle, the bulk of the protein produced remains in situ. Leucine ^{14}-C incorporation was used as an index of protein biosynthesis in goldfish muscle.

MATERIALS AND METHODS

Goldfish (Carassius auratus) about 10 - 12 g in weight (2 - 3 in. length) were used. Fish were kept at 25°C in a constant temperature room and were fed daily with commercial baby fish food.

Experimental fish were maintained in aquaria containing aerated tap water at 23°C, an acclimation period of at least 3 days was allowed prior to their use in the experiment. Food was withdrawn 24 hours prior to experimental work.

Fish were treated with insulin, alloxan and tolbutamide separately as described below:-

INSULIN 0.16 I.U/Fish (NOVO ACTRA, NOVO INDUSTRI A/S COPENHAGEN, DENMARK) was injected intramuscularly.

ALLOXAN MONOHYDRATE (SIGMA CHEMICAL COMPANY) was dissolved in 0.9% saline and injected within 2 - 3 minutes to avoid deterioration. The administered dose was 600 mg/Kg at the volume of 0.3 ml.

TOLBUTAMIDE (as sodium salt) (RASTIONON HOECHST) was prepared in double distilled water and injected intramuscularly.

The dose of tolbutamide was 10 mg/Kg.

Control fish were injected with 0.9% saline. Anaesthetics were not used.

Leucine ^{14}-C (uniformly labelled, 270 mci/m moles) was purchased from THE RADIOCHEMICAL CENTRE, AMERSHAM. It was diluted with 0.9% saline and the appropriate dose of radioactivity (1μC/100 g body weight) was injected intraperitoneally, 48 hours after the administration of either insulin, alloxan or tolbutamide. Exactly 1 hour later, fish were killed by a blow on the head and 100 mg portions of muscle were quickly removed and homogenized immediately in 5 ml of 10% of trichloroacetic acid (TCA) to precipitate tissue proteins. After centrifugation, a 0.2 ml aliquot of the supernatant fluid was diluted with 10 ml of a scintillation mixture containing 600 ml p-dioxane, 100 ml anisole, 100 ml 1, 2-dimethoxyethane, 4.8 g 2, 5-diaphenyl-oxazole and 120 mg 1, 4-bis-2(4-methyl-5-phenylozazolyl) benzene. The radioactivity in this sample was measured (Kostypo & Schmidt, 1963) for 10 minutes on a β-counter (BECKMAN L.5. 230) with a counting efficiency of 63 - 68%.

The TCA precipitates of the muscle homogenates were allowed to dry, dissolved in 1 ml of hyamine hydroxide and then mixed with 10 ml of the scintillation fluid (Kostyo & Schmidt, 1963) and counted as described above. The radioactive uptake was expressed in terms of dpm/mg tissue.

EFFECT OF INSULIN

The incorporation of leucine ^{14}-C into protein bound amino-acid of goldfish after insulin treatment was significantly higher than contol values. The significant difference was ($P<0.001$) as shown in Table 11.

Table 11 shows that there was a significant decrease in the incorporation of leucine ^{14}-C into the free-amino-acids compared to control values. The significant difference was ($P<0.001$).

When a low dose of insulin (0.04 I.U/Fish) was injected, a significant increase in the incorporation of leucine ^{14}-C into the protein-bound amino-acid was observed. The significant difference was ($P<0.01$) as shown in Table 11. Results indicate that the incorporation of leucine ^{14}-C into the free-amino-acids was reduced significantly by $P<0.01$ after a low dose of insulin treatment (Table 11).

EFFECT OF ALLOXAN

Alloxan had no significant effect on the incorporation of leucine ^{14}-C into goldfish muscle.

EFFECT OF TOLBUTAMIDE

After tolbutamide treatment, the incorporation of leucine ^{14}-C into protein-bound amino-acid was significantly higher than control values. The significant difference was $P<0.01$. However, there was a significant decrease ($P<0.01$) in the leucine ^{14}-C incorporation into the free-amino-acid of goldfish muscle (Table 12).

DISCUSSION

The above results suggest that insulin might exert an acute in vivo effect of protein biosynthesis in goldfish skeletal muscle at 23°C 48 hours after the injection. Leucine ^{14}-C incorporation was increased in the protein-bound amino-acids in goldfish muscle 1 hour after the administration of a tracer. But there was a significant decrease in the incorporation of leucine ^{14}-C into the free-amino-acids. There was a proportional correlation between the fall in leucine ^{14}-C incorporation into the free-amino-acid of goldfish muscle and the increase in incorporation of leucine ^{14}-C into the protein-bound amino-acids following insulin injection. Similar observations were reported by Jackim & Laroche (1973) concerning Fundulus heteroclitus muscle. These workers demonstrated that leucine ^{14}-C incorporation was increased by insulin, but not changed significantly by exercise, darkness, fish size or sex.

The incorporation of labelled amino-acid into diaphragm protein of alloxan-diabetic rats was significantly depressed and later restored to the normal level by treatment with insulin (Krahl, 1953; Manchester & Young, 1960a). In the current study, alloxan was used to make the fish alloxan-diabetic and to examine alloxan's effect on the incorporation of leucine ^{14}-C into goldfish muscle. However, alloxan had no effect on the incorporation of leucine ^{14}-C into goldfish muscle protein. This may be due to the fact that in the goldfish, unlike mammals, alloxan has no active role to play in the incorporation of leucine ^{14}-C in muscle protein.

With tolbutamide, as with insulin, there was a proportional correlation between the fall in incorporation of leucine ^{14}-C into the free-amino-acid and the increase in leucine ^{14}-C incorporation into the protein-bound amino-acid of goldfish muscle. It is suggested that the administration of tolbutamide increased the release of insulin from the endocrine pancrease. The increased availability of insulin resulting from the action of tolbutamide increased the incorporation of leucine ^{14}-C into protein bound amino-acids and decreased the incorporation into free-amino-acids of goldfish muscle.

VALIDITY OF THE USE OF AMINO-ACID INCORPORATION STUDIES AS A MEASURE OF PROTEIN SYNTHESIS

It has been tactically assumed that the incorporation of labelled amino-acids into protein can be used as a measure of protein synthesis. This assumption needs further consideration and, at the outset, it must be freely admitted that, in the experiments so far, the incorporation of amino-acid can not yet be unequivocally equated with the synthesis of specific biologically active proteins. Indeed experimental work with diaphragm incorporation is not unlikely to represent a net synthesis of new protein, greater in quantity than that already present. Although only a single amino-acid was added to the medium, the other amino-acids required for protein synthesis were already present in the muscle. Nevertheless in a tissue whose total protein content was not increasing, the maintenance of a steady state in the presence of continuous turnover of protein meant that a continuous synthesis of protein must have been taking place.

Workers in this field are aware of the problems concerning the incorporation of labelled amino-acids into the protein, and evidence is beginning to accumulate which offers direct or circumstantial support to the assumption implicit in experiments of this type, that the incorporation of amino-acid is a reflection of true protein synthesis (Campbell, Greengard & Kernat, 1960; Bates & Simpson, 1959).

MECHANISM OF THE ACTION OF INSULIN IN STIMULATING AMINO-ACID INCORPORATION

The action of insulin in stimulating the transfer of labelled amino-acids can be interpreted in various ways. Firstly, in the presence of insulin the rate of total protein synthesis is unchanged, but the rate of transfer of labelled amino-acids from the incubation medium to the site of protein synthesis is accelerated. The proportion of labelled to unlabelled molecules incorporated therefore increases without a rise in the total number of molecules actually being incorporated. Secondly, insulin may induce a rise in the total amount of protein synthesized, the increased rate of incorporation of labelled amino-acids being a direct reflection of additional synthesis. A combination of both of these is also possible.

SUMMARY

(1) Leucine ^{14}-C incorporation was increased in muscle of goldfish at 23°C, 48 hours after injection of insulin.

(2) There was no change in the incorporation of leucine ^{14}-C into goldfish muscle at 23°C in the presence of alloxan.

(3) Tolbutamide increased the incorporation of leucine ^{14}-C into goldfish muscle at 23°C, 48 hours after injection.

CHAPTER VI

EFFECT OF INSULIN ON THE TOTAL RNA AND TOTAL DNA CONTENT OF GOLDFISH (CARASSIUS AURATUS) MUSCLE

INTRODUCTION

Nucleic acids play an important role in the regulation of protein synthesis. The various molecular species of nucleic acid impinge at several steps in the intracellular synthesis of protein. For that reason, it seemed logical to investigate the effect of insulin on nucleic acid synthesis, bearing in mind the possibility that nucleic acid synthesis might be the intracellular locus of insulin action on protein biosynthesis. Wool (1960, 1963) was able to show that insulin increased the incorporation of radioactivity from C^{14}- adenine and C^{14}- glucose into nucleic acid. The effect was solely on the RNA fraction; incorporation into DNA was not affected. Moreover, a new increase in RNA was obtained in diaphragms incubated with insulin. In addition to its action on diaphragm muscle, insulin also stimulated the synthesis of RNA and DNA by explants from the hearts of 13-day-old chick embryos (Leslie, 1952) and the synthesis of RNA by human fibroblast and kidney cells in tissue culture (Leslie, Fulton & Sinclair, 1957). Insulin favoured the incorporation of radioactivity from labelled glucose into adipose tissue (Carruthers, Winegard, 1962). Insulin has also been shown to increase the incorporation of carbon from labelled adenosine into adenine of RNA and DNA in bone marrow (Necheles, 1962).

The rate of protein synthesis in a tissue will probably be affected by (a) the total number of ribosomes present, (b) the proportion of ribosomes in the polysomes, and (c) regulation of the rate of movement

of ribosomes along the messenger. Although in diabetes the RNA content of several tissues declines, (b) and (c) will be more important in the short-term regulation.

Several tissues from diabetic animals, most notably in skeletal muscle, showed decreased rate of protein synthesis and were characterized by having a smaller proportion of their ribosomes contained in polysomes (Wool; Stirewalt, Kurihara, Low, Bailey & Oyer, 1968). The administration of insulin rapidly led to an increase in the proportion of ribosomes in polysomes - even when RNA synthesis was not occurring. Thus insulin promoted initiation, the process of attachment of ribosomes to messenger RNA. How this was affected by insulin was not known, but it seemed unlikely that inherent difference existed between ribosomes from normal and from diabetic tissues (Castles, Rolleston, Wool, 1971; Leader, Wool & Castles, 1971).

The studies described below were performed to assess the effect of insulin on the total RNA and total DNA content of goldfish muscle. Alloxan effects on the total RNA and total DNA concentration of goldfish muscle were investigated. Tolbutamide as potent insulin secretogogue was used, and its effects on the total RNA and the total DNA content of goldfish muscle were observed.

As regards the presence of RNA and DNA in the muscle of fish, the discovery of the carp muscle protein, nucleotropomyosin, marked the first clear-cut demonstration of the presence of RNA in fish muscle (Hamoir, G., 1951). More recently, RNA has been

demonstrated in concentration of 42-142µg/gm in the muscle of Pacific Ocean fish (Bluhm & Tarr, 1957).

Initial attempts to determine DNA in fish muscle extracts met with failure (Hamoir, 1951; Tarr, 1953), probably due to the comparatively low concentration present, and the fact that cell damage was apparently required to liberate nucleic acids from the muscle cells (Love, 1955). The highest concentration of DNA obtained from cod muscle press juice was observed to be 0.628 mg/100 ml (Love, 1955).

MATERIALS AND METHODS

Fish used were goldfish (Carassius auratus) about 10 - 12 g in weight (2 - 3 in length) obtained from WOLVERHAMPTON AQUATICS, WOLVERHAMPTON, STAFFS.

Experimental fish were kept at 25°C in a constant temperature room, with constant illumination and aeration. Every two days the tanks were thoroughly washed out with clean tap water at the appropriate temperature to remove all excreta and uneaten food. Fish were fed daily with commercial baby fish food (TETRA WERKE MELLE, WESTERN GERMANY).

Prior to experimental work, fish were brought to the laboratory and maintained in experimental aquaria containing aerated tap water approximately at the temperature of 23°C. An acclimation period of at least 3 days prior to the initiation of the experimental studies was

used. Food was always withheld 24 hours prior to experimental work.

Fish were treated with insulin, alloxan and tolbutamide separately as follows:-

0.16 I.U/Fish INSULIN (NOVO ACTRAPID, NOVO INDUSTRI A/S COPENHAGEN, DENMARK) was injected intramuscularly.

ALLOXAN MONOHYDRATE (SIGMA CHEMICAL COMPANY) was dissolved in 0.9% saline and injected within 2 to 3 minutes to avoid deterioration. The administered dose was 600 mg/Kg at the volume of 0.3 ml.

TOLBUTAMIDE (as sodium salt) (RASTIONON HOECHST) was prepared in double distilled water and injected intramuscularly. The dose of tolbutamide was 10 mg/Kg body weight.

Control fish were injected with 0.9% saline. Anaesthetics were not used.

