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

A thesis presented in partial fulfilment
of the requirements for the degree
MASTER OF TECHNOLOGY
in Biotechnology
At Massey University
Palmerston North
New Zealand

JANIS ELIZABETH SWAN

1971

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.

ACKNOWLEDGEMENTS.

I wish to thank my supervisor, Professor R. L. Earle, for his guidance, constructive criticisms and enthusiasm during this research project.

Financial assistance from Tasman Vaccine Laboratory Limited, Upper Hutt, is gratefully acknowledged.

I am also grateful for the assistance given by Dr. A. Kirton, Ruakura Animal Research Centre, Hamilton, and his assistants for collection of the pancreas glands.

Mr. R. J. Trott, Radiotherapy Department, Palmerston North Hospital, for carrying out the radioimmunoassay.

Mr. M. S. Carrie, Canterbury Frozen Meat, Christchurch, for the translation of Russian references, and Mr. Gilberg, Food Technology Department, Massey University, for translation of Swedish references.

Mr. R. Fenwick, Food Technology Department, Massey University, and Dr. S. Antonsen, Nordisk Insulinlaboratorium, Denmark, for doing the disc electrophoretograms.

The staff of the Food Science and Biotechnology Faculty, Massey University, for their helpful advice and aid.

Mrs. E. Self for her competent typing.

Mr. M. R. Patchell, Poultry Research Centre, Massey University, for his advice on statistical analysis.

Stephen, my husband, for his encouragement and patience.

TABLE OF CONTENTS

		PAGE.
SUMMARY		ii
ACKNOWLEDGMENTS		iii
LIST OF TABLES		vi
LIST OF FIGURES		vii
LIST OF PLATES		vii
CHAPTER		
	INTRODUCTION	1
	AIMS OF THE PRESENT STUDY	2
I	THE PANCREAS, INSULIN AND DIABETES MELLITUS	3
	A THE PANCREAS	3
	a Situation	3
	b Structure	3
	c Pancreatic Enzymes	4
	d Pancreatic Hormones	4
	B INSULIN	6
	a Molecular Weight	7
	b Primary Structure	7
	c Tertiary Structure	8
	d Synthetic Insulin	11
	e Proinsulin	11
	C DIABETES MELLITUS	12
II	INSULIN YIELDS AND EXTRACTION METHODS	15
	A INSULIN CONTENT OF THE PANCREAS	15
	a Representation of Insulin Content	15
	b Factors Affecting Insulin Content	15
	B EXTRACTION METHODS	22
	C INDUSTRIAL PRODUCTION OF INSULIN	25
III	ASSAY SYSTEMS FOR INSULIN	26
	A UNIT OF INSULIN	26
	B BIOASSAY SYSTEMS	26
	a Rabbit Assay	26
	b Mouse Assay	26
	c In vivo Assays	27
	c In vitro Assays	27

PAGE.

C RADIOIMMUNOLOGICAL METHODS 28

D CHROMATOGRAPHIC METHODS 29

E FIBRIL METHODS 29

F OTHER METHODS 30

G COMPARISON OF ASSAY METHODS 30

IV PANCREAS COMPOSITION AND INSULIN CONTENT OF NEW ZEALAND SHEEP 33

A SHEEP PANCREAS IN NEW ZEALAND 33

a Introduction 33

b Source of Glands 35

c Analysis 35

d Results 36

e Discussion 41

B COMPARISON OF EXTRACTION METHODS 41

a Introduction 41

b Methods Investigated 42

c Materials 46

d Discussion 46

C ASSAY METHODS 49

a Introduction 49

b Methods Investigated 50

D YIELDS OF INSULIN FROM NEW ZEALAND SHEEP PANCREAS 52

a Method

b Discussion

E CRYSTALLINE INSULIN 59

CONCLUSIONS 61

POSSIBILITIES FOR FURTHER INVESTIGATIONS 62

APPENDICES.

