AN INVESTIGATION OF THE EXTRACTABLE INSULIN LEVELS AND PANCREAS WEIGHTS OF NEW ZEALAND SHEEP

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SUMMARY.

The average yields of insulin and weights of pancreas from New Zealand sheep with age have been determined to ascertain the economic possibility of large-scale production of insulin. Acid-alcohol extractions of the pancreas glands was used to extract the insulin and three methods of obtaining insulin from the alcoholic extract were investigated. Insulin yields using the ether counter-current concentration of the extract or the alginic acid adsorption of insulin from the extract were four to six times that obtained by vacuum concentration of the extract.

A double antibody radioimmunoassay with human insulin antiserum was used to measure the insulin activity of the alcoholic extracts. The yields of insulin from sheep pancreas were 0.2 to 2.0 international units per gram of pancreas, and the yield tended to decrease as the sheep aged. Other assay methods investigated were paper chromatography, bioassay and radioimmunoassay of crude insulin.

Crystalline sheep insulin of the rhombohedral type were obtained and shown to have similar electrophoretic properties to that of crystalline beef insulin.

Pancreas weight increased as the animal matured. A decrease in pancreas weight was observed in five year old sheep. In the age range 42 days to five years, six different growth periods were observed. The yields of insulin extractable from a single pancreas are fifteen to fourty international units, the average yield being twenty international units per pancreas glands.

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INTRODUCTION.

In 1921 Banting and Best were the first workers to successfully obtain the active substance insulin from the pancreas. The dramatic clinical benefits of the hormone were immediately apparent and insulin has become the most important commercially produced protein in the medical field. It also has a unique place in protein chemistry investigations as it was the first protein to be crystallised, the amino acid sequence determined and a biologically active molecule synthesized chemically.

Methods for producing insulin were developed by Scott and Best at the Connaught Laboratories, Toronto, in the early 1920's. The Insulin Committee set up by the Connaught Laboratories selected Eli Lilly and Company as the first pharmaceutical company to manufacture insulin. Scott and Best developed methods for the preparation of insulin, including large-scale production methods and these methods were sent to interested manufacturers throughout the world at regular intervals (Best 1960).

Active work on insulin has continued both at Connaught Laboratories and at the Banting and Best Institute in Toronto. The more recent work from these groups has been on the physiological effects of insulin, modes of insulin action, and factors affecting secretion of insulin by the pancreas. Many other laboratories throughout the world are also investigating similar aspects of insulin.

After preliminary work on insulin extraction in the 1920's most of the work on insulin extraction has been done by pharmaceutical companies. Many of the results on the extraction process, and factors affecting yields of insulin obtained have not been published. The ease of extraction, high yields and the availability of large quantities of glands have led to the extensive use of pig and beef pancreas for commercial production of insulin. Whale and fish pancreas glands have also been used for insulin manufacture but pancreas from other animals such as buffalo, sheep, goats or horses have not been used for the commercial production of insulin due to the lower yields obtained, or to large quantities of glands not being readily available.

The structure of insulin differs between species (Table I). Development of insulin antibodies by the human body varies with the structure of administered insulin. As pig insulin has a similar structure to

that of human insulin, antibody development is minimized, making pig insulin preparations more desirable than beef insulin. Sheep insulin has a structure more like beef insulin than pig or human insulin.

AIMS OF THE PRESENT INVESTIGATION.

In New Zealand large numbers of sheep and lambs are killed annually (Table IV). Large quantities of pancreas glands could easily be collected from the carcasses, and commercial production of insulin from sheep pancreas may be feasible if the yield of insulin is satisfactory and the price of the pancreas is suitable (Ferguson, 1970). Little work has been published on insulin yields from sheep pancreas. Reported yields from sheep pancreas vary from 0.7 International Units (i.u.) per gramme (gm) of pancreas gland to 8.2 i.u./gm (Table II). Willes et al (1969a, 1969b) investigated the cytological localisation of insulin and the insulin concentration on the foetal sheep pancreas. The insulin concentration increased with age of the foetus to a peak average value of 5 i.u./gm just prior to birth, but the insulin concentration in lamb and sheep pancreas glands studied in the same investigation decreased with age.

Several factors are known to affect the yield of insulin from pancreas glands. One of the major factors, other than the processing method, is the age of the animal. Insulin concentration changes with age have been shown to occur in beef (Fisher and Scott, 1934), human (Jorpes and Rastgeldi, 1953) and mouse (Sneyd, 1964) pancreas. Insulin levels in sheep pancreas glands, and the variation of pancreas gland weight with age has not been investigated except in the limited study of Willes et al (1969b).