ANALYTICAL TECHNIQUE

Preparation of Ribonucleic Acid Standard

10 mg (corrected for moisture) of CALF LIVER RNA was dissolved in 0.3N potassium hydroxide and diluted to 10 ml. A standard curve of RNA concentration vs. optical density was prepared using this stock solution diluted with perchloric acid (PCA) to a final concentration of approximately 0.2N PCA.

Preparation of Deoxyribonucleic Acid Standard

10 mg (corrected for moisture) of CALF THYMUS deoxyribonucleic acid was dissolved in 0.3N potassium hydroxide and diluted to 10 ml. A standard curve of DNA concentration vs. optical density was prepared

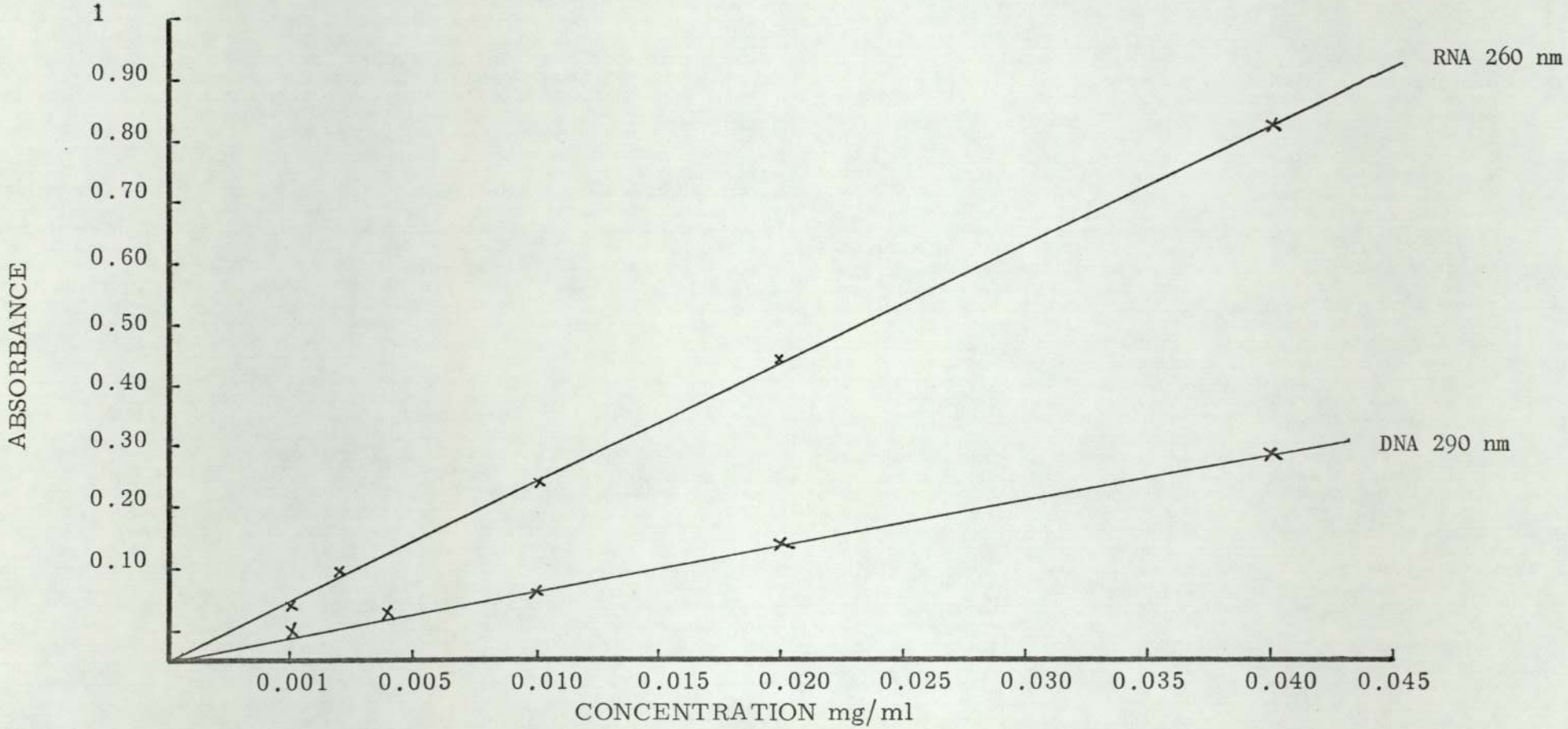
using this stock solution diluted with PCA to a final concentration of approximately 0.2N PCA.

PROCEDURE

Fish were killed by a blow on the head 48 hours after administration had been started, and approximately 100 mg portions of tissue were quickly removed and homogenized in cold distilled water to 0.5 ml solution and frozen at -4°C. The samples were thawed, and 8 ml of cold 10% trichloroacetic acid (TCA) solution was added to 0.5 ml aliquot of homogenate. The precipitate formed was separated from the supernatant fluid by centrifugation at 2000 rev./min. and 4°C. This precipitate was subsequently washed twice with 5 ml of cold 10% TCA. To remove the residual TCA as well as some of the lipid, the precipitate was extracted with 5 ml of a 95% ethanol solution that was saturated with sodium acetate. Additional lipid extractions were performed with 5 ml of a 3 - 1 ethanol-ethyl-ether mixture and 5 ml of anhydrous ethyl ether. The excess ether was removed by drying the precipitate for 5 - 10 minutes. While the precipitate was still moist 4 ml of 0.3N KOH solution was added and the samples were incubated for 60 minutes at 37°C in a constant temperature oven. The resulting KOH hydrolyzate was used for total RNA and total DNA determination.

RNA and DNA were separated by adding 1 ml of cold 60% perchloric acid (PCA) solution to the remaining 2 ml of KOH hydrolyzate. All samples were centrifuged, and an aliquot of the supernatant fluid was

FIG. 13 STANDARD CURVE OF RNA AND DNA



carefully removed for DNA determination. The precipitate was washed twice with 5 ml of cold 5% PCA and stored in 95% ethanol until the final total DNA determinations were performed.

Total RNA concentrations were measured by diluting a 1 ml aliquot of the PCA supernatant fluid to 10 ml and reading this solution in a Beckmann D.B. Spectrophotometer (BECKMAN INSTRUMENTS LTD.) at the wavelength of 260nm. To estimate the total tissue RNA in the sample, these readings were compared with a standard curve which had been obtained from a purified sample of RNA. (Fig. 13.)

The total DNA concentration was determined by adding 1.5 ml of 0.5N PCA to the isolated PCA precipitate and hydrolyzing the nucleic acid after 45 minutes. After centrifugation, the supernatant was poured into a tube and the precipitate was washed twice with 1 ml of 0.5N PCA. These extracts and washes were diluted to 10 ml and read on a Beckman D.B. Spectrophotometer at a wavelength of 290 nm. To calculate the total sample DNA, the readings were compared to a standard curve which had been prepared from a purified sample of standard DNA. (Fig. 13.)

OBSERVATIONS AND RESULTS

Tissue Analysis

EFFECT OF INSULIN

Total RNA and total DNA concentrations in goldfish muscle at 23°C 48 hours after injection are shown in Fig. 14 & 17.

The total RNA content of insulin-treated muscle was higher than

FIG. 14 EFFECT OF INSULIN ON TOTAL RNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)

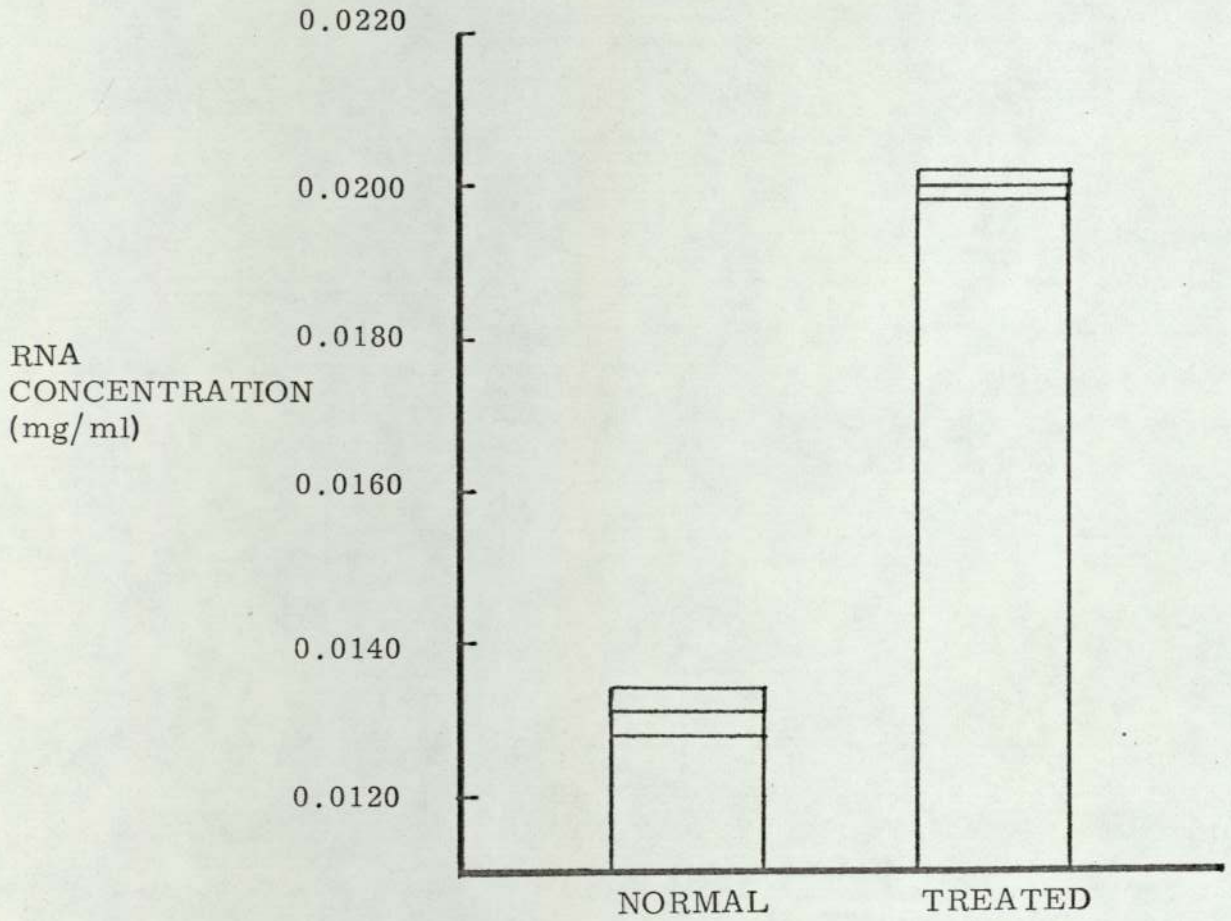
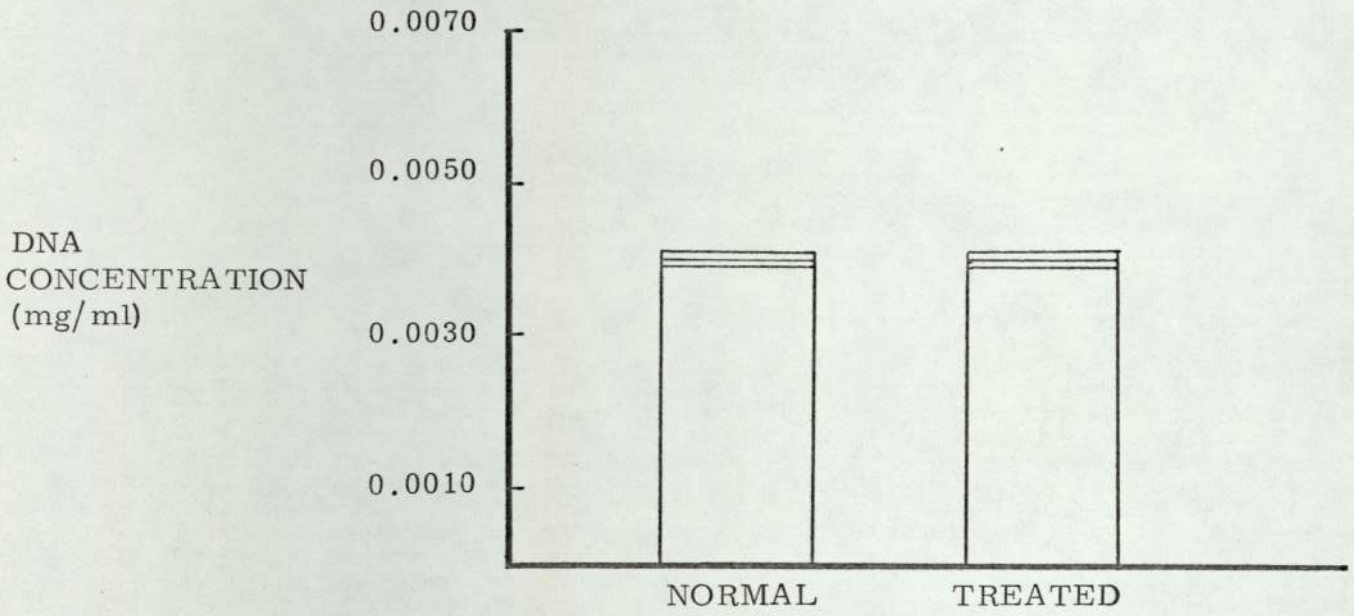


FIG. 17 EFFECT OF INSULIN ON TOTAL DNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)



the content of untreated muscle. The mean values were 0.0200 ± 0.003 mg/ml and 0.0131 ± 0.0008 mg/ml respectively. There was no observable change in total DNA concentration of goldfish muscle after insulin treatment. The normal mean total DNA concentration for goldfish muscle was 0.0040 ± 0.0001 mg/ml.