1 PANCREAS WEIGHTS 63

2 METHODS OF ANALYSIS 65

3 STATISTICAL ANALYSIS OF SHEEP PANCREAS WEIGHTS 68

4 RADIOIMMUNOASSAY METHOD 72

5 METHODS INVESTIGATED FOR THE CRYSTALLISATION OF SHEEP INSULIN 73

6 DISC POLYACRYLAMIDE GEL ELECTROPHORESIS 75

7 TWO-PHASE COUNTERCURRENT EXTRACTION OF THE ALCOHOLIC EXTRACT 76

REFERENCES CITED 78

LIST OF TABLES

TABLE		PAGE
I	Species Differences in Insulin Amino Acid Sequence.	9
II	Insulin Levels in Animal Pancreas Glands.	16
III	Comparison of Assay Systems	31
IV	Meat Slaughtering in New Zealand Export Works and Abattoirs.	34
V	Regression Equations: Sheep Pancreas Weight with Age.	37
VI	Comparison of Reported and Calculated Pancreatic Weights.	38
VII	Regression Equations: Sheep Pancreas Weight with Age.	39
VIII	Analysis of Pancreas Composition.	40
IX	Effect of Extract pH on Insulin Yield.	43
X	Insulin Yields from New Zealand Sheep Pancreas (Assayed from crude Insulin Samples).	53
XI	Insulin Yields from New Zealand Sheep Pancreas (Assayed from Alcoholic Extracts).	56
XII	Pancreas Insulin Concentration of Young Lambs and Adult Sheep.	55
A1.I	Sheep Pancreas Weight with Age.	63
A1.II	Average Pancreatic Weights of Sheep with Age.	65
A3.I	Regression Data: Sheep Pancreatic Weight with Age.	68
A3.II	Comparison of Regression Coefficients for Pancreas Weights of Lambs 100-140 Days-old and Sheep 425-490 Days-old.	69
A3.III	Analysis of Variance of Pancreas Weights from Sheep during the Winter Period.	70
A3.IV	Comparison of Regression Coefficients for Pancreas Weights of Sheep 42-140 Days-old from Earle and this Study.	71

LIST OF FIGURES

FIGURE		AFTER PAGE
1	Diagram of the Sheep Pancreas.	3
2	Diagram of a Pancreatic Islet and Surrounding Acini.	3
3	Amino Acid Sequence of Sheep Insulin.	7
4	Weight of Sheep Pancreas with Age.	36
5	Moisture, Fat and Protein Composition (wet basis) of Sheep Pancreas with Age.	39
6	Relationships of Protein, Fat and Ash Composition (dry basis) in Sheep Pancreas.	40
7	Schematic Diagram of Insulin Yields in Two Extraction Methods	48
8	Insulin Yields from Sheep Pancreas with Animal Age.	57
A4.1	Standard Curves for Radioactivity (counts/min) with Concentration (μU/ml) of Sheep and Human Insulins for Radioimmunoassay of Insulin using Human Insulin Antiserum.	72

LIST OF PLATES

PLATE		AFTER PAGE
1	Disc-gel-electrophores of Insulin, Proinsulin and Glucagon.	60
2	Disc-gel-electrophoresis of Insulin.	60
3	Crystalline Sheep Insulin.	60
4	Crystalline Sheep Insulin.	60

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.