The aims of the present study are to:

1. Study analytical methods for extracting insulin and select a suitable method for investigating insulin levels in sheep pancreas.
2. Ascertain the amount of extractable insulin in sheep pancreas and obtain a relationship between the extractable insulin content in sheep pancreas glands and the age of the sheep.
3. Relate the results obtained for insulin content to other analyses in other laboratories.
4. Obtain a correlation between the pancreas weight and the age of sheep.
5. Obtain crystalline sheep insulin.

CHAPTER I.

THE PANCREAS, INSULIN AND DIABETES MELLITUS.

A. THE PANCREAS.

a. Situation: The pancreas is an irregularly shaped pale-pink organ covered by a thin layer of connective tissue which does not form a definite fibrous capsule. It is attached to the liver and the diaphragm and lies between the stomach and the duodenum. Because of its shape, the pancreas gland is usually described as having a head, body and tail. A duct through which the pancreatic juice is secreted leaves the posterior part of the right border of the gland, and in the sheep this duct joins the alimentary tract at the bile duct. These relationships are shown in Figure 1.

b. Structure: The pancreas is a compound gland, having both exocrine and endocrine functions.

1. Exocrine cells: The Exocrine portion of the pancreas is composed of pyramidal acinar cells forming lobules, which are bound together by loose connective tissue rich in capillaries. The apices of several cells converge to a central lumen.

The acinar cell has a large spherical nucleus and the basal two-thirds of the cell is characterised by rough-surfaced endoplasmic reticulum. The apical third of the cell is occupied by a large Golgi complex and numerous zymogenic granules in various stages of maturation. When stimulated, the zymogen granules secrete their enzymic contents, usually in an inactive form, into the lumen. The contents from the many lumina drain to the pancreatic duct and are secreted into the digestive system.

2. Endocrine cells. The endocrine portion of the pancreas is usually called the islets of langerhans. The islets are aggregations of irregular masses of pale cells scattered between the acinar cells, and separated from the acini by a thin layer of reticulum. The volume of islets in the pancreas can vary, but usually is about 1% - 2% of the total gland volume. The islets are twice as numerous in the tail as in the body and head of the gland in the human pancreas. (Netter, 1965). In certain species, e.g. fish, the islets and acini are spatially separate.