Fig. 20 shows a dose response curve of insulin with the total DNA content of goldfish muscle at 23°C, 48 hours after injection.

EFFECT OF ALLOXAN

The total RNA and total DNA concentrations of goldfish muscle at 23°C, 48 hours after injection, are shown in Fig. 16 & 19. The value of total RNA content of alloxan treated muscle was less than that for untreated fish. The mean values were 0.0071 ± 0.0004 mg/ml and 0.0132 ± 0.007 mg/ml respectively. There was no change in the total DNA content of muscle after alloxan administration.

EFFECT OF TOLBUTAMIDE

The total RNA and total DNA content of goldfish muscle treated with tolbutamide at 23°C 48 hours after injection is shown in Fig. 15 & 18. The total RNA concentration of treated muscle was higher than that found in untreated muscle. The mean values were 0.0173 ± 0.0004 mg/ml and 0.0130 ± 0.0005 mg/ml respectively. There was no change in DNA content after tolbutamide treatment.

FIG. 20 EFFECT OF INSULIN ON RNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)

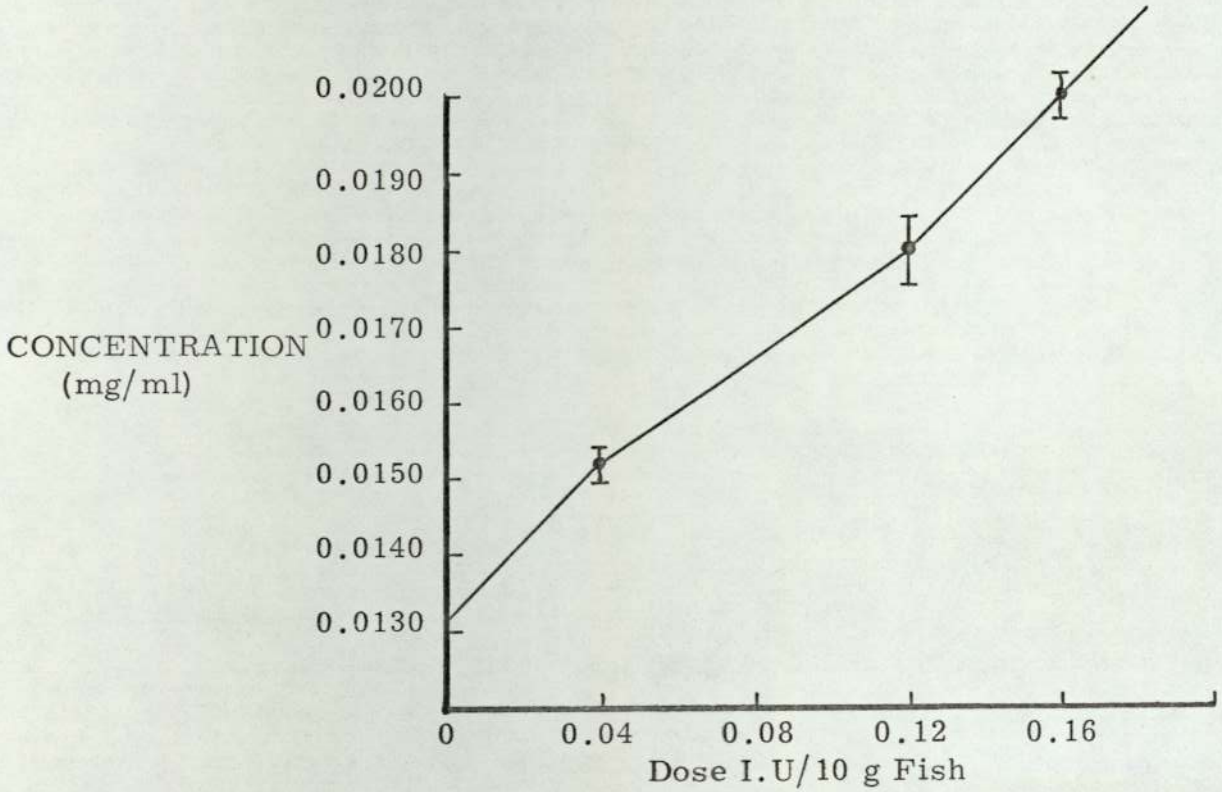


FIG. 16 EFFECT OF ALLOXAN ON TOTAL RNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER INJECTION)

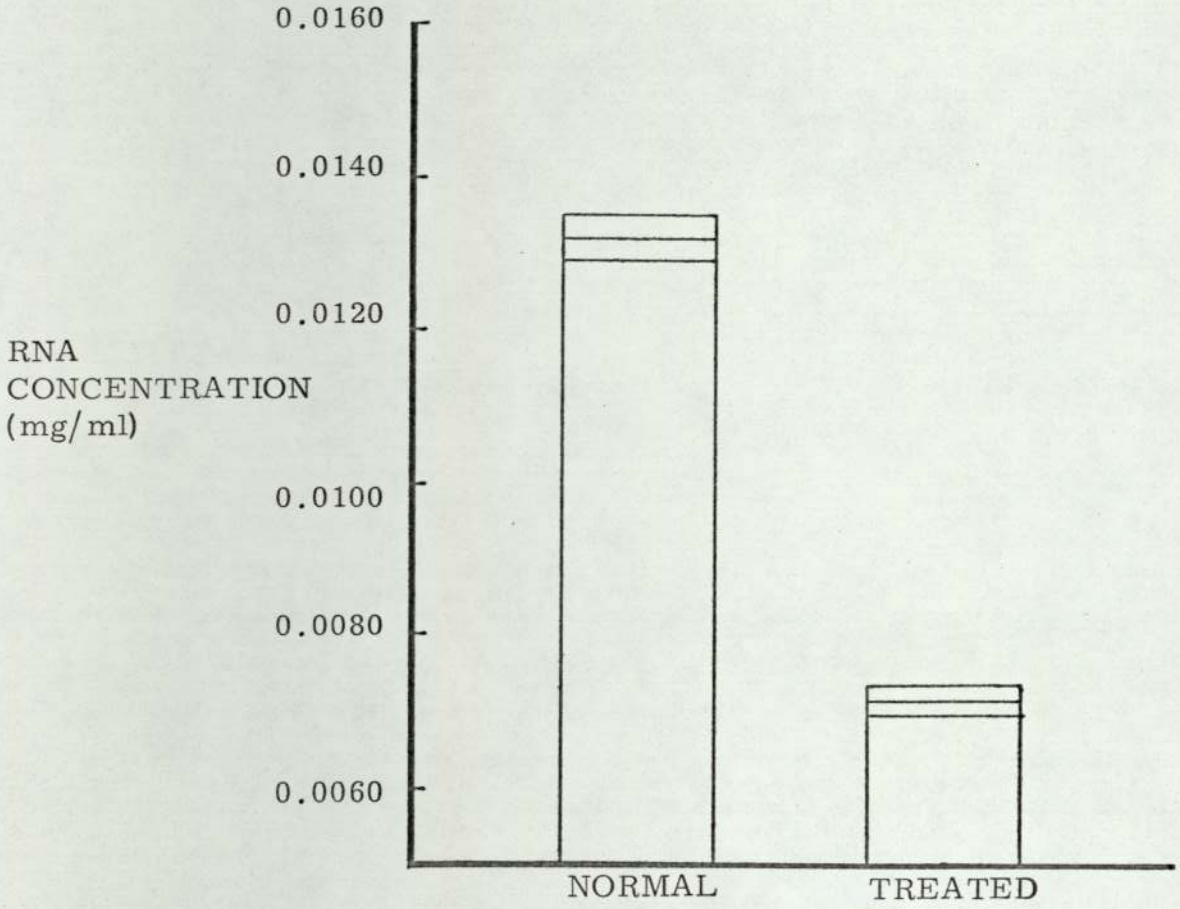


FIG. 19 EFFECT OF ALLOXAN ON TOTAL DNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)

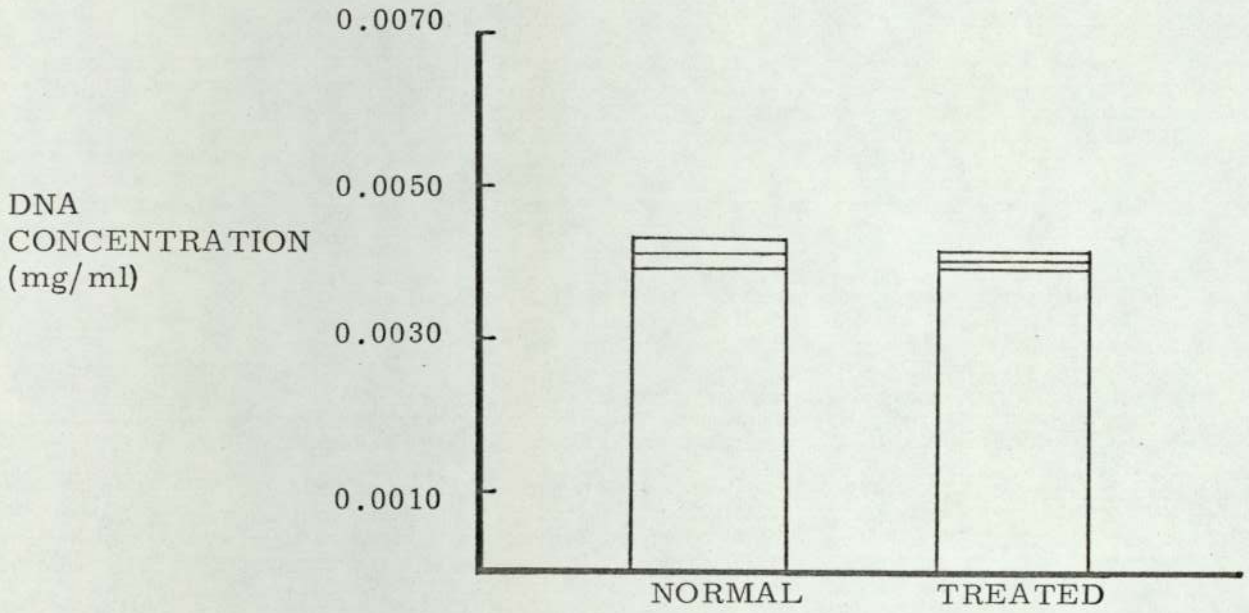


FIG. 15 EFFECT OF TOLBUTAMIDE ON TOTAL RNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)