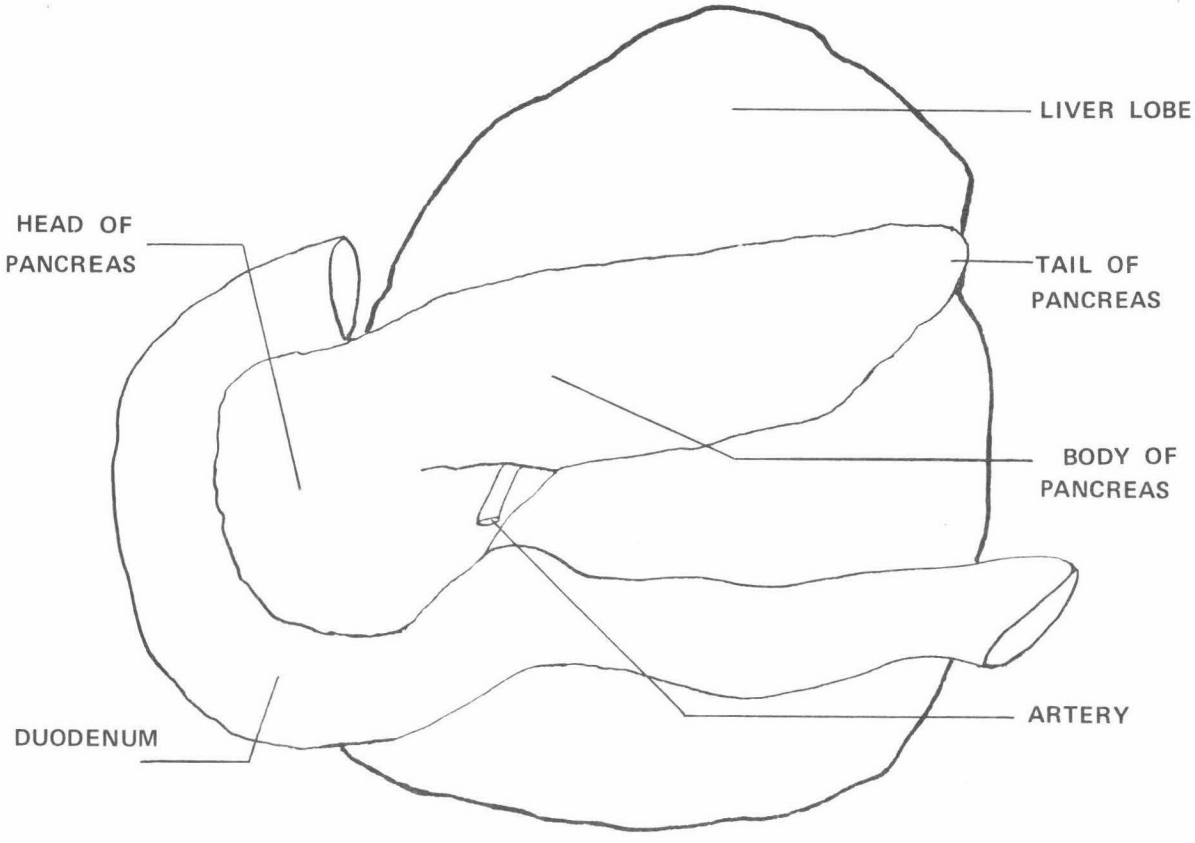


Figure 1: DIAGRAM OF THE SHEEP PANCREAS

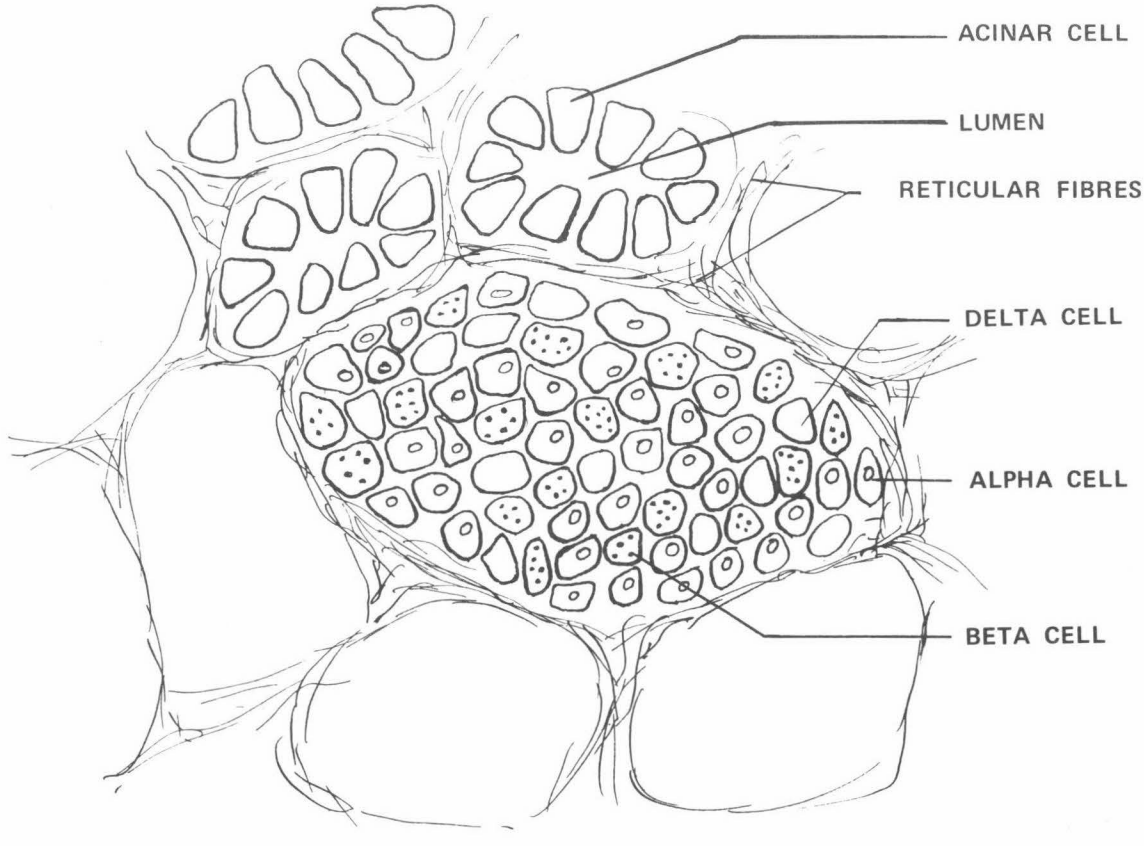


Figure 2: DIAGRAM OF A PANCREATIC ISLET AND SURROUNDING ACINI.

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.