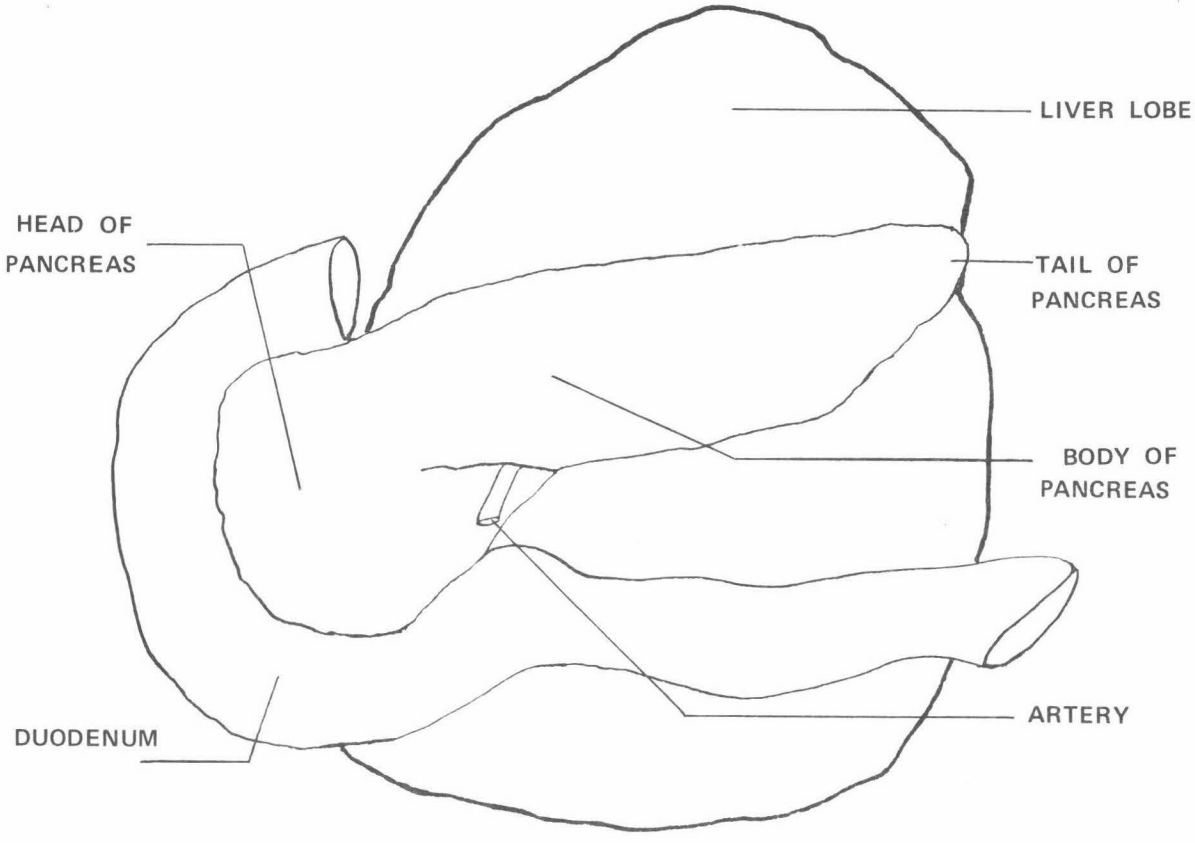


Figure 1: DIAGRAM OF THE SHEEP PANCREAS

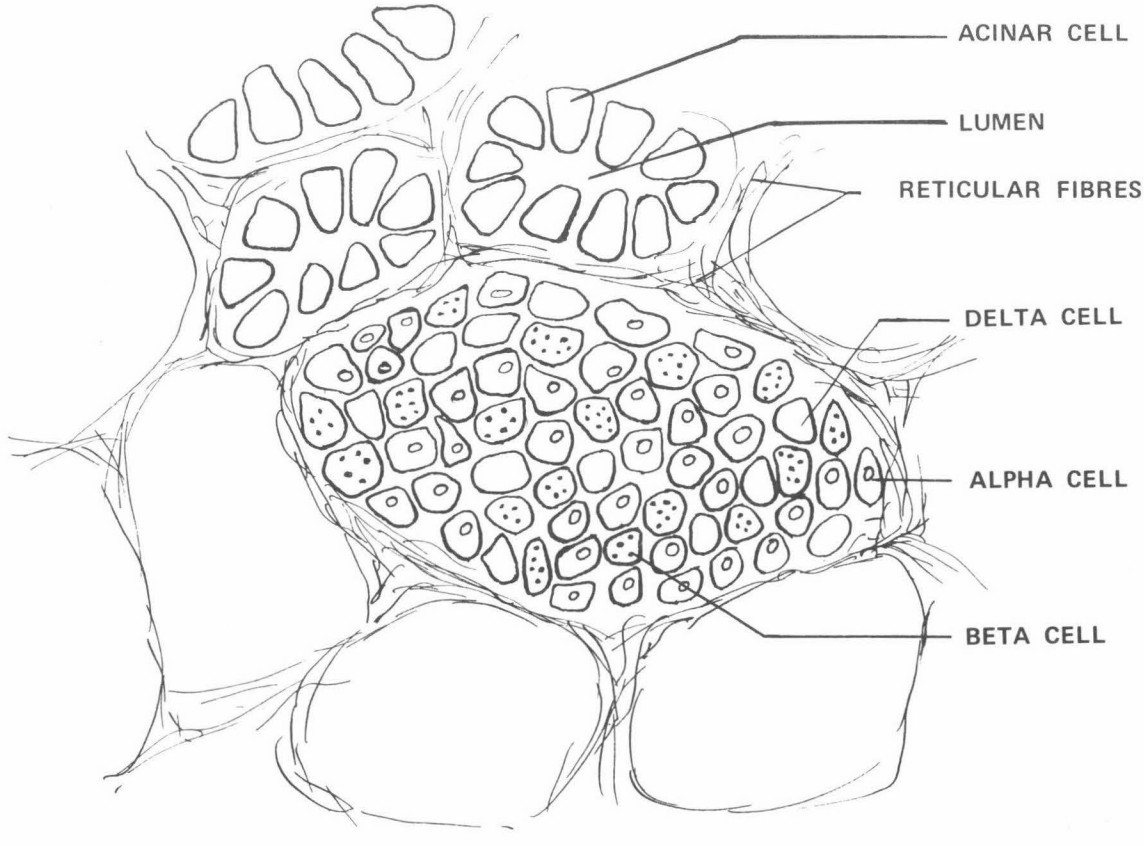


Figure 2: DIAGRAM OF A PANCREATIC ISLET AND SURROUNDING ACINI.

The islet cells contain many granules, and different types of cells can be distinguished by the variation in density, shape and ultra-structure of the granules.

Alpha cells are relatively large, and contain many spherical highly electron-density granules. The granules are alcohol insoluble and are thought to secrete glucagon. Alpha cells do not differ much in appearance between species.

Beta cells are smaller and occur more frequently than the alpha cells. Beta cells contain many irregular sized granules of medium density. The granules are alcohol soluble and secrete insulin. Species difference in the structural appearance of the secretory granules is well documented (Boquist, 1970).