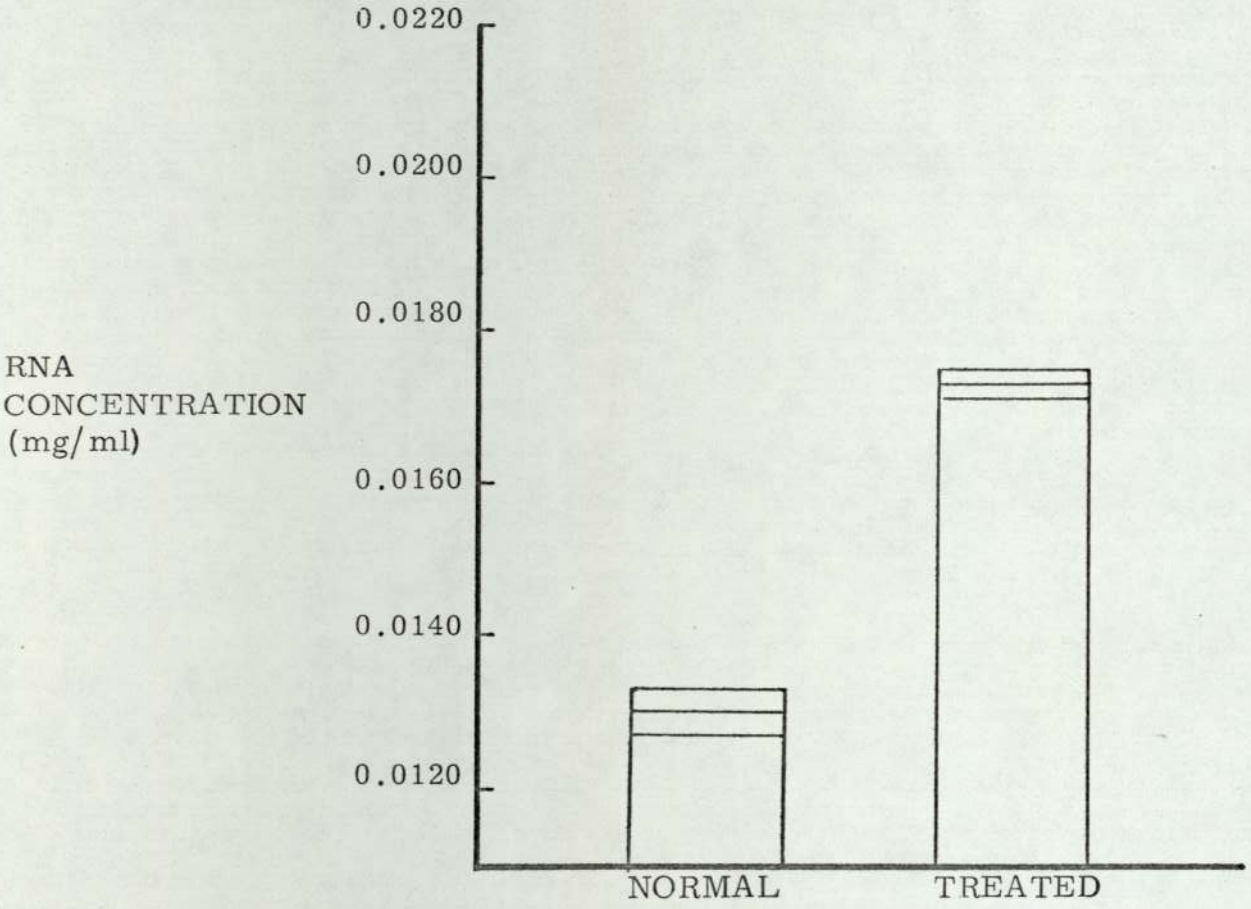
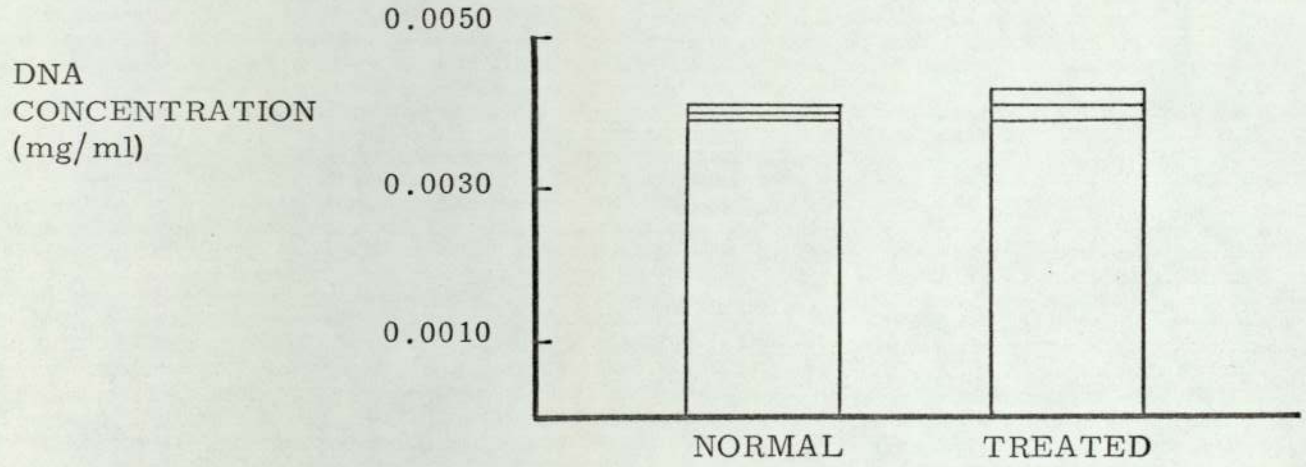


FIG. 18 EFFECT OF TOLBUTAMIDE ON TOTAL DNA CONTENT OF GOLDFISH MUSCLE (48 HOURS AFTER SINGLE INJECTION)



DISCUSSION

The specificity of the template is determined by the nucleotide sequence of m RNA and the protein itself is released from the m RNA tempelate . The sites of active protein synthesis are also rich in RNA and that increased number of RNA particles could be attributed to increased synthetic activity of the cells. In the present study, insulin increased the total RNA content in the muscle of goldfish at 23°C 48 hours after injection. It is suggested that the synthetic activity of the cell was increased, and increased concentration in RNA content was thought to be due to increased protein synthesis. Alloxan decreased the total RNA content of goldfish muscle at 23°C 48 hours after injection. One might suggest that, after alloxan treatment, there was a deficiency of circulating insulin and probably because of this the total RNA content in the muscle was effected. Possibly, the synthetic activity of the cell was decreased and so the total RNA content was also decreased, and this tended to decrease protein synthesis.

Tolbutamide increased the total RNA content of goldfish muscle at 23°C 48 hours after injection. It is generally accepted that hypoglycemic effect of sulfonylurea compounds is mediated by the release of increased amounts of insulin from the pancreas (Maingay, Ruyter, Touber, Croughs, Schopman and Lequin, 1967). Yalow et al (1962) have shown, using the immunochemical assay for insulin, that the administration of tolbutamide to normal human subjects produces an increased circulating level of serum insulin. Tolbutamide injection,

acting via increased insulin levels, might have elevated the total RNA content of goldfish muscle. The increased total RNA content of goldfish muscle may be consonant with increased protein synthesis.

Data from experimental work with ribosomes from insulin treated animals have provided a great deal of support for several of the theories of insulin action (Wool and Munro, 1963). No matter by which mechanism insulin increases protein synthesis in muscle, it would seem unlikely that an increase in protein synthesis could account for the several other metabolic effects of the hormone. It has been shown by a number of investigations (Eboue-Bonis, Chambaut, Volfin & Clauser, 1963; Carlin, Hechter, 1964; Castles, 1964) that puromycin in a concentration almost completely suppresses protein synthesis in muscle, but does not interfere with the action of the hormone in increasing glucose uptake, amino-acid transport or nucleic acid synthesis. Thus, the stimulation of these several processes seems to be independent of an increase in protein synthesis. Just as the insulin-mediated increase in protein synthesis is independent of an increase in glucose or amino-acid transport, so the reverse is also true, that an increase in substrate transport is independent of an increase in protein biosynthesis.

SUMMARY

(1) The total RNA concentration of goldfish muscle treated with insulin increased markedly at 23°C, 48 hours after injection. Total DNA concentration was not changed by insulin treatment.

(2) Alloxan lowered the total RNA concentration of goldfish muscle at 23°C, 48 hours after injection. There was no change in the total DNA concentration of goldfish muscle after alloxan treatment.

(3) Tolbutamide administration for 48 hours produced an increase in the total RNA concentration of goldfish muscle. No change in total DNA concentration was observed after tolbutamide treatment.

CHAPTER VII

THE EFFECT OF INSULIN ON THE FREE AMINO-ACID CONCENTRATIONS OF GOLDFISH (CARASSIUS AURATUS) MUSCLE

INTRODUCTION

The free amino-acid fraction of teleost muscle has been the subject of considerable research in recent years because of the growing importance of fish as a source of animal protein in human nutrition (reviews by Tarr, 1958, Cowey, 1965) and variations in free amino-acid patterns have been shown to occur. Jones (1959) measured the composition of the free amino-acid fraction in the muscle of the lemon sole for a 3-year period, and found that the concentrations of the different amino-acids varied greatly, some of them seasonally. He suggested that these variations were the result of the type of food, starvation, temperature, sex and gonad maturation. A recent detailed study on the free amino-acid pattern in Atlantic salmon (Salmo salar) muscle, was conducted on migrating marine and fresh water adults and on young fresh water parr and older smolt (Cowey et al, 1962; Cowey & Parry, 1963). They found a gradual change in the concentration of free-amino-acids in the muscle of maturing salmon, which contrasted with the sudden change in fatty acid composition.

However, Halver & Shanks (1960) found that Chinook salmon (Oncorhynchus tshawytscha) require orginine, histidine, isoleucine, leucine, lysine, methionine, phenylalamine, threonine, trypotophan and valine for normal growth. Alanine, aspartic acid, cystine, glycine, glutamic acid, proline, serine, hydroxyproline and tyrosine were not required for growth. From this data they concluded that

the essential amino-acids of teleosts such as Chinook salmon were identical to those of man and many other vertebrates.

The problem of whether or not insulin effects the transport of amino-acids has been, as Wool (1965) has said, a vexatious one. Almost without exception, what has so far been done has been to examine the problem with the aid of radioactively labelled amino-acids. Under these conditions, the results obtained represented the accumulation of radioactivity in the cell, and this may not have given a true picture of the actual concentration of the amino-acid there. Clearly, a multiplicity of fates may befall a natural amino-acid once it has found its way into the cell interior. It may be incorporated into protein or utilized in transamination or deamination reactions. It may even leave the cell, returning back into the medium. However, the incorporation of labelled amino-acids into the muscle protein has been demonstrated by several workers to be independent of glucose transport (Wool & Krahl, 1959). Recently, Jackim & Laroche (1973) demonstrated that leucine incorporation was increased by insulin in Fundulus heteroclitus muscle. This present study described below was performed to evaluate the effect of insulin, alloxan and tolbutamide on free amino-acid concentrations in goldfish muscle.

MATERIALS AND METHODS

Goldfish (Carassius auratus) about 10 - 12 g in weight (2 - 3 in. length) were obtained commercially from the BULL RING SHOPPING CENTRE, BIRMINGHAM. Fish were maintained at 25°C with constant illumination and aeration. Fish were fed daily with commercial baby fish food (TETRA-WERKE, MELLE, WESTERN GERMANY). An acclimation period of at least 3 days was allowed in experimental laboratory aquaria prior to the initiation of experimental work. Food was withheld 24 hours prior to the experiments.

Fish were treated with insulin, alloxan and tolbutamide as described below:-

INSULIN 0.16 I.U/Fish (NOVO ACTRAPID, NOVO, INDUSTRI A/S COPENHAGEN, DENMARK) was injected intramuscularly.

ALLOXAN MONOHYDRATE (SIGMA CHEMICAL COMPANY) was dissolved in 0.9% saline and injected immediately to avoid deterioration. The administration dose was 600 mg/Kg at the volume of 0.3 ml.

TOLBUTAMIDE (as sodium salt) (RASTIONON HOESCHST) was prepared in double distilled water and injected intramuscularly. The dose of tolbutamide was 10 mg/Kg.

Control fish were injected with 0.9% saline.

ANALYTICAL TECHNIQUE

Fish were killed by a single blow on the head 48 hours after injection, and approximately 100 mg portions of muscle were quickly removed and homogenized in 2 ml of 10% trichloroacetic acid (TCA). The precipitate was separated from the supernatant fluid by centrifugation. 1 ml of the supernatant fluid was diluted to 10 ml with sodium citrate buffer PH2.2 (originally developed by Moore & Stein, 1958). By using the long column of the Amino-acid Analyser (EVANS ELECTRO SELENIUM LIMITED) the concentrations of acidic and neutral amino-acids were determined in 1 ml of the 10X diluted solution. The short column of the Amino-acid Analyser was used to estimate the basic amino-acid concentrations in an aliquot taken from the 10X diluted solution. Buffer pressure and Ninhydrin pressure were adjusted as appropriate. A standard mixture of amino-acids (EVANS ELECTRO SELENIUM LIMITED) was diluted ten times and treated in exactly the same was as the samples. The measurement of individual amino-acid concentration was calculated as follows:-

The concentration of each amino-acid present in a sample was indicated by the integrated area of the peaks representing it on the chromatogram. This was assessed by the height/width method and results were expressed in μmoles/ml. $(\frac{HXW}{C})$ when H is the total height, W the total width, and C the actual amino-acid concentration from the standard mixture of amino-acids.

However, one cannot estimate tryptophane and cystine with this Amino-acid Analyser (EVANS ELECTRO SELENIUM LIMITED).

OBSERVATIONS AND RESULTS

EFFECT OF INSULIN

The free amino-acid levels in muscle of goldfish at 23°C 48 hours after insulin injection (0.16 I.U/Fish) were reduced significantly compared with control values, Table 18. The reductions in the concentrations of threonine, lycine and histidine was highly significant ($P<0.001$). Glutamic acid, glycine and alanine levels were significantly reduced ($P<0.01$). The proline, leucine and arginine levels were significantly reduced ($P<0.02$). The levels of the remaining amino-acids were unchanged.

In the presence of a minimum dose of insulin (0.04 I.U/Fish) threonine and histidine were reduced significantly ($P<0.01$) (Table 19). The levels of glutamic acid, alanine and lycine were significantly reduced ($P<0.02$). The levels of the remaining amino-acids were unchanged.

EFFECT OF ALLOXAN

Alloxan significantly reduced the levels of threonine, histidine ($P<0.001$) and proline ($P<0.02$) in goldfish muscle.