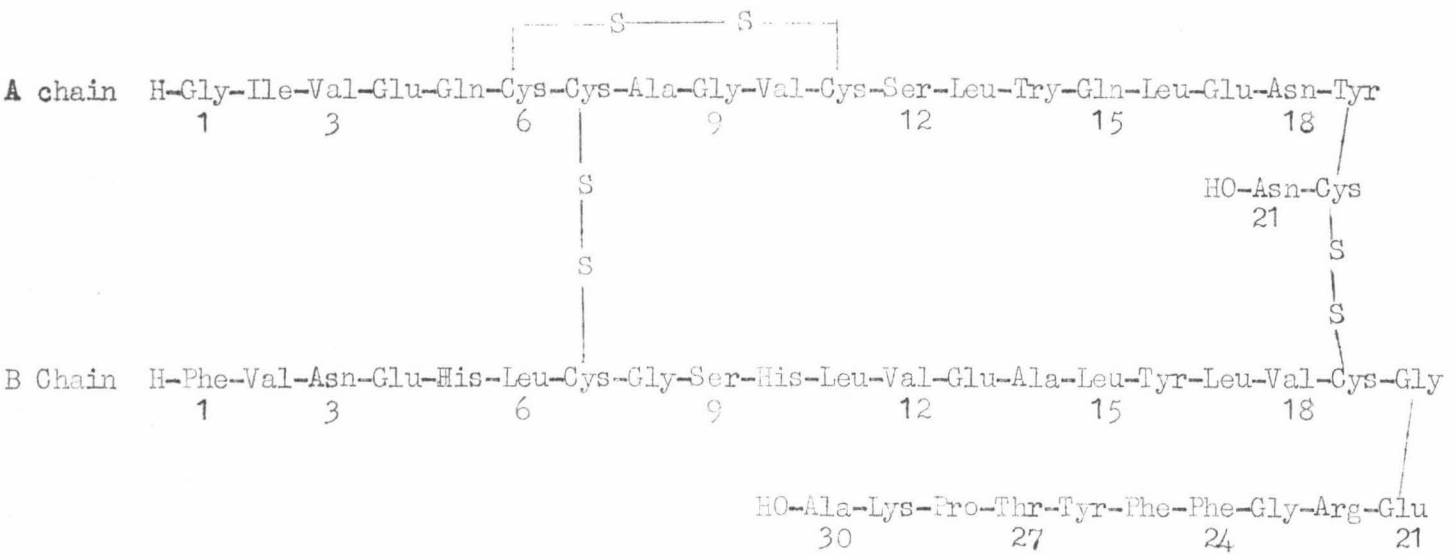
a. Molecular weight. After several investigations, the smallest unit of insulin, a monomer, has been found to have a molecular weight of 5700 (Kupke and Linderstrom-Lang, 1954). Except under special conditions, the stable unit in aqueous acidic solutions is the dimer (Low and Einstein, 1960).

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).

CHAPTER II.

INSULIN YIELDS AND EXTRACTION METHODS.

A. INSULIN CONTENT OF THE PANCREAS.

a. Representation of Insulin Content.

The insulin content of the pancreas is dependent upon the concentration of insulin in the islets cells and the total volume of the beta cells. Factors which change the insulin content of the pancreas may affect the beta cell volume in the pancreas or the insulin content of the beta cell. Simultaneous measurement of beta cell volume and insulin content of the pancreas, although investigated in the mouse and mouse foetus (Dixit et al, 1964), is not easily done and other methods of expressing insulin content are used.

Yields of insulin from pancreas have been expressed as units of insulin per kilogram of body weight, units of insulin per unit weight of pancreas or units of insulin per animal or group of animals. Insulin yields may also be expressed as weight of insulin e.g. milligrams instead of units, but the activity of the insulin must be known. None of the methods of presenting the results are satisfactory under all circumstances. If the pancreas of a species is diffusely distributed in the body any expression on the basis of weight of pancreas becomes of doubtful value because variable amounts of fat and other materials are usually present in the gland. Expressions of insulin content on the basis of body weight are useful but frequently there is no comment to indicate whether the reference is the initial or final weight of the test animal. The comparison of insulin content based on final body weight can be misleading as under varying conditions, e.g. diet, the insulin content of the pancreas need not bear any specific correlation to body weight (Best et al, 1939b).

b. Factors Affecting Insulin Content.

1. Species. The total insulin content of the pancreas varies greatly between species depending both on insulin concentration in the beta cells and pancreas size. The concentration of insulin in the gland, expressed as units of insulin per unit weight of pancreas, is more constant. Normal insulin values have been reported for several fish and mammalian species (Table II).

TABLE II.

INSULIN LEVELS IN ANIMAL PANCREAS GLANDS.

ANIMAL	UNITS/GRAM PANCREAS	WORKER	DATE.	EXTRACTION METHOD.	ASSAY METHOD.
BIRDS					
Chicken	0.76	Redenbaugh et al	1926	Alcohol-H_2SO_4	Bioassay
"	0.65	Jephcott	1931b	Alcohol-HCl	Bioassay
"	0.2-0.5	Kimmel et al	1968	Alcohol-H_2SO_4	Paper Chromatography
Duck	0.53	Jephcott	1931b	Alcohol-HCl	Bioassay
MAMMALS.					
Mouse	1.7	Marks & Young	1940	-	Mouse
adult	2.0	Renold et al	1960	-	Adipose Tissue
adult	1.8	Dixit et al	1962	Alcohol-acid	Adipose Tissue
young	6.0	Dixit et al	1964	Alcohol-acid	Adipose Tissue
2 months	5.4	Sneyd	1964	Alcohol-acid	Adipose Tissue
4 months	5.6	"	"	"	and
6-11 months	2.8	"	"	"	Diaphragm
Rat	2.1	Best et al	1939a	Alcohol-HCl	Mouse
"	0.6	Soong	1940	Alcohol-HCl	Bioassay
"	1.3	Marks & Young	1940	Alcohol-HCl	Mouse
"	1.0	Griffiths	1941	Alcohol-HCl	Mouse
Rabbit	7.8	Marks & Young	1940	Picrate-acetone	Mouse
"	9.5	"	1940	Alcohol-HCl	"