Delta cells have not been found in all species investigated. They are small cells, with pale cytoplasm and do not appear to have any granules.

c. Pancreatic Enzymes: The enzymes present in the pancreatic juice and in the zymogen granules of the acinar cell include proteases, lipases, amylases and nucleases. The protolytic enzymes are present as inactive precursers e.g. trypsinogen, chymotrypsinogen, procarboxypeptidase. The relative concentrations of the enzymes differ in each species, being dependent on the diet of the animal. The enzymes present in the pancreatic juice and zymogen granules of bovine (Keller et al, 1958; Keller and Cohen, 1961) and human (Preston and Kukral,1962; Keller and Allan, 1967; Allan et al, 1970) pancreas have been investigated.

The enzymic activity of pancreas glands from pigs (Avrameas, 1966), young pigs (Lewis et al, 1957; Hudman et al, 1957), lambs (Walker,1959); calves (Huber et al, 1961; Gorrill and Thomas, 1967) has been investigated. Schingoethe et al, (1970) determined the size and proteolytic activity of the pancreas from cattle, sheep, chickens, rats and mice of different ages.

d. Pancreatic Hormones: Two hormones are present in the pancreas-insulin and glucagon. From indirect evidence it has been deduced the alpha cells produce glucagon and the beta cells produce insulin.

The relationship of the alpha cells to glucogen production rests on fairly general evidence. The distribution of extractable glucagon from various portions of the pancreas parallel the relative abundance of alpha cells, and these cells stain with fluorescent antibodies, specific to glucagon.

The beta cells are thought to be the site of insulin synthesis, storage and secretion because of:-

(i) the strict parallelism between the abundance of granules and the content of insulin.

(ii) the observation that beta granules are the only cellular structures of the islet to which the specific insulin antibodies can be attached.

(iii) the effect of alloxan, which produces diabetes by injuring the beta cells without visible changes in the alpha-cell system.

Zinc is present in the islet tissue in comparatively large amounts but may not be a necessary, integral part of molecule's hormonal actior (Netter, 1965). Zinc may be the complexing agent which maintains the beta granule in aggregated forms.

1. Insulin. The hormonal action of insulin includes lowering of the blood sugar level and promotion of the synthesis of glucagon, fat and protein in specific cells. If insulin is absent in the body or not present in an active form the blood sugar level rises, as the sugar cannot be transported across specific cell walls and membranes. Insulin does not initiate processes in the body but influences the rate of some enzymic processes.

The mechanism of insulin biosynthesis, secretion and action is only partially understood and recent evidence suggest that the complexity of insulin action is greater than previously thought. From the results of several studies (Falkmer and Wilson, 1967; Wessells and Cohen, 1967) it appears that pancreatic cells are present in species at least as primitive as some tunicates, with immunologically insulin-like material being found in snails, starfish and the earliest vertebrates. Beta cell development and insulin secretion in the pancreas has been demonstrated in the fetus of mice (Dixit et al, 1964), lambs (Willes et al, 1969a) and human (Grillo and Shima, 1966). The insulins, as a group of proteins, seem to be essential for the survival of most species because of their unique role in controlling the storage or utilisation of food in the body.

Many factors can affect the synthesis and secretion of insulin from the pancreas. Due to the uncertainty of insulin action and factors affecting insulin secretion there have been many recent papers

on the biological control synthesis, storage, and secretion of insulin (Buchanan et al, 1969; Crockford et al, 1969; Milner and Hales, 1969; Steiner, 1969; Renold, 1970).

2. Glucagon controls the breakdown of liver glycogen and increases the blood sugar level. It is often present as an impurity in commercial insulins. As the primary structure of the amino acids in glucagon differ from the primary structure of insulin it is concluded that glucagon is not a degredation product of insulin. The hormonal action of glucagon has not been investigated as fully as insulin but many factors affect glucagon secretion and the metabolic pathways affected by glucagon are not fully known (Frohman, 1969).

There is no definite proof that other substances such as lipocaic, vagotonin and kallekrein are secreted from the pancreas or are pancreatic hormones (Houssay, 1958). New studies using comparative histophysiological techniques on the islets of Langerhans may show that a hormone, at present not identified, may be produced by the delta cells of the pancreas (Epple, 1969).