EFFECT OF TOLBUTAMIDE

The levels of lycine and histidine in goldfish muscle (23°C) were significantly reduced ($P<0.001$) after tolbutamide injection (Table 20). The levels of threonine ($P<0.01$), proline, alanine, leucine and arginine were significantly reduced with the levels of ($P<0.02$). The remaining amino-acids were unchanged.

SUMMARY

(1) Insulin significantly suppressed the concentrations of threonine, lycine, histidine, glutamic acid, glycine, alanine, proline leucine and arginine in goldfish muscle at 23°C, 48 hours after the injection.

(2) Alloxan treatment significantly lowered the concentrations of threonine, histidine and proline in goldfish muscle at 23°C.

(3) The administration of tolbutamide significantly reduced the concentrations of threonine, lycine, histidine, proline, alanine, leucine and arginine in goldfish muscle at 23°C, 48 hours after the injection.

CHAPTER VIII

INSULIN SECRETION IN RESPONSE TO HIGH AND LOW PROTEIN INGESTION IN RAINBOW TROUT (SALMO GAIRDNERI)

INTRODUCTION

For studies on insulin biosynthesis, the goosefish or angler fish (Lophius piscatorius) is especially advantageous since it yields up to 150 mg of islet tissue per fish (Bauer, Lindal and Lazarow, 1965). The biosynthetic studies with insulin on this preparation and the detailed methods used, have been extensively reviewed (Bauer et al, 1965; Lazarow, 1963). The rate of incorporation of labelled amino-acid into insulin of goosefish islet tissue incubated in vitro has shown to increase progressively with increasing time of incubation (Bauer, Lazarow, 1961). The process was dependent upon amino-acid concentration in the medium and on the presence of oxygen. Radioactivity from labelled glucose was likewise incorporated into goosefish islet insulin and many of the constituent amino-acids were derived from the glucose added to the medium (Bauer et al, 1965). Patent & Foa (1971) demonstrated in toadfish that leucine stimulated insulin secretion in the presence of glucose. They suggested that glucose and leucine might act synergistically in their preparations, as they do in mammalian islets (Malaisse & Malaisse, Lague, 1965) In the rabbit, leucine was apparently the only amino-acid capable of stimulating insulin release in the absence of glucose (Milner, 1969), although the evidence on this point was conflicting (Edgar, Rabinowitz & Merimee, 1969). Patent and Foa (1971) found that arginine plus

glucose did not stimulate insulin release, whereas, according to Edgar et al (1969) this amino-acid stimulated insulin release in mammals.

Floyd, Fajans, Conn, Knopf and Rull (1966) demonstrated that, in healthy subjects, the ingestion of a protein meal released a large increase in the plasma insulin level, and have concluded that this was an important physiological phenomenon. Tashima and Cahill (1968) found that protein meals, but not oral glucose, lead to significantly increased insulin levels in the toadfish. They suggested that, in toadfish, amino-acid and not glucose might serve as the major stimulus for insulin secretion.

The role of insulin on protein metabolism in the muscle of goldfish has been described previously in this Thesis. The purpose of this study was to demonstrate the secretion of insulin in response to protein ingestion in rainbow trout.

MATERIALS AND METHODS

Rainbow trout (Salmo gairdneri) were obtained from BIBURY TROUT FARM, GLOUCESTERSHIRE. There were about 250 to 300 g in weight and 10 - 11 in. length.

Fish were kept at 10°C to 12°C in large tanks with continuously running well-aerated water. Sometimes, carbon filters were incorporated into the inflow system of water supply to check the variations of chlorine content of the tap water. Commercially available beta floating trout pellets were given daily to the animals (COOPER NUTRITION PRODUCTS LTD., WITHAM, ESSEX). Every four to five days the tanks were thoroughly cleaned to remove all excreta and uneaten food.

Prior to the experimental work, fish were divided into two tanks containing continuously running aerated tap water, the one for high protein fed animals and the second for low protein fed animals. An acclimation period of at least 3 days prior to the initiation of the experiment was used. The fish were weighed individually in each tank.

Two diets were formulated using different levels of protein. The one as high protein diet containing 54% protein, and the second as low protein diet containing 24% protein. Each diet was prepared in the following way:-

HIGH PROTEIN DIET

Dry Ingredients	%
Fish Meal	70
Wheat Middlings	14
Soya Bean Oil	5
Vitamin Premix	10
Durabond Binder	1

LOW PROTEIN DIET

Dry Ingredients	%
Fish Meal	30
Alpha Cellulose	40
Wheat Middlings	14
Soya Bean Oil	5
Vitamin Premix	10
Durabond Binder	1

Water was added to the dry mix at the rate of 150 ml/100 g in each diet.

The vitamin premix was made of pure vitamins added to Wheat Middlings as a carrier. The quantity of vitamins added were such that the quantities in the finished diet were in the following proportions (after Halver, 1957):-

Vitamin	mg/100 g finished diet
Thiamin (pure)	5
Riboflavin (97%)	20
Pyridoxine (pure)	5
Choline chloride (50%)	500
Nicotinic acid (pure)	75
Inositol (pure)	200
Biotin (1%)	0.5
Folic acid (pure)	1.5
L. ascorbic acid (pure)	100
Vitamin B_{12} (100/Kg)	0.01
Menadione (K) (pure)	4
Alpha tocopherol acetate (E) (25%)	40

In addition, the following were added to give quantities shown in the finished diet:-

Vitamin D_3 activity 132 I.U

Vitamin A activity 660 I.U

All vitamins were mixed into dry wheat middlings carrier except B_{12} which was added to the finished diet with water.

The fish meal, wheat middlings and alpha cellulose were mixed together in the dry state until a uniform mix was obtained. The vitamin premix was then added and mixed thoroughly with soya bean oil. A suitable quantity of a deep red dye - Permecol - was dissolved in water and added to the diet to give a pink colour. Durabond binder

was then added with water until a stable consistency was obtained. The diets so prepared were then extruded through a Prestige sponge mincer to make pellets. The latter were left to dry at room temperature for at least 48 hours. In each of the diets the source of protein used was fish meal, (72% protein) and wheat middlings (16% protein). By varying the amount of fish meal by substituting with alpha cellulose, two diets were prepared with two different protein levels. Each diet contained the same amount of carbohydrate (mainly wheat middlings), the same amount of fat (soya bean oil), and the complete complement of vitamins. The percentage protein in each diet was estimated using the Macrokjeldahl method. Fish were fed daily at the feeding level of 1% of the body weight with each of the diets.

BLOOD SAMPLING

Blood was obtained from the ductus cuvieri, which runs subcutaneously, immediately beneath the operculum, and is easily accessible. The vein is surrounded by loose connective tissue, which necessitates a careful puncturing of the vessel. The fish were killed by a single blow on the head after 19 days of feeding, and weighed individually. Blood samplings were performed by puncturing the ductus cuvieri as shown in Plate 9, using a heparinized needle and syringe. The blood sample was immediately centrifuged and the plasma separated off. The cystic duct and surrounding islet tissue was removed for histological examination.

PLATE 9

HISTOLOGICAL TECHNIQUE

Histological examination of the islets of Langerhans was carried out as follows:-

Pancreatic tissue was fixed for 24 hours in a Bowin's fluid, dehydrated through graded alcohols, cleared in xylene and embedded in paraffin wax. Serial sections of 5μ thickness were cut using a Cambridge microtome, (CAMBRIDGE INSTRUMENT CO. LTD., CAMBRIDGE, ENGLAND) and stained with hematoxylin and eosin.

Islets for photography were selected and coloured photomicrographs were taken of a Zeiss Mk.I Photomicroscope (CARL ZEISS, DEGENHARDT & COMPANY LTD. LONDON) using Ektochrome EHB 135-20 (KODAK, LONDON), bulb temperature 3200^{o}C.

RADIOIMMUNOASSAY USED IN PRESENT STUDY

The insulin radioimmunoassay procedure adopted for the present study was the double antibody method of Hales & Randle (1963 a, b). Characteristic of this method is the use of a second antibody, rabbit anti-r-globulin serum to precipitate the insulin antibody complex. The process is termed immunoprecipitation. In this way, the antibody bound insulin may be separated from the free labelled insulin present in the final reaction mixture, using an antibody specific to insulin. The insulin in the sample competes with added radioactive insulin for reaction with the antibody specific to insulin. The insoluble insulin antibody complex is separated off and measured for radioactivity. The level of radioactivity is related in an inverse manner to the amount of insulin present in the sample.

REAGENTS

Buffer solutions were prepared with deionized water (ELGASTAT, ELGA PRODUCTS LTD.) because the quality of ordinary distilled water is inconsistent and may give rise to variations in the amount of insulin bound to the antibody precipitate.

Buffer A, a phosphate buffer (40 mmol, PH 7-4) containing bovine serum albumin (0.51% w/v) and sodium ethyl mercurithiosalicylate (thiomersalate) (0.6 mmol) was used to dilute the iodinated insulin. The buffer was prepared by dissolving 5.1 g bovine serum albumin, 6.2 g $Na\ H_2\ Po_4,\ 2H_2O$ and 0.25 g thiomersalate in a litre of deionized water. The solution was adjusted to PH 7-4 with aqueous NaOH and diluted with deionized water to a final volume of 1 litre. The PH was checked and readjusted to PH 7-4 with further NaOH if necessary. It was stored at 2^{o} - $4^{o}C$.

Buffer B was an isotonic phosphate buffer comprised of buffer A plus 0.9% (w/v) NaCl. This buffer was used to dissolve and dilute the standard insulin and to dilute assay samples when this was required, and was stored at 2^{o} - $4^{o}C$.

Buffer C was a high protein buffer consisting of buffer A and horse serum in equal proportions, used for washing the antibody precipitate. The horse serum (No. 5, WELLCOME REAGENTS LTD., BECKENHAM) was used immediately after purchase and rarely required filtering.

The insulin antibody and the labelled insulin preparations were obtained from the RADIOCHEMICAL CENTRE, AMERSHAM, in a commercially available insulin immunoassay kit.

The antibody was supplied in a preprecipitated (insulin binding) reagent, having been prepared for the RADIOCHEMICAL CENTRE by WELLCOME REAGENTS LTD. It was stored at 2^{o} - 4^{o}C in a dry state. After reconstitution with deionized water (8 ml per vial as supplied) storage at 4^{o}C for three days was permissible, but the material must not be frozen and thawed more than once. Purified codfish insulin used as standard was kindly supplied by the INSTITUTE OF MARINE BIOCHEMISTRY (NATURAL ENVIRONMENT RESEARCH COUNCIL) ABERDEEN, SCOTLAND. Purified bovine insulin was used as tracer. The bovine insulin labelled with I^{125} was prepared for the Radiochemical Centre by Wellcome Reagents Ltd. Each vial contains 0.1 µg of iodinated insulin dissolved in 5 ml of buffer A. The working solution was prepared by adding 1 ml of the reagent with the aid of a micro-syringe, to 7 ml of buffer A. 0.1 ml of the diluted mixture will contain 250 picogram of iodinated insulin. The working solution was kept at 2^{o} - 4^{o}C for not more than a week, or deep frozen for longer periods. It was not re-frozen after thawing.

Purified codfish insulin was dissolved in buffer B, and solutions containing 10, 6, 4, 3, 2, 1 and 0.5 ng/ml were prepared by serial dilution for the construction of a standard curve.

PROCEDURE

The complete procedure was performed in polystyrene tubes, 10 mm diameter and 64 mm long, fitted with polythene stoppers (HOPKINS & WILLIAMS LTD., ROMFORD). All reactants were added in aliquots of 100 µl using an Eppendorf Marburg micropipette

equipped with interchangeable plastic tips (A.V. HOME & CO. LTD., LONDON).