Rabbit	5.2	Griffiths	1942	Alcohol-HCl	Mouse
Dog	0.8-1.5	Nothmann	1925	Alcohol-H_2SO_4	Rabbit
"	2.1	Takeuchi	1928	Alcohol-H_2SO_4	Rabbit
"	2.6	"	"	Picrate-acetone	"
"	3.8	Jephcott	1931b	Alcohol-HCl	Bioassay
"	5.0-5.5	Murray & Waters	1932	Alcohol-HCl	Bioassay
"	3.4	Best et al	1939b	Alcohol-HCl	Mouse
"	3.3	Marks & Young	1940	Alcohol-HCl	Mouse
Cat	2.0	Baker et al	1924	Picrate-acetone	Rabbit
"	0.7	Takeuchi	1928	Picrate-acetone	Rabbit
"	2.5	Jephcott	1931b	Alcohol-HCl	Bioassay
"	1.7	Scott & Fisher	1938a	Alcohol-HCl	Mouse
"	2.2	Marks & Young	1940	Alcohol-HCl	Mouse
"	1.0	Davoren	1962	Alcohol-HCl	Paper Chromatography
Guinea Pig	0.08	Marks & Young	1940	Alcohol-HCl	Mouse
"	0.23	"	1940	Picrate-acetone	"
Cattle	0.6-0.8	Fisher	1923	Alcohol-acid	Rabbit
adult	0.1	"	"	"	"
young	1.0	"	"	"	"
"	0.7-1.2	Wernicke	1924	Alcohol-HCl	Bioassay
"	1.5-2.5	Somogyi et al	1924	Alcohol-H_2SO_4	Rabbit
"	0.25	Dodds & Dickens	1924a	Formic acid	Rabbit
"	1.8	"	1924b	-	Rabbit

Cattle	0.9-1.5	Dudley & Starling	1924	Alcohol-$NaHCO_3$	Rabbit
"	2.5	Baker et al	1924	Picrate-acetone	Rabbit
"	4.4	Moloney & Findlay	1924	Alcohol	Bioassay
"	1.8	Fenger & Wilson	1924	Alcohol-HCl	Rabbit
"	2.0	Langecker & Wichowski	1925	Alcohol-acid	Bioassay
"	1.8-2.2	Scott & Best	1925	Alcohol-H_2SO_4	Bioassay
"	1.8-2.5	Blatherwick et al	1927	Alcohol-H_2SO_4	Rabbit
"	1.3	Takeuchi	1928	Picrate-acetone	Rabbit
"	1.3	Kaulbersz	1930	Alcohol-H_2SO_4	Rabbit
"	1.4	"	"	Alcohol-$NaHCO_3$	"
"	1.4	"	"	Water-HCl	"
"	1.4	"	"	Water-$NaHCO_3$	"
"	3.0-3.5	Jephcott	1931a	Alcohol-HCl	Bioassay
6-8 weeks	11.4	Fisher & Scott	1934	Alcohol-HCl	Mouse
2-yrs.	4.8	"	"	"	"
9=yrs.	1.8-2.2	"	"	"	"
"	3.6	Homan	1952	Alcohol-acid/NaCl	Bioassay
"	1.3-1.9	Maxwell & Hinkel	1952	Alcohol-H_3PO_4	"
"	0.9-1.5	"	"	Alcohol-H_2SO_4	"
"	0.8-1.0	"	"	Alcohol-HCl	"
"	1.8-2.0	"	1954a	Alcohol-oxalic acid	Mouse
"	0.99	"	"	Alcohol-formic acid	"
young	10	Light & Simpson	1956a	Alcohol-HCl	Paper Chromatography

Pig	1.6-3.1	Dodds & Dickens	1924b	W ter, formic acid formaldehyde	Rabbit
"	5.5	"	"	Picrate-acetone	"
"	3.0	Clough et al	1924	Water-HCl	Rabbit
"	3.0-4.3	Baker et al	1924	Picrate-acetone	Rabbit
"	1.7-1.9	Fenger & Wilson	1924	Alcohol-HCl	Rabbit
"	2.1	Takeuchi	1928	Alcohol-H_2SO_4	Rabbit
"	1.3	"	"	Picrate-acetone	"
"	2.0	Jephcott	1931b	Alcohol-HCl	Bioassay
"	1.6-3.1	Maxwell & Hinkel	1952	Alcohol-H_3PO_4	Bioassay
"	0.9-1.7	"	"	Alcohol-formic acid	"
"	2.1	"	1954	Alcohol-oxalic acid	Mouse
Sheep	1.0	Baker et al	1924	Picrate-acetone	Rabbit
"	1.8	Fenger & Wilson	1924	Alcohol-HCl	Rabbit
"	0.7	Jephcott	1931b	Alcohol-HCl	Bioassay
"	0.3	Katkovskii & Slvarts	1961	Alcohol-acid	-
1 day	8.2	Willes et al	1969b	Alcohol-HCl	Radioimmunoassay
1 week	6.2	"	"	"	"
2 weeks	5.6	"	"	"	"
14 weeks	3.0	"	"	"	"
2-6 yrs.	1.2	"	"	"	"
Horse	1.5	Baker et al	1924	Picrate-acetone	Rabbit
"	1.4	Takeuchi	1928	"	Rabbit
"	2.0	Jephcott	1931b	Alcohol-HCl	Bioassay