B. INSULIN.

Chemical analysis of insulin was in the preliminary stages in late 1924. The protein nature of insulin was not recognised in early investigations (Best and Macleod, 1923; Piper et al, 1924). This may have been because the insulin available at that time was not very pure, having an activity of only 12 i.u./mgm (Best, 1960), or because the insulin solutions were too dilute for a positive protein reaction to be detected. A large number of different methods for extraction and purification were investigated to obtain the pure hormone. The experiments of Abel (1926) led, in time, to the conclusion that insulin is a complicated protein, composed of amino acids and devoid of any extraneous active groups. This concept could not be reconciled with the contemporary theories on protein structure and the scientific community was extremely reluctant to accept Abel's evidence and believed that his insulin crystals must consist of the true hormone of unknown chemical nature adsorbed upon a protein carrier. Thus, the early discussions on the chemistry of insulin parallel the controversy that arose during the same period over the chemical identity of enzymes. The work of Abel in the crystallisation of insulin can only be appreciated in retrospect as the importance of insulin in the advancement of the chemistry of

biological substances was only gradually apparent.

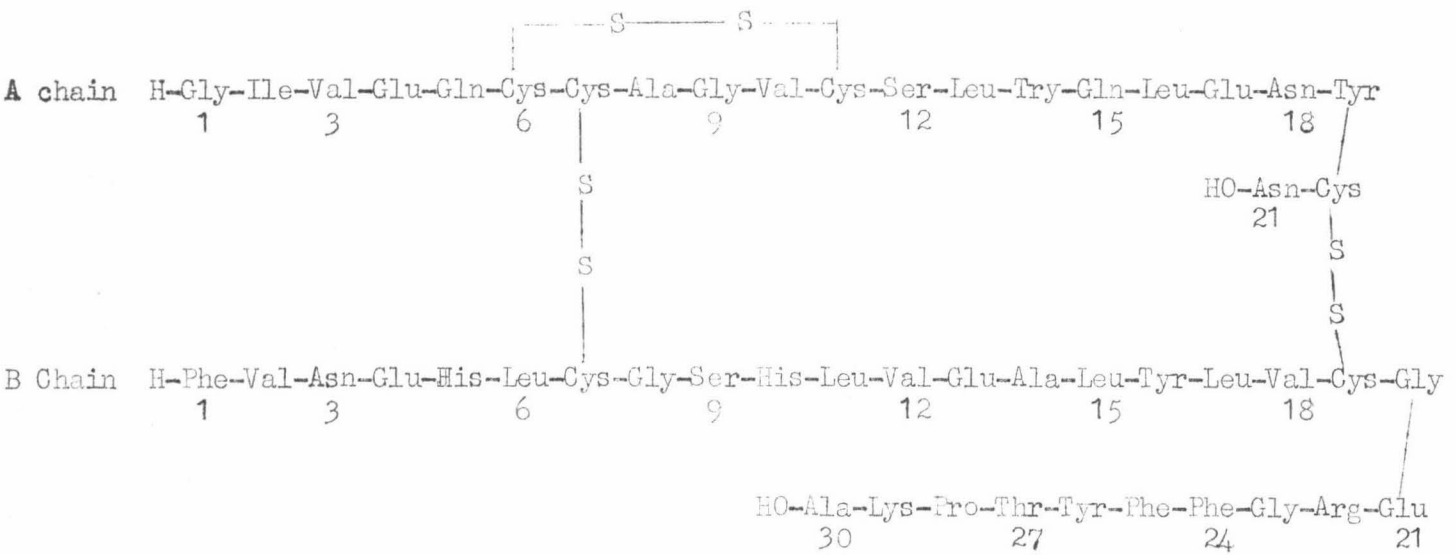
a. Molecular weight. After several investigations, the smallest unit of insulin, a monomer, has been found to have a molecular weight of 5700 (Kupke and Linderstrom-Lang, 1954). Except under special conditions, the stable unit in aqueous acidic solutions is the dimer (Low and Einstein, 1960).

b. Primary structure. In 1935 Jensen and Evans demonstrated that phenylalanine was one of the terminal amino acids of insulin. This was the first time the position of an amino acid in a protein had been demonstrated. By 1943 the basic principles of protein chemistry were firmly established. The differences in both the physical and biological properties of proteins was thought to be due to differences in the arrangements of the amino acids. Chibnall (1942) studied insulin in considerable detail. He found that insulin had a high content of free alpha-amino acids indicating that the protein was composed of relatively short chains.

In the ten year period 1945 - 1955 Sanger and his colleagues developed the new methods of dinitrophenyl amino acid end-group labelling, enzymic breakdown of proteins, and acid hydrolysis to identify the positions of specific amino acids in proteins. They applied these methods to insulin and elucidated the structure of the phenylalanine chain (Sanger and Tuppy, 1951) and the glycyl chain (Sanger and Thompson, 1953). Using disulphide interchange reactions (Ryle and Sanger, 1955) the position of the disulphide bonds was found. The primary structure of beef (Ryle et al, 1955), pig and sheep (Brown et al, 1955), horse and whale (Harris et al, 1956) insulins were found by the same group of workers. The amino acid sequence of sheep insulin is given in Figure 3.