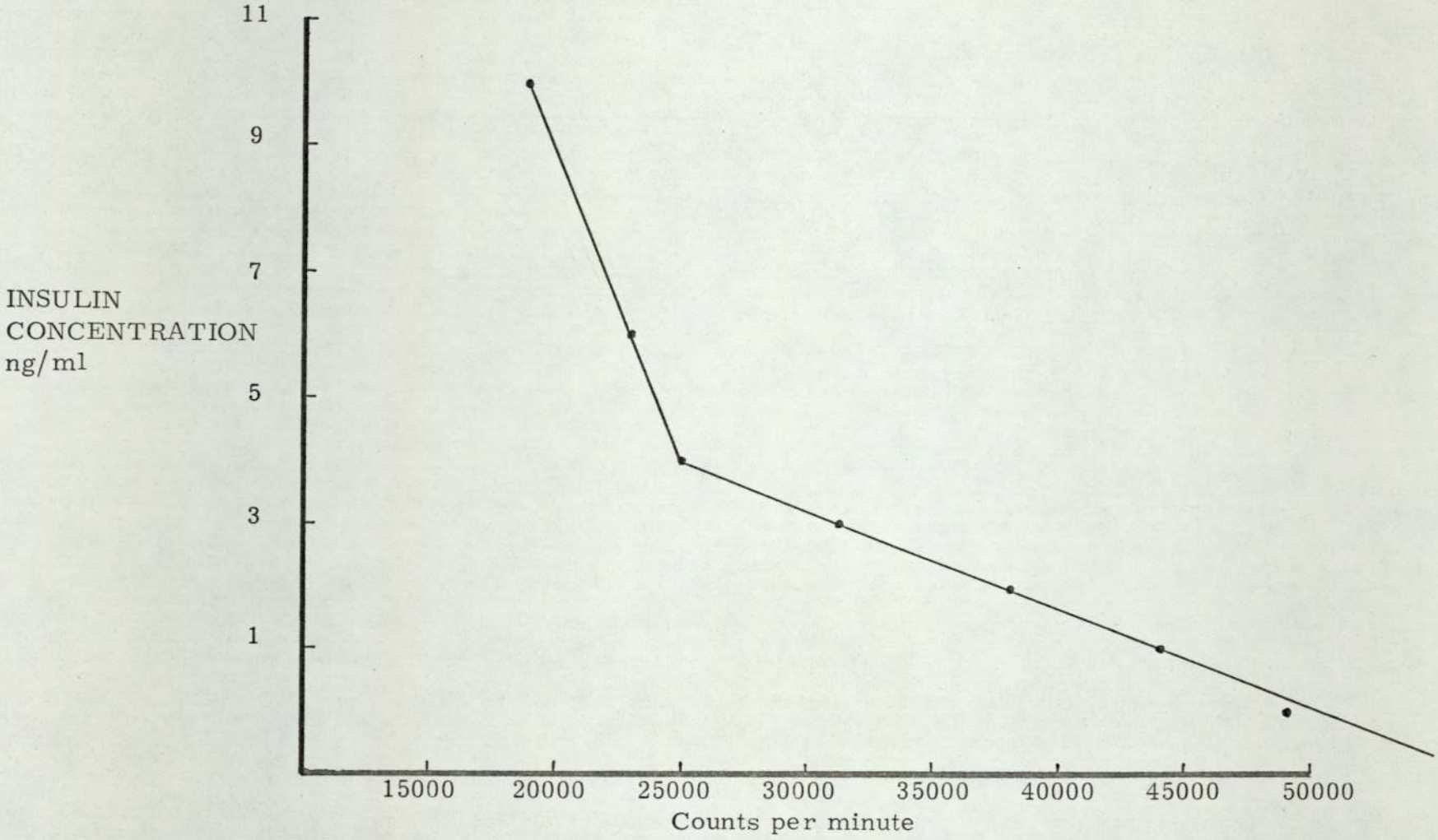
The essential features of the assay are described below:- Unlabelled insulin (standard or assay sample) was initially reacted with the insulin binding reagent (precipitated insulin antibody). Duplicates or triplicates of the same sample were determined in certain experiments to improve accuracy. Triplicate sets of codfish insulin standards (0.5, 1, 2, 3, 4, 6 and 10 n.g/ml) zero and blank tubes were incubated in assay. The zero tube contained buffer B in place of the unlabelled insulin in order to evaluate the amount of radioactivity of the insulin antibody complex in the absence of unlabelled insulin. The blank (or control) tubes contained buffer A in place of the insulin binding reagent in order to evaluate the washing procedure of the antibody precipitate. The initial reactants were mixed using a vortex mixer (WHIRLIMIXER, FISONS SCIENTIFIC APPARATUS LTD., LOUGHBOROUGH) and incubated in a refrigerator at $4^{o}C$ for 12 hours.

Following the initial incubation, a 100μl aliquot of tracer hormone, comprising 250 pg of I^{125}-labelled bovine insulin was introduced, and the reactants were vortex mixed, and subsequently incubated for 24 hours at $4^{o}C$. Triplicate samples of labelled insulin were also added to empty tubes and retained at $4^{o}C$ without further additions. These tubes were used to determine the total count of tracer hormone added. At the end of the second (24 hours) incubation, the interaction of the labelled and unlabelled hormones with the antibody was assumed to be completed.

After the second incubation period, the precipitated antibody-bound insulin and the free labelled insulin were separated by centrifugation for 1 hour at 4°C using an MSE Mistral 4L refrigerated centrifuge (MEASURING AND SCIENTIFIC EQUIPMENT LTD., LONDON) at 1,500 rev/min. The supernatant was decanted off by everting and draining the tubes. The precipitate remaining in the tube was washed with 1 ml of buffer C at 4°C and the contents of the tube were vortex mixed. The sequence of centrifugation, decantation and washing was repeated twice to ensure the removal of all free insulin. After the third washing, each tube was recentrifuged, after which the supernatant was decanted and the precipitate allowed to dry at room temperature for 12 hours. The radioactivity of the precipitate was counted for 10 minutes using a gamma scintillation spectrophotometer gamma set 500, (ICN TRACERLAB DIVISION, BELGIUM). The counts were corrected for background and decay. Duplicate or triplicate determinations were averaged and any grossly aberrant counts rejected. The final values were converted to ct/min.

A standard curve was constructed as a semi-logarithmic plot to obtain a linear expression. The log of the insulin concentration was plotted against the respective averaged corrected counts per minute. A typical standard curve is shown in Fig. 21.

FIG. 21 TYPICAL STANDARD CURVE OF CODFISH INSULIN



OBSERVATIONS AND RESULTS

The plasma insulin level was significantly elevated after 19 days of feeding with high protein diet, compared with controls, and low protein values. The significant difference was $P < 0.001$. The mean plasma insulin level of the control group (34% protein) was 2.40 ± 0.03 n.g/ml, compared to 4.00 ± 0.07 n g/ml for the high protein fed animals (54% protein) and 2.26 ± 0.14 n.g/ml for the low protein fed animals (24% protein).

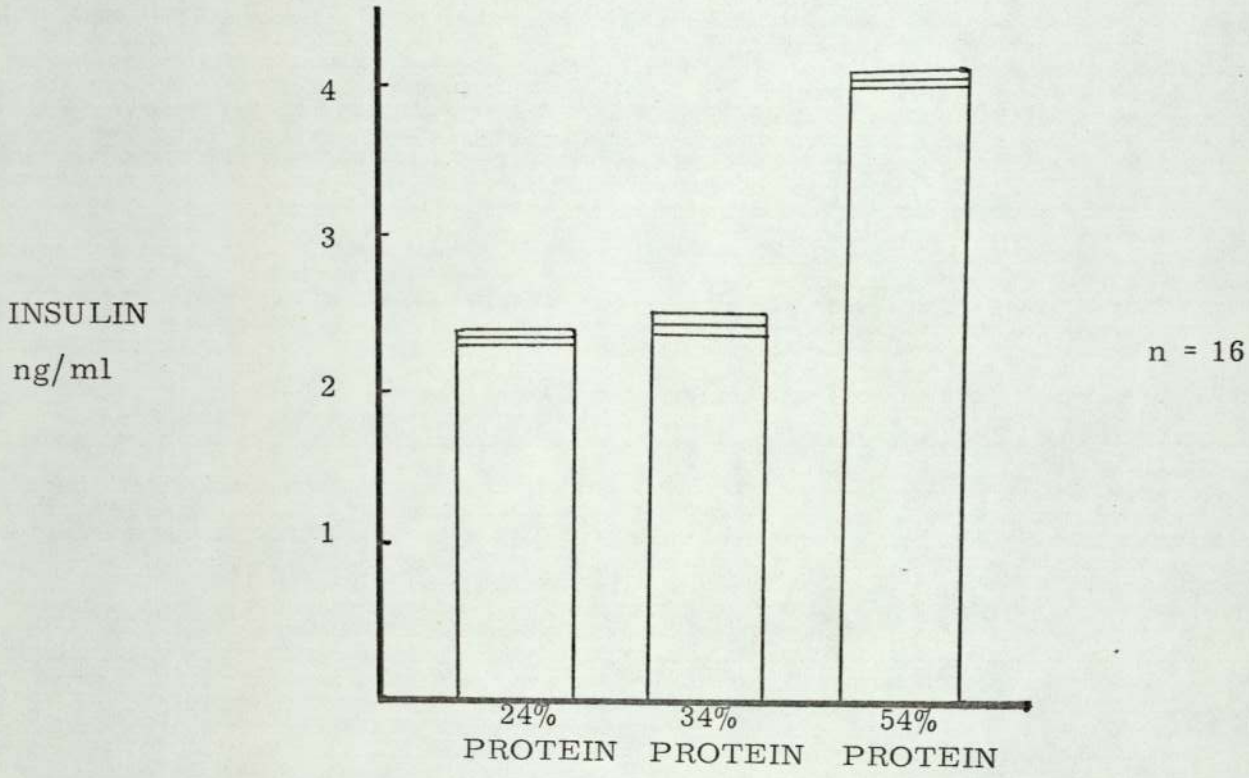
There was an 8% increase in the mean body weight of the animal fed on the high protein diet after 19 days. The mean body weight of animals feeding on the low protein diet was decreased by only 2% after 19 days of feeding.

Morphological changes were not observed in the islets of Langerhans of fish fed on either high or low protein diet.

DISCUSSION

The role of insulin in increasing total protein synthesis in mammals is well established (Sniper 1968). Floyd, Fajans, Conn, Knopf and Rull (1966) demonstrated that in healthy subjects the ingestion of a protein meal resulted in a large increase in the plasma level of insulin and have concluded that this was an important physiological phenomenon. Floyd, Fajans, Conn et al (1966) later concluded that intravenous administration of essential amino-acid induced the secretion of insulin. The phenomenon of amino-acid induced release of insulin did not depend upon the presence of leucine in the infusion mixture. The intravenous

FIG. 22 PLASMA INSULIN OF TROUT AFTER 19 days FEEDING



administration of individual essential amino-acids also induced the release of insulin; however, there were large differences among the individual amino-acids in their capacities to stimulate insulin secretion.

The literature reveals little information on the rate of protein synthesis in fish. Insulin stimulates protein synthesis and enhances the transport of amino-acids into the muscle of goldfish, as described previously in this Thesis. Patent & Foa (1971) showed that leucine stimulated insulin release to an extent comparable to that of glucose in toadfish islets. Tashima & Cahill (1968) found that a protein meal, but not an oral glucose load, significantly increased circulating insulin levels in toadfish and suggested that, in this fish, amino-acids and not glucose might serve as the major stimulus for insulin secretion. Similarly in this present study with rainbow trout, a high protein diet caused an increase in the plasma insulin level, which might be interpreted to reflect an increase in the rate of insulin secretion. It is possible that the liberation of amino-acids into the gastro-intestinal tract during the digestion of the protein or the absorption of amino-acids might enhance insulin secretion. In addition, there was an 8% increase in the mean body weight of trout fed on the high protein diet. It might be suggested that insulin increased the body weight of trout by synthesizing more muscle protein.

SUMMARY

(1) The ingestion of a high protein diet for 19 days resulted in a significant increase in the plasma insulin level of rainbow trout.

(2) The mean body weight was increased in fish fed on high protein diet, while there was a decrease in the mean body weight of fish fed on the low protein diet.

(3) There were no morphological changes in the islets of Langerhans of trout feeding on either a high or a low protein diet.

GENERAL DISCUSSION

Insulin is known to influence many biological processes, but its role in the control of protein metabolism is not as widely appreciated or understood as its effects on glucose or fat metabolism. While the blood glucose level has served as an index of the effect of insulin on carbohydrate metabolism and free fatty acid levels as an index of fat metabolism, until recently no such simple, easily measured, index of protein metabolism was available. Although the use of changes in free amino-acid concentrations as an index of protein metabolism has been used for a number of years, efforts in this field have been hampered by difficulties in methodology. Recently, investigations concerning the relationship of insulin with protein and amino-acid metabolism have provided one of the most active areas of research. Work in this area has produced sufficient evidence to formulate a tentative hypothesis to explain the physiological role of insulin in fish (Tashima & Cahill, 1968; Jackim & Laroche, 1973).

The work presented in this Thesis has investigated certain aspects of the action of insulin in controlling the overall metabolism of nitrogen in goldfish. On the basis of the experimental work described in this Thesis, it appears that the marked increase in ammonia production induced by insulin and tolbutamide was the result of the hormone stimulating some aspects of tissue metabolism, probably protein metabolism. Recently it has been shown that excreted ammonia is derived mainly from that already circulating through the gills and

delivered there as preformed ammonia (Goldstein et al, 1964). As far as the action of insulin is concerned, one might postulate two possible mechanisms of action. Insulin might facilitate the entry of amino-acids into the cell, in which case the increased intracellular substrate would result in increased ammonia production. On the other hand, it might enable ammonia to leave the cell more quickly, thus allowing the deamination reaction to proceed faster.