Monkey	2.5	Jephcott	1931b	Alcohol-HCl	Bioassay
Chimpanzee	11.2	Marks & Young	1940	Alcohol-HCl	Mouse
Man	0.24	Pollak	1926	-	Bioassay
"	0.84	Takeuchi	1928	-	Rabbit
"	1.7	Scott & Fisher	1938b	Alcohol-HCl	Mouse
<6 months	8.1	Jorpes & Rastgeldi	1953	Alcohol-HCl	Rabbit
> 40 yrs.	3.66	"	"	"	"
70-86 yrs.	4	"	"	"	"
"	0.8	Mirsky et al	1963	Alcohol-HCl/NaCl	Adipose Tissue & Radioimmunoassay
"	2.2	Kimmel & Pollock	1967	-	-
Whale Sperm	3	Jorpes	1950	-	-
Blue	2.2	"	"	-	-
Finback	1.6	"	"	-	-
Humpback	1.1	"	"	-	-
"	0.5	Shibata	1953	Me_2CO-HCl	Bioassay
"	0.8	Kimotsuki et al	1959	Alcohol-HCl	Bioassay
Sheep/Beef 80:20	0.7	Vasavada et al	1964	Alcohol-H_2SO_4	Mouse
65:35	0.8	"	"	"	"
FISH					
various spp.	150-190	Toyama et al	1941	-	-
various spp.	70-220	Nagasawa	1968	Picrate-acetone	Rabbit

Levels for insulin content in fish are much higher than in mammals as the acinar and islet cells are separate organs and fish insulin content, can be compared with mammalian insulin levels if the ratio of islet to acini tissue is known. For humans, 21 years old the ratio islet:acini is 0.0162 (Ogilvie, 1937) and for Wistar rats 0.011 (Richardson and Young, 1937). The ratio for mice at different ages has also been obtained (Dixit et al, 1964).

2. Age. The insulin content of the pancreas is related to age, as the size of the pancreas, ratio of islet and acini, and the concentration of insulin in the beta cells change with age. Increase in the insulin concentration, units of insulin per unit weight of tissue, have been observed in foetal pancreatic tissue from human (Grillo and Shima, 1966), sheep (Willes et al, 1969b), rat and rabbit (Grillo, 1964), chicken (Grillo, 1961), mouse (Dixit et al, 1964) and beef (Fisher and Scott, 1934) during gestation. At or near birth a peak in insulin concentration is usually reached, followed by a decrease in insulin concentration with increase in age. This effect has been observed in beef (Fisher and Scott, 1934), mouse (Dixit et al, 1964), man (Jorpes and Rastgeldi, 1953) and sheep (Willes et al, 1969b), although an increase in insulin content with age has been observed in Wistar rats (Griffiths, 1941).

3. Sex. The relationship of sex and insulin content in the pancreas has not been reported in the literature, except for the guinea pig (Davidson and Haist, 1964) and human (Jorpes and Rastgeldi, 1953; Wrenshall and Bogoch, 1955) pancreas glands.

4. Seasonal variations. Fisher and Scott (1934) observed that better yields of insulin were obtained from beef pancreas in the winter months. Ostling (1928) observed the same effect in pigs and young domestic animals.

5. Diet. Diet has been found to affect insulin secretion and insulin content of the pancreas. Best et al, (1939b) showed that the insulin content of the rat pancreas is reduced by fasting, decrease in calorie intake of a balanced diet, and by feeding high fat levels. The reduction of insulin content appeared to be related to the deficiency of carbohydrate, or carbohydrate-forming substances in the diet, although diets rich in carbohydrate did not appreciably increase the insulin

content of the pancreas above the normal level.

6. Collection of glands. The enzymes present in the pancreas can inactivate the insulin. Formerly fresh pancreas glands were used for insulin extraction, the glands being collected, trimmed of fat, and processed as soon after killing the animal as possible. A high pancreatic fat level, as in pig pancreas, makes extraction difficult, and pancreas from thin animals have been recommended (Sordelli, 1927).

Present-day collection methods advocate freezing of the glands as soon as possible and within half an hour after killing the animal. Glands can then be stored until needed for processing. Wrenshall et al, (1957a) found that extractable insulin levels in beef pancreas increased to a peak value if aged for eight to twelve days at 6°C or for eighteen hours at 25°C. Insulin levels then decreased, most probably due to enzymic action. No increase in extractable insulin was observed in glands frozen immediately after slaughter, thawed one hour later and aged (Wrenshall et al, 1957b).

Other methods for preservation of pancreas if freezing is not available include picric acid or phenol solutions for fish islet preservation (Tarteno, 1948), drying (Katkovskii et al, 1946), salt preservation (Eingorn, 1946), acidified alcohol containing preservative, or acidified salt solutions (Barollier, 1954), and azeotropic defatting and drying of the glands (Levin, 1970). Processing conditions may need to be modified depending upon the preservation method used.