Since then the primary structure of insulins of the following species have been obtained: human (Nicol and Smith, 1960); sperm and sei whale (Isihara et al, 1958); cod (Wilson and Dixon, 1961); fin whale (Hama et al, 1964); elephant, goat, rabbit, guinea-pig and chicken (Smith, 1966).

The A- or glycyl chain of insulin has twenty-one amino acids with an interal disulphide bridge in the $A_7 - A_{11}$ position. Most of the amino acid sequence differences between species occurs in the amino acids within this disluphide bridge. Cattle, pig, sheep, horse and whale insulins have an identical amino acid sequence except in the positions



From : Sober, 1968.

Figure 3.

AMINO ACID SEQUENCE OF SHEEP INSULIN.

A_8, A_9, A_{10}. The B- or phenylalamine chain has thirty amino acids. Species differences in the primary structure of the B-chain can occur in most of the positions. (Table I).

Except for the notable exceptions of the guinea-pig structure, the insulins of the mammalian species studied differ from a common insulin structure by only a few amino acids in selected positions. The changes in sequence produce no apparent change in biological activity, although the more distant the insulin structure of administered insulin from the insulin of the recipient animal, the less the biological activity (Falkmer and Wilson, 1967) e.g. guinea-pig insulin is only one quarter as effective in cattle as in the guinea-pig. Chicken insulin is as active as crystalline beef, pork and sheep insulins in an 'in vitro' rat diaphrgm bioassay, but is more effective in intact chickens than equivalent amounts (by weight) of mammalian insulins (Hazelwood et al, 1968).

From the limited number of amino acids involved in the species difference of insulins a possible sequence of mutations from a central insulin structure has been developed (Smith, 1966). As the number of insulin structures known is small, not much reliance can be placed on the pathway developed. All mammalian sequences known, except guinea-pig insulin, can be derived from the central structure by a single step mutation, or a small number of mutations. The rat is the only mammal, to date, from which more than one insulin has been isolated, although multiple insulins can occur among fishes (Smith, 1966).

The relationship of the physiological action of insulin to its chemical structure has been investigated, but little is known as yet. The disulphide bonds are necessary for the biological activity of insulin. Degredative studies have shown that the carboxyl terminal alanine of the B-chain, and the amino groups of asparagine on the carboxyl terminal of the A-chain are not important for determining the biological activity of insulin (Carpenter, 1966).

c. Tertiary structure. Abel obtained crystalline insulin late in 1925. He used a series of isoelectric precipitations, brucine adsorption and solvent extractions to obtain the crystals (Abel, 1926). After his initial success Abel failed to obtain insulin crystals from different batches of pancreatic extracts. The reasons for the failure, and the difficulty of obtaining crystals was thought to be due to changes in the extraction method of crude insulin from pancreas, or to hydrolysis

TABLE I.

Species Differences in Insulin Amino Acid Sequence.

	A CHAIN								B CHAIN										
	A_4	A_8	A_9	A_{10}	A_{12}	A_{13}	A_{14}	A_{18}	B_3	B_4	B_{10}	B_{14}	B_{17}	B_{20}	B_{21}	B_{22}	B_{27}	B_{29}	B_{30}
Man Pig, Dog	Glu	Thr	Ser	Ile	Ser	Leu	Tyr	Asn	Asn	Gln	His	Ala	Leu	Gly	Glu	Arg	Thr	Lys	Thr
Fin Whale																			Ala
Rabbit																			Ser
Guinea Pig									Ser	Arg	Asn	Thr	Ser	Gln	Asp	Asp	Ile		Asp
Cow		Ala		Val															Ala
Sheep		Ala	Gly	Val															Ala
Goat		Ala	Gly	Val															Ala
Horse			Gly	Ile															Ala
Elephant			Gly	Val															
Rat 1	Asp	Ala	Gly	Thr	Thr	Arg	His	Ser	Lys										Ser
Rat 2	Asp								Lys									Met	Ser
Sperm Whale	Asp																		Ala
Sei Whale		Ala		Thr															Ala
Chicken		His	Asn	Thr					Ala	Ala							Ser		Ala

From : Sober, 1968.

of the protein, but these reasons were later found to be wrong. It has been found that zinc, or related heavy metals such as nickel, cadium or cobalt, is necessary for crystallising insulin (Scott, 1934). Polymerization of insulin in aqueous solution only occurs with difficulty if metal ions, especially zinc ions, are not present (Marcker and Graae, 1961) and crystals from which the heavy metal has been removed can not be successfully recrystallised. Nucleation and growth characteristics of insulin crystals have been studied (Schlichtkrull, 1956; 1960; Marcker, 1960; Jeffrey and Coates, 1966).