Both insulin and tolbutamide markedly increased the amino-acid nitrogen content of goldfish muscle while alloxan, on the other hand, decreased the amino-acid nitrogen content. Insulin and tolbutamide also increased the total nitrogen content of goldfish muscle, but alloxan reduced this in the muscle. It might be assumed, therefore, that insulin may be playing a definite role in the actual transport mechanism for amino-acids into the cell. The reduction in free amino-acids as well as in total nitrogen content of goldfish muscle after alloxan treatment might be due to the reduced availability of circulating insulin. However, the rate of protein biosynthesis essentially depends upon the rate at which the protein synthesizing mechanism is working and/or the rate at which amino-acids are transported across the cell membrane from the extracellular fluid into the muscle cell. So the action of insulin might be to directly stimulate the protein synthesizing mechanism or to increase the transport of amino-acids across the cell membrane, or possible to exert an effect on both the processes.

Leucine-^{14}C- incorporation was used as an index of protein metabolism because the use of radioactivity labelled amino-acids and the study of their transport and incorporation into protein provided a useful method for the investigation of the effects of insulin and the mechanism of its action of protein metabolism. Leucine incorporation was increased by insulin and tolbutamide into protein bound amino-acids, and there was a decrease in the leucine incorporation into the free amino-acid fraction of goldfish muscle. There seemed to be a proportional correlation between the fall in leucine-^{14}C incorporation into free amino-acids and the increase in incorporation of leucine-^{14}C into the protein bound amino-acid fraction following the injection of insulin and tolbutamide.

However, the specificity of the temp late is determined by the nucleotide sequence of mRNA and the protein itself is released from the mRNA temp late. The sites of active protein synthesis are also rich in RNA and an increased number of RNA particles can be att ributed to increased synthetic activity of the cells (Jacob & Monad, 1961). Insulin and tolbutamide have been shown to increase the total RNA concentration of goldfish muscle. It is suggested that the synthetic activity of the cell was increased, and the increased concentration of RNA reflected an increased protein synthesis. One might suggest that, after alloxan treatment, there was a deficiency of insulin, and because of this the total RNA content in the muscle might have been effected. The synthetic activity of the cell appeared to be decreased.

In addition, the total RNA content of muscle was also decreased, supporting the tendency for decreased protein synthesis.

Unexplicably, insulin and tolbutamide appeared to decrease the free amino-acid contents of goldfish muscle when they were analysed individually. But insulin and tolbutamide increased the free amino-acid content of muscle when examined for total muscle amino-acid nitrogen content. It is difficult to understand why insulin appeared to suppress the levels of free amino-acid when examined individually by an amino-acid analyzer.

Tashima & Cahill (1968) found that a protein meal, but not an oral glucose load, significantly increased the circulating insulin level in toadfish, and suggested that amino-acids and not glucose might serve as the major stimulus for insulin secretion in this species. Similarly, in the present study with rainbow trout, a high protein diet produced an elevated circulating plasma insulin level. This acute change in the circulating insulin might be interpreted as an increase in the rate of insulin secretion from the islets. It is possible that the liberation of amino-acids into the gastro-intestinal tract during the digestion of protein or the absorption of amino-acid might enhance the secretion of insulin. In addition, there was an observable increase in the mean body weight of trout fed on high protein diet. It might be suggested that insulin acted to increase the body weight of trout by initiating the synthesis of an increased amount of muscle protein.

APPENDIX TO GENERAL DISCUSSION

This Thesis is concerned with the influence of insulin on nitrogen metabolism in teleost fish. It has been tried to treat the subject in its own right, but clearly too, one must look to see to what extent there are parallels with effect of the hormone on carbohydrate and fat metabolism and to what extent the various effects are interdependent. The need to decide whether stimulation of protein synthesis is or is not dependent on a simultaneous stimulation of glucose utilization/transport. Thus one can ask to what extent the transport hypothesis which has been of such value in understanding the effects of hormone on sugar uptake is equally applicable to consideration of the uptake of amino-acids and to what extent the high protein diet contributes to the release of insulin from the beta cells of the pancreas.

An observation that has proved of crucial importance in the development of the concept of a direct control by insulin of protein synthesis was first made by Krahl (1952, 1953). But, however, in teleost fish, Seshadri (1959), Tashima and Cahill (1968), Patent and Foa (1971) and recently Jackim and Laroche (1973) first made a similar observation about the role of insulin in protein metabolism. When Tashima and Cahill (1968) made these observations it was of course well established that insulin stimulates the uptake of glucose by muscle and uptake of amino-acids into the protein, and it was not

unreasonable to conclude that these two effects (uptake of glucose and uptake of amino-acids) were in some way related. The question remains as to how. The stimulating influence of protein meal for release of insulin was clearly demonstrated in the absence of added glucose as in its presence (Tashima and Cahill, 1968). Moreover, since the influence of insulin was clearly shown in the absence of glucose in the supporting medium as in its presence (Manchester and Young, 1958a; Wool and Krahl, 1959a). The action of insulin of amino-acids incorporation does not appear to depend on the stimulation by the hormone of glucose uptake. Moreover the presence of glucose in the medium does not result in consistent enhancement of incorporation or affect the response in incorporation to insulin. This indicates that availability of energy derived from glucose catabolism is not specifically important for protein synthesis or more importantly its enhancement by insulin. Thus one sees here an example of the parallel between effects of insulin on carbohydrate and protein metabolism, on the one hand increasing uptake of sugar and its conversion to glycogen, and on the other increasing uptake of amino-acid as reflected in increased incorporation into protein which takes place independently of enhancement of sugar metabolism. Are the two effects really independently mediated or do they contain common actions different from or more fundamental than those usually measured? The converse observation of the independence of insulin action on sugar uptake from effects on protein metabolism is impossible to make by excluding amino-acids altoghether because they are bound to be present in the tissue.

However, inhibition of protein synthesis by the presence of puromycin does not prevent enhancement of sugar entry by insulin (Eboue-Bonis et al, 1963). The enhancement of glucose uptake by insulin obviously provides a boost to glycogen synthesis even if it is not the entire explanation. To what extent is enhancement of amino-acid entry into cellular space of muscle the cause of increased incorporation of amino-acid into protein? Undoubtedly, insulin can increase the uptake of certain amino-acids by muscle, but despite intensive investigation the significance of this observation is very confused. So, in trying to trace a parallel between glycogen and protein synthesis, there are obviously very important differences. For example, stimulation of glycogen synthesis is chemically measured and not dependent on use of isotopes as in the case of amino-acids.

However, to observe the role of insulin in nitrogen metabolism in teleost fish, the dose of insulin used in all the experiments described in this Thesis is well within the range of what is at present believed to be the insulin content of plasma of normal goldfish (Carassius auratus) Patent and Foa (1971). From the foregoing account, it appears that the increased incorporation of leucine-^{14}C in the muscle of goldfish by insulin as reported here is independent of its effects on glucose. Similarly, the release of insulin from trout pancreas after high protein meal is also possibly not the consequence of any change in glucose. It has already been described in the Thesis in detail that insulin has an unpredictable action on the blood sugar of fish.

Moreover, the very lowest dose of insulin was used in two different experiments in an attempt to avoid the possibility of the effect of insulin on the glucose. One experiment was conducted at different dose levels to determine the effect of insulin on the total nitrogen in the muscle of goldfish, and the second one again at different dose levels to determine the total RNA and total DNA in the muscle of goldfish. These two experiments clearly show that insulin has an effect on protein metabolism.

In the present study we have examined the effects of insulin on amino-acid nitrogen in goldfish and the influence of high protein diet in the release of insulin from the beta cell of the fish pancreas in trout. From this, it seems logical to conclude that there is a relationship in the amount of insulin released from the fish pancreas and the concentration of amino-acids in the blood. This suggests a more important role of insulin in protein metabolism, particularly in carnivorous forms.

However, this does not permit us to minimize the significance of insulin in glucose metabolism, where rather contradictory information is available. These apparently contradictory and unpredictable findings seem to originate from dose level, species specificity of the hormone and biological half-life of the administered insulin, as too is the initial blood glucose of the animal under test. Thus, insulin-induced hypoglycaemia may be more difficult, or even impossible, to detect in those species which have low resting levels of blood glucose.

It appears that the role of insulin in fish metabolism also depends on mobility, feeding habits and the phylogenetic position of the species

under test. For example, as in glucose metabolism the levels of blood glucose vary with the mobility of the fish, and effect of insulin is also variable. In bottom-dwelling scorpion fish Scorpaena porcus, in which hypoglycaemia lasts longer more than in the active sea-bass, Spicara smaris (Gray (1928) and Gray and Hall (1930) had earlier suggested that insulin sensitivity may be dependent upon mobility. It appears from the literature that there is a trend for the reduction of hypoglycaemia from the cyclostomes through the cartilaginous fish to teleost. Moreover, diet or habit are also likely to be very important in dictating the role of insulin in fish metabolism.

As in glucose metabolism, the effect of administered insulin in protein metabolism also seems to be dependent upon feeding habits and evolutionary status of the species. In the goldfish and trout, with its high protein intake, it is perhaps not unexpected that insulin is seen to have an important role in the control of nitrogen metabolism in these fish. From this point of view, further studies are needed to show the role of insulin in protein metabolism in fish. For example, it will be more helpful to investigate on these lines, firstly having these things in mind, the phylogenetical position of the animal, mode of life and feeding habits. One can do more investigations by feeding carnivorous fish with non-protein diet, and protein diet to herbivorous fish and then to observe their response to the release of insulin. Secondly, to measure insulin after feeding the animal with high carbohydrate diet to compare this with the results of high protein feeding. Lastly, the most important is that, since the previous studies, which are mainly based upon administration of exogenous insulin and use

of alloxan to observe the function of beta cells are subjected to criticism due to non-physiological effects. Therefore it would be more informative if further investigations are made on protein metabolism after total isletectomy. Isletectomy in some species does not seem to be difficult as the islets are aggregated as visible masses with little exocrine tissue. (Falkmer and Matty, 1966a).

Further, more emphasis should be laid on the measuring of pancreatic hormones under different metabolic conditions, rather than observing the effect of administered hormone on various metabolic parameters which have a multi-hormonal control.

TABLE 1

THE EFFECT OF INSULIN ON AMMONIA PRODUCTION BY GOLDFISH IN THE AMBIENT WATER

(0.016 I.U/100 G) mg/100 g fish/24 hr ± S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
Mean resting level	11.5 ± 1.3		
After 24 hours	–	18.6 ± 1.8	P<0.02
After 48 hours	–	24.6 ± 1.3	P<0.01
After 72 hours	–	15.5 ± 1.3	
Resting level	–	12.1 ± 1.3	

TABLE 2

THE EFFECT OF INSULIN ON TOTAL NITROGEN EXCRETION OF GOLDFISH IN THE AMBIENT WATER

(0.016 I.U/100 G) mg/100 g Fish/24 hr ± S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
Mean resting level	21.5 ± 1.3		
After 24 hours	–	28.1 ± 1.2	P<0.02
After 48 hours	–	32.5 ± 1.7	P<0.01
After 72 hours	–	25.0 ± 0.8	
Resting level	–	23.0 ± 0.6	

TABLE 3

THE EFFECT OF TOLBUTAMIDE ON AMMONIA PRODUCTION BY GOLDFISH IN THE AMBIENT WATER

(10 mg/Kg of Body Weight) mg/100 g Fish/24 hr ± S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
Mean resting level	11.0 ± 1.0		
After 24 hours	–	15.6 ± 1.3	P<0.05
After 48 hours	–	18.5 ± 1.0	P<0.02
After 72 hours	–	12.2 ± 1.2	
Resting level	–	12.1 ± 1.0	

TABLE 4

THE EFFECT OF ALLOXAN ON AMMONIA PRODUCTION BY GOLDFISH IN THE AMBIENT WATER

(600 mg/Kg dissolved in 0.9% saline)

mg/100 g Fish/24 hr ± S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
Mean resting level	11.6 ± 1.3		
After 24 hours	–	11.6 ± 1.0	N.S.
After 48 hours	–	11.5 ± 1.0	N.S.

N.S. = Not significant.

TABLE 5

THE EFFECT OF INSULIN ON AMINO-ACID NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER

(0.16 I.U/Fish) μg/100 mg, Wet Weight $\pm$ S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
After 48 hours in MUSCLE	69.2 $\pm$ 2.1	104.5 $\pm$ 4.3	$P < 0.001$
After 48 hours in LIVER	59.7 $\pm$ 1.2	62.2 $\pm$ 2.1	N.S.

N.S. = Not significant.

TABLE 6

THE EFFECT OF TOLBUTAMIDE ON AMINO-ACID NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER

(10 mg/Kg of Body Weight) μg/100 mg, Wet weight $\pm$ S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
After 48 hours in MUSCLE	68.3 $\pm$ 3.7	88.4 $\pm$ 2.5	$P<0.01$
After 48 hours in LIVER	59.6 $\pm$ 3.6	61.3 $\pm$ 3.7	N.S.

N.S. = Not significant

TABLE 7

THE EFFECT OF ALLOXAN ON AMINO-ACID NITROGEN CONTENT OF GOLDFISH MUSCLE AND LIVER

(600 mg/Kg dissolved in 0.9% saline)

μg/100 mg, Wet Weight $\pm$ S.D

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
After 48 hours in MUSCLE	69.5 $\pm$ 3.7	45.3 $\pm$ 2.1	$P < 0.01$
After 48 hours in LIVER	58.7 $\pm$ 2.1	56.8 $\pm$ 1.2	N.S.