B, EXTRACTION METHODS.

Although many methods of extraction of insulin exist most procedures are still based on the acidified alcohol extraction used by Banting and Best. Most extraction methods use Scott and Fisher's (1938a) modifications of Jephcott's procedure (1931a) with slight modifications.

The main extraction methods are:

1. Acidified alcohol. Insulin is soluble in acidified ethanol and denatured ethanol (ethanol containing methanol) aqueous solutions. Variations of ratios of solvent to gland tissue are used but usually the glands are extracted in two to three times their weight of solvent. The glands are usually minced while frozen into the solvent. Thawing of the glands is carefully avoided as the proteolytic enzymes will rapidly destroy the insulin. This action does not occur very rapidly in the

intact gland during the 0 - 20 hours after death when the insulin is separated from the enzymes of the acini by cellular structures. These histological structures are usually destroyed by the actions involved in freezing and thawing (Jorpes and Rastgeldi, 1953).

Organic or inorganic acids can be used to acidify the extract to pH 2- 4. In earlier methods sulphuric or hydrochloric acid was used and the insulin yield could be optimised by adjusting acid strength and type as well as the concentration of alcohol (Somogyi et al, 1924; Jephcott, 1931a; Romans, 1954). Recent methods advocate orthophosphoric acid as the extract is more easily handled due to some unstated property of the acid (Maxwell and Hinkel, 1954b). Organic acids which have been used for acidification include oxalic, acetic, formic, propionic, butyric (Maxwell and Hinkel, 1954a) and lactic (Katkovskii et al, 1962).

Addition of salts, such as sodium chloride, can improve yields by twenty percent (Romans, 1954).

2. Alkaline alcohol. Dudley and Starling (1924) claimed improved yields of insulin if alcohol-sodium bicarbonate solutions were used for extractions, and although similar methods were used in the early 1950's, (Indian Institute of Science, 1952; Peterson, 1953) this method is rarely used as the proteolytic activity of the pancreas is not as easily suppressed under alkaline conditions as acid conditions.

3. Picrate. Picrate in acetone, alcohol or water can be used to extract insulin directly from pancreatic tissue (Dudley, 1924). Picrate-protein complex is precipitated, and the insulin-picrate is easily extracted by differential dissolution from the precipitate.

Other extraction systems used for insulin include perfusion with acidified water (Clough and Murlin, 1943), aqueous extraction of defatted, powdered glands (Santenoise, 1961) and acidified carbon tetrachloride (Domba and Tarsai Ceg, 1949).

The pancreas glands are usually extracted with more than one lot of clean solvent depending on the solid to liquid ratio. Fixed or moving bed counter-current extraction of the pancreatic glands has not been used commercially. After extraction the tissue and insulin-containing extract are separated, usually by centrifugation, although filtration may be used. The solid is usually discarded, although it can be used

for animal feeds or for extraction of the pancreatic enzymes(Federiksen, 1951; Thompson, 1954; Janholm and Holm, 1962).

Vacuum concentration at temperature below 40°C (Romans et al, 1940), ether addition (Pettinger, 1958) or countercurrent ether extraction (Randall, 1964) are methods which have been used to concentrate the insulin extract. Fat in the concentrate may be removed by filtration, centrifugation or solvent extraction.

The crude insulin is usually salted out using sodium chloride or ammonium sulphate, although other protein precipitation methods may be used such as iso-electric precipitation at pH 5.3 - 5.7, or acetone addition. Crude insulin with an activity of 1 - 2 i.u./mgm can be purified to insulin of 25 i.u./mgm using various protein purification methods such as isoelectric precipitation, chromatographic and ion-exchange techniques, and recrystallization. (Abel, 1926; Romans et al, 1940; Randall, 1964).

4. Ion-exchange Resins. The use of adsorption techniques on the manufacture and purification of insulin from alcoholic extracts avoids vacuum distillation, separation and processing of the lipids (involving potentially explosive mixtures), and fractional salting out, all of which contribute to the decrease in the yield of insulin. Several workers have investigated the use of ion-exchanges in insulin manufacture.

Alginic acid can quantitatively take up insulin from a 65% ethanolic solution at pH 2.8 - 3.4. The bulk of the fats remain in the alcoholic extract and the ethanol can be recovered by distillation. The insulin is eluted from the alginic acid with hydrochloric acid. 20 - 30% increases in yields of insulin as compared with the conventional insulin process have been observed (Jorpes and Rastgeldi, 1960). DEAE- cellulose can adsorb 75% of the insulin from an alcoholic extract at pH 6, with 98% recovery of insulin from the cellulose when eluted with hydrochloric acid (Volini and Mitz, 1962). Carboxy-methyl cellulose has also been used for insulin production (Wellcome Foundation, 1966). Katkovskii et al, (1969a) observed a 39% increase in yield over conventional methods using SDV-3T ion-exchange resin, and a reduction from 30% to 5% in the loss of insulin in the processing stages, extraction to crystallisation. Sulphonated cation exchangers have been found to be superior to cellulose

ion exchange resins and natural cation exchange resins in the production of crystalline insulin (Katkovskii et al, 1969b).