Recent studies (Marcker and Graae, 1962; Adams et al, 1969) have confirmed that the lattice-like structure of the most common, or rhombohedral form of crystalline zinc-insulin depends upon the bonds created by a variable number of zinc ions. The amount of zinc and the stage at which it is added affect the ease of crystallisation. Orthorhombic, monoclinic, and rhombo-dodecahedral forms of insulin crystals have also been observed (Schlichtkrull, 1956).

Although insulin was crystallised by Abel in 1926, data from the electron density maps leading to the secondary and tertiary structure of crystalline insulin have only recently been published (Adams et al, 1969). The insulin hexamer is a compact, oblate spheriod formed by co-ordination of three insulin dimers around two zinc ions. Two crystallographically independent molecules can be differentiated in the electron density maps. They each have a similar internal organisation but are not quite identical to each other. In each molecule the A-chain is a compact unit with the B-chain wrapped around it. The closest series of contacts between the two insulin molecules occurs between the extended B-chain residues B_{23} - B_{28}.

Some of the residues which are associated with the biological activity are found on the surface of the hexamer. The unusually high reactivity of the disulphide bonds in native insulin is thought to depend on the integrity of the native molecule suggesting that the disulphide bonds are involved in the hormonal action (Massaglia et al, 1968).

Insulin will form fibrils if a slightly acidic solution is heated (Blatherwick et al, 1927). These fibrils will revert to crystalline insulin if treated with alkali (Waugh, 1948). The fibrils are thought to be formed by endwise linking of the monomer units, perhaps through the guinidinyl groups of arginine. (Philips and Mercer, 1953).

d. Synthetic insulin. The total synthesis of biologically active sheep, beef and human insulin has been accomplished in several laboratories (Zahn and Zabel, 1962; Katsoyannis et al, 1963; Kung et al, 1965). Beef insulin was obtained in crystalline form with a biological activity identical to that of native beef insulin. The du Vigneaud method of of synthesis was used; Peptide fragments were condensed, the thiol groups being protected by benzyl groups. The benzyl groups were then removed by treatment with sodium in liquid ammonia and the thiol groups oxidised by air. The yield in each of the reaction steps is small and would not be suitable for the synthesis of insulin on a commercial scale. Overall yields are in the order of 0.02 - 0.7% of the initial reactants. The most difficult step is the recombination of the A and B chains. Yields obtained from this reaction are 1 - 10% of the theoretical yields although Katsoyannis and Tometsko (1966) obtained 60 - 80% yields in a modified process. Solid-phase synthesis of bovine insulin (Marglin and Merrifield, 1966) gave higher yields.

A new method of synthesis could be:

1. Synthesis of the peptide chains on a solid support.
2. Replacement of the benzyl groups by other protective groups that can be removed more gently than the sodium/liquid ammonia reaction.
3. Combining a pre-oxidized A-chain with a sulphonated B-chain (Zahn, 1966).

The total synthesis of insulin by such a technique may allow the development of the total synthesis of proteins of greater molecular weight than insulin, and make possible the commercial production of synthetic insulin.

e. Proinsulin. The biosynthesis of insulin through the larger molecule, proinsulin, was first demonstrated in human islet cell adenomata (Steiner et al, 1967). Subsequent experiments with normal islet tissue from other species have confirmed that proinsulin is the precurser of insulin, and it has also been separated from commercial insulins. (Steiner et al, 1968).

Proinsulin is a single polypeptide chain of molecular weight about 9000, beginning at the amine (N) - terminus at the B-chain sequence of insulin and ending with the A-chain sequence. The A-chain and B-chain are joined by an additional polypeptide (C-peptide) of 30 (bovine -

Steiner et al, 1968) or 33 (porcine - Chance et al, 1968) amino acids. Two proinsulin structures have been isolated from the rat pancreas (Clark and Steiner, 1969). Proinsulin is thought to facilitate the formation of disulphide linkages of the insulin molecule, (Steiner and Clark, 1968) as proinsulin can spontaneously reform its native structure from a random coil structure and is converted to insulin by limited trypsin action.

Proinsulin is produced on the ribosomes of the beta cells but is usually transformed to insulin within the islet cell for storage and subsequent release. Under some circumstances small amounts of proinsulin can be secreted from the islets.

C. DIABETES MELLITUS.

One of the hormones required by the mammals for regulation of some metabolic cycles is insulin. Insufficient insulin, or destruction of the insulin producing cells in the body causes a wasting disease - diabetes mellitus.

The date of the first description of diabetes is ancient, but unknown, although claims have been made on behalf of China, India and Egypt as home of the first discoverer of the disease (Young, 1960). Brunner, in 1682, and Cowley in 1788, described conditions suggestive of diabetes mellitus.