N.S. = Not significant.

TABLE 8

THE EFFECT OF INSULIN ON THE TOTAL NITROGEN CONTENT OF GOLDFISH MUSCLE

(0.16 I.U/Fish) mg/100 mg tissue

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED
After 48 hours in MUSCLE	2.4	3.6

NX 6.25 for protein.

TABLE 9

THE EFFECT OF TOLBUTAMIDE ON THE TOTAL NITROGEN CONTENT OF GOLDFISH MUSCLE

(10 mg/Kg of Body Weight) mg/100 mg tissue

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED
After 48 hours in MUSCLE	2.3	3.2

NX 6.25 for protein.

TABLE 10

THE EFFECT OF ALLOXAN ON THE TOTAL NITROGEN CONTENT OF GOLDFISH MUSCLE

(600 mg/Kg dissolved in 0.9% saline)

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED
After 48 hours	2.5	1.4

NX 6.25 for protein.

TABLE 11

IN VIVO EFFECT OF INSULIN ON LEUCINE-^{14}C-INCORPORATION INTO THE PROTEIN OF MUSCLE OF GOLDFISH

Fish were injected with insulin. After 48 hours they were then injected intraperitoneally with Leucine-^{14}C (1μC/100 g body weight) and sacrificed 1 hour later. The values given for leucine incorporation are $\pm$S.D.

(0.16 I.U/Fish) D.M.P./mg

Number of animals = 5

	CONTROLS	TREATED	P
F.A.A.	2768 $\pm$ 71	1575 $\pm$ 75	P<0.001
P.B.A.A.	477 $\pm$ 21	1631 $\pm$ 73	P<0.001

Counting efficiency = 65-70%
Counting period = 10 minutes

(0.04 I.U/Fish) D.M.P./mg

Number of animals = 5

	CONTROLS	TREATED	P
F.A.A.	2761 $\pm$ 80	1985 $\pm$ 31	P<0.01
P.B.A.A.	467 $\pm$ 31	1230 $\pm$ 23	P<0.01

Counting efficiency = 65-70%
Counting period = 10 minutes

TABLE 12

IN VIVO EFFECT OF TOLBUTAMIDE ON LEUCINE-^{14}C INCORPORATION INTO PROTEIN OF MUSCLE OF GOLDFISH

Fish were injected with tolbutamide. After 48 hours they were then injected intraperitoneally with leucine-^{14}C (1 μC/100 g body weight) and sacrificed 1 hour later. The values given for leucine incorporation are $\pm$ S.D.

(10 mg/Kg Body Weight) D.M.P./mg

Number of animals = 5

	CONTROLS	TREATED	P
F.A.A.	2753 $\pm$ 73	2159 $\pm$ 15	$P<0.01$
P.B.A.A.	581 $\pm$ 30	1196 $\pm$ 22	$P<0.01$

Counting efficiency = 65-70%
Counting period = 10 minutes

TABLE 13

IN VIVO EFFECT OF ALLOXAN ON LEUCINE-^{14}C INCORPORATION INTO PROTEIN OF MUSCLE OF GOLDFISH

Fish were injected with alloxan. After 48 hours they were then injected intraperitoneally with leucine-^{14}C (1μC/100 g body weight) and sacrificed 1 hour later. The values given for leucine incorporation are $\pm$S.D.

(600 mg/Kg dissolved in 0.9% saline)

Number of animals = 5

	CONTROLS	TREATED	P
F.A.A.	2759 $\pm$ 71	2750 $\pm$ 70	N.S.
P.B.A.A.	575 $\pm$ 27	583 $\pm$ 31	N.S.

N.S. = Not significant.

Counting efficiency = 65-70%
Counting period = 10 minutes

TABLE 14

THE EFFECT OF INSULIN ON THE TOTAL RNA AND TOTAL DNA CONTENT OF GOLDFISH MUSCLE

(0.16 I.U/Fish) mg/ml ± S.D.

Number of animals = 6

TIME AFTER STARTING After 48 hours:-	CONTROLS	TREATED	P
RNA	0.0131 ± 0.0008	0.0200 ± 0.0003	P<0.01
DNA	0.0040 ± 0.0001	0.0040 ± 0.0002	N.S.

N.S. = Not significant.

TABLE 15

THE EFFECT OF TOLBUTAMIDE ON THE TOTAL RNA AND TOTAL DNA CONTENT OF GOLDFISH MUSCLE

(10 mg/Kg Body Weight) mg/ml ± S.D.

Number of animals = 6

TIME AFTER STARTING After 48 hours:-	CONTROLS	TREATED	P
RNA	0.0130 ± 0.0005	0.0173 ± 0.0004	P<0.02
DNA	0.0040 ± 0.0001	0.0041 ± 0.0003	N.S.

N.S. = Not significant.

TABLE 16

THE EFFECT OF ALLOXAN ON THE TOTAL RNA AND TOTAL DNA CONTENT OF GOLDFISH MUSCLE

(600 mg/Kg dissolved in 0.9% saline) mg/ml ± S.D.

Number of animals = 6

TIME AFTER STARTING	CONTROLS	TREATED	P
After 48 hours:-			
RNA	0.0132 ± 0.0007	0.0071 ± 0.0004	P<0.01
DNA	0.0040 ± 0.0003	0.0040 ± 0.0002	N.S.

N.S. = Not significant.

TABLE 17

INSULIN SECRETION IN RESPONSE TO HIGH AND LOW PROTEIN INGESTION IN RAINBOW TROUT AFTER 19 DAYS OF FEEDING

Insulin Concentration n g/ml ± S.D.

PROTEIN CONTENT OF DIET	PLASMA INSULIN CONCENTRATION	P
34% Protein (Normal diet)	2.40 ± 0.03	
24% Protein (Low protein diet)	2.26 ± 0.14	
54% Protein (High protein diet)	4.00 ± 0.07	P<0.001

TABLE 18

EFFECT OF INSULIN ON FREE AMINO-ACID CONCENTRATIONS OF GOLDFISH MUSCLE 48 HOURS AFTER INJECTION

(0.16 I.U/Fish) μmoles/100 mg Wet Weight $\pm$ S.D.

Number of animals = 6

AMINO-ACIDS	CONTROLS	TREATED	P
ASPARTIC	0.011 $\pm$ 0.001	0.011 $\pm$ 0.001	N.S.
THREONINE	0.097 $\pm$ 0.002	0.005 $\pm$ 0.001	P$<$0.001
SERINE	0.084 $\pm$ 0.010	0.066 $\pm$ 0.001	N.S.
GLUTAMIC	0.066 $\pm$ 0.010	0.004 $\pm$ 0.001	P$<$0.01
PROLINE	0.049 $\pm$ 0.010	0.005 $\pm$ 0.001	P$<$0.02
GLYCINE	0.206 $\pm$ 0.060	0.123 $\pm$ 0.034	P$<$0.01
ALANINE	0.126 $\pm$ 0.026	0.066 $\pm$ 0.020	P$<$0.01
VALINE	0.032 $\pm$ 0.002	0.018 $\pm$ 0.001	N.S.
METHIONINE	0.010 $\pm$ 0.001	0.011 $\pm$ 0.001	N.S.
ISOLEUCINE	0.022 $\pm$ 0.002	0.020 $\pm$ 0.001	N.S.
LEUCINE	0.037 $\pm$ 0.003	0.003 $\pm$ 0.001	P$<$0.02
LYCINE	0.217 $\pm$ 0.047	0.123 $\pm$ 0.033	P$<$0.001
HISTIDINE	0.259 $\pm$ 0.048	0.096 $\pm$ 0.054	P$<$0.001
ARGANINE	0.048 $\pm$ 0.006	0.006 $\pm$ 0.001	P$<$0.02

N.S. = Not significant.

TABLE 19

THE EFFECT OF INSULIN ON FREE AMINO-ACID CONCENTRATIONS OF GOLDFISH MUSCLE 48 HOURS AFTER INJECTION

(0.04 I.U/Fish) μmoles/100 mg Wet Weight ± S.D.

Number of animals = 6

AMINO-ACIDS	CONTROLS	TREATED	P
ASPARTIC	0.011 ± 0.001	0.010 ± 0.001	N.S.
THREONINE	0.095 ± 0.002	0.047 ± 0.010	P<0.01
SERINE	0.080 ± 0.010	0.060 ± 0.001	N.S.
GLUTAMIC	0.066 ± 0.010	0.019 ± 0.001	P<0.02
PROLINE	0.049 ± 0.030	0.011 ± 0.001	N.S.
GLYCINE	0.212 ± 0.080	0.136 ± 0.010	N.S.
ALANINE	0.126 ± 0.026	0.080 ± 0.010	P<0.02
VALINE	0.031 ± 0.020	0.036 ± 0.020	N.S.
METHIONINE	0.010 ± 0.001	0.010 ± 0.001	N.S.
ISOLEUCINE	0.022 ± 0.004	0.021 ± 0.001	N.S.
LEUCINE	0.034 ± 0.002	0.003 ± 0.001	N.S.
LYCINE	0.216 ± 0.027	0.181 ± 0.040	P<0.02
HISTIDINE	0.258 ± 0.048	0.160 ± 0.010	P<0.01
ARGININE	0.048 ± 0.006	0.039 ± 0.001	N.S.

N.S. = Not significant.

TABLE 20

THE EFFECT OF TOLBUTAMIDE ON FREE AMINO-ACID CONCENTRATIONS OF GOLDFISH MUSCLE 48 HOURS AFTER INJECTION

(10 mg/Kg Body Weight) μmoles/100 mg Wet Weight $\pm$ S.D.

Number of animals = 6

AMINO-ACIDS	CONTROLS	TREATED	P
ASPARTIC	0.011 $\pm$ 0.001	0.009 $\pm$ 0.001	N.S.
THREONINE	0.099 $\pm$ 0.004	0.036 $\pm$ 0.008	$P<0.01$
SERINE	0.086 $\pm$ 0.010	0.082 $\pm$ 0.001	N.S.
GLUTAMIC	0.066 $\pm$ 0.010	0.052 $\pm$ 0.004	N.S.
PROLINE	0.049 $\pm$ 0.003	0.016 $\pm$ 0.001	$P<0.02$
GLYCINE	0.222 $\pm$ 0.070	0.153 $\pm$ 0.010	N.S.
ALANINE	0.126 $\pm$ 0.026	0.088 $\pm$ 0.020	$P<0.02$
VALINE	0.032 $\pm$ 0.002	0.021 $\pm$ 0.002	N.S.
METHIONINE	0.010 $\pm$ 0.001	0.013 $\pm$ 0.001	N.S.
ISOLEUCINE	0.022 $\pm$ 0.004	0.019 $\pm$ 0.001	N.S.
LEUCINE	0.037 $\pm$ 0.003	0.008 $\pm$ 0.002	$P<0.02$
LYCINE	0.209 $\pm$ 0.037	0.079 $\pm$ 0.014	$P<0.001$
HISTIDINE	0.250 $\pm$ 0.045	0.103 $\pm$ 0.019	$P<0.001$
ARGININE	0.048 $\pm$ 0.006	0.004 $\pm$ 0.001	$P<0.02$

N.S. = Not significant.

TABLE 21

THE EFFECT OF ALLOXAN ON FREE AMINO-ACID CONCENTRATIONS OF GOLDFISH MUSCLE 48 HOURS AFTER INJECTION

(600 mg/Kg dissolved in 0.9% saline)

μmoles/100 mg Wet Weight ± S.D.

Number of animals = 6

AMINO-ACIDS	CONTROLS	TREATED	P
ASPARTIC	0.011 ± 0.001	0.010 ± 0.001	N.S.
THREONINE	0.096 ± 0.002	0.013 ± 0.001	P<0.001
SERINE	0.088 ± 0.010	0.080 ± 0.002	N.S.
GLUTAMIC	0.066 ± 0.010	0.045 ± 0.001	N.S.
PROLINE	0.049 ± 0.003	0.018 ± 0.001	P<0.02
GLYCINE	0.212 ± 0.070	0.154 ± 0.007	N.S.
ALANINE	0.128 ± 0.026	0.102 ± 0.003	N.S.
VALINE	0.032 ± 0.002	0.027 ± 0.001	N.S.
METHIONINE	0.012 ± 0.001	0.010 ± 0.001	N.S.
ISOLEUCINE	0.022 ± 0.004	0.013 ± 0.001	N.S.
LEUCINE	0.037 ± 0.003	0.026 ± 0.001	N.S.
LYCINE	0.217 ± 0.047	0.210 ± 0.032	N.S.
HISTIDINE	0.254 ± 0.040	0.106 ± 0.009	P<0.001
ARGININE	0.048 ± 0.006	0.030 ± 0.001	N.S.

N.S. = Not significant.

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