In 1869 Langerhans described islets of cells in the pancreas which were not connected to the pancreatic ducts. He did not assign any specific function to them. About the same period Heddon in physiological studies of the pancreas, established that the islets were a "scattered" gland of internal secretion.

Lageusse in 1883 suggested that the islets of Langerhans may secrete a hypoglycemic factor, but the definite relationship of the pancreas to diabetes mellitus was not established until 1889 when von Mehring and Minkowski showed that diabetes could be induced in a dog by removing the pancreas. (Netter, 1965).

Many rigorous studies to extract the active principle from the pancreas, with no apparent success were made in the 30 years following Minkowski's results. The belief that the pancreas secreted a hormone, the deficiency of which caused diabetes, was so strong that the early failures did not discourage the search for the hormone. In 1909 de Meyer suggested the name "insuline" for this substance from the pancreas

and Banting and Best (1922) used this name for the active principle they obtained from calf and dog pancreas, which was successful in treating diabetic dogs.

Many different diabetes melliturias and related melliturias have been classified (Bloodworth, 1968). Insulin deprivation can occur both experimentally and clinically. Inherited diabetes accounts for a large proportion of diabetes mellitus, although pancreatectomy, endocrine disorders, insulin inhibitors and antibodies in the body, and chemical destruction of the beta cells or the pancreas can cause melli-tirias. In all cases the insulin sensitive tissue (skeletal, muscle, heart, adipose and fibroblasts) are effectively deprived of insulin necessary for the transport of metabolites across cell membranes, and regulation of metabolic processes. Glucose accumulation in the blood is the most apparent result of an insulin deficiency. When the tolerance for glucose is reached, the renal threshhold for glucose is exceeded causing glycosuria, polyuria and loss of minerals from the body. Metabolic changes in insulin-sensitive tissues eventually affect non-insulin sensitive tissues such as the brain. Ketosis, acidosis, weight loss, and a negative nitrogen balance due to protein breakdown, occurs. If the insulin deficiency is not compensated diabetic coma and death will eventually occur.

The incidence of known diabetes is $\frac{1}{2}$ - 1% of the population. In community surveys a higher proportion of the population has been found to be diabetic. A study by Birmingham medical practitioners found that 10% of the randomly selected group showed diabetic glucose tolerance curves (Malins, 1961). Diabetics can be treated with insulin (40% of known diabetics), oral hypoglycemic drugs (30%), or by careful regulation of the diet (30%). Suggestions have been made that treatment should be: insulin 10%; tablets, 10%; diet, 80% (Nabarro, 1960).

Two groups of insulin substitutes are used for treating diabetics. Sulphonamide derivatives stimulate insulin secretion and may inhibit glucose secretions from the liver. Biguanides may cause inhibition of oxidative metabolic paths, enhancing lactic acid production, as well as reducing the reaction of insulin with inhibitors present in the blood. Both classes of oral hypoglycemic drugs require a source of insulin - endogenous or exogenous. Thus, their use is limited to patients who can still secrete insulin, or exogenous insulin must be administered.

Insulin must be administered by injection as, due to its protein nature, it is inactivated by enzymes in the digestive tract. New insulin products have been developed to modify the short, rapid action of regular amorphous, or zinc-insulin preparations. The rapid insulin action is due to the quick solubilisation of insulin of the body fluids.

The basic protein, protamine, obtainable from salmon sperm, will decrease the solubility of insulin. Protamine zinc insulin has an even longer and more reproducible activity than protamine insulin. Careful control of protamine and crystaline insulin ratios, so that no free insulin or protamine is present results in the commercial Neutral Protamine Hagedorm (NPH) insulin. NPH insulin has an activity intermediate between protamine zinc insulin and regular insulin. A relatively insoluble insulin can be prepared if high concentrations of zinc ions are used e.g. ten times the amount required for regular crystalisation techniques. The product can be crystalline and therefore long-acting, (ultralente) or in an amorphous or micro-crystalline form (semilente). Different activity profiles can be obtained by using different ratios of ultra and semi-lente (Shangraw and Lamy, 1969).

Insulin excess (hyperinsulin) can be caused by excessive manufacture, release of endogenous insulin or administration of exogenous insulin. The decrease in blood sugar concentration stimulates adrenaline production and deprives the brain cells of glucose, producing convulsions, shock, anxiety and weakness. Prolonged or repeated hypoglycemic attacks will produce neuronal damages. The consequences of insulin excess are due to the hypoglycemia produced, and not to the direct actions of insulin excess. Insulin dosages, and administration of oral hypoglycemic drugs must, therefore, be carefully calculated to minimize the chances of hypoglycemia or hypoglycemic attacks(Netter, 1965).