C. INDUSTRIAL PRODUCTION OF INSULIN.

Many factors can affect the yields of insulin obtained in commercial production. Beef, especially calf pancreas, are preferred as high yields (10,000 i.u. insulin/kilo of pancreas) can be obtained, processing is not difficult and beef and calf pancreas can be collected from slaughter houses in large quantities. Pig pancreas can also be collected in large quantities from slaughter houses, especially in the United States of America, but due to the high fat content of the glands they are not as easily processed.

Pancreas from small animals e.g. sheep or goats are rarely used mainly because large quantities are not available in the countries where large scale processing of insulin is undertaken. Almost all insulin manufactured in Japan was obtained from fish and whale pancreas until the early 1960's when production ceased as manufacturing costs were greater than for insulin obtained from domestic animals (Nagasawa, 1968). If an insulin extraction plant is going to be erected the pancreatic insulin content of animals readily available must be investigated. Vasavada et al, (1964) investigated the insulin content of buffalo, goats and sheep from slaughter houses in India.

Many factors in the process e.g. temperature, acidity, solvent, can affect the yields obtained. New methods usually include decreases in extraction time and a decrease in heat processing, e.g. the use of ion exchange resins, to help increase insulin yields. As many factors affect the yields of insulin optimisation of a process is usually obtained by trial and error. (Jephcott, 1931a; Romans, 1954).

CHAPTER III.

ASSAY SYSTEMS FOR INSULIN.

A. UNIT OF INSULIN.

A direct and specific chemical quantitative method for determining the potency of insulin is not yet possible as the relationship of insulin activity (or effect) with insulin structure is not known. Insulin assays usually involve observation of one or more of the hormonal effects of insulin in animals, or animal tissues, in comparison with a standard preparation. More precise but less specific chemical and immunological assay systems have been developed for insulin. Thus many different assay systems for insulin are available (Stewart, 1960; Smith, 1962).

The standard or reference insulin used in the assays is the Fourth International Standard established by the World Health Organisation in 1959 (Bangham and Mussett, 1959). The standard is defined as having a potency of 24.0 International units per milligram. The International unit of insulin is defined as the activity contained in 0.04167 milligrams of the standard preparations.

B. BIOASSAY SYSTEMS.

a. Rabbit Assay.

The blood sugar level of rabbits and other mammals will decrease (hypoglycemic reaction) if insulin is administered. In 1922 Banting and Best defined the Rabbit unit for insulin as being the smallest dose of insulin which would cause the blood sugar level in a rabbit to decrease to 0.45 milligrams per millilitre within four hours of administering insulin. As there is variation in the response to insulin between rabbits, and in the same rabbit, the cross-over test for the assay was developed (Marks, 1925). A linear regression between the logarithm of the dose of insulin and decrease in blood sugar exists (Stewart, 1960). Lacy (1941) developed a cross-over technique in which the unknown could be assayed in rabbits in terms of one dose of standard solution. Results from the bioassay must be analysed statistically to eliminate bias and allow for variation between rabbits.

b. Mouse Assay.

Although mice are not sensitive to large doses of insulin at normal environmental temperatures, they exhibit characteristic hypogly-

cemic convulsions if kept at elevated temperatures (25° - $37^{\circ}C$). There is a linear relationship between the number of animals convulsing and the logarithm of the dose level.

Temperature of the assay (Jerke et al, 1966), storage temperature of the mice (Sellar and Smart, 1959) and diet (Stewart, 1960) can affect the sensitivity and the precision of the assay. Results must be analysed statistically, four mice having the same statistical contribution to the assay as one rabbit (Smith, 1962). The advantages of the mouse assay compared with the rabbit assay include the speed with which the results are obtained, less labour, and that the mice can be housed much more easily than rabbits.

c. In vivo Assays.

The sensitivity of animals to insulin, in assays based on the hypoglycemic reaction, is increased if endocrine glands are removed. Assays have been developed using laboratory animals from which the hypophysis and adrenals have been removed, and the pancreatic insulin cells destroyed chemically e.g. alloxan-diabetic, hypophysectomised, (ADH) rats (Anderson et al, 1947), ADH adrenallated (ADHA) rats and mice (Anderson et al, 1957), and hypophysectomized alloxaned (HA) rats and mice (Beigelman, 1960). For a limited range of insulin dose there is a linear regression between blood sugar concentration and the logarithm of the insulin dose.

d. In vitro Assays.

Insulin-like activity can be detected at low levels using isolated tissue. The term insulin-like activity is used as unknown factors, present in plasma, can influence glucose uptake by tissues, and a discrepancy exists between insulin-like activity and biologically active insulin detected in a mouse or rabbit assay.

1. Diaphragm Assay.

Insulin-like activity will affect the uptake of glucose and the synthesis of glycogen by the diaphragm when placed in a suitable buffer solution. As the diaphragm is small it can only be divided into two parts without losing its sensitivity to insulin and it is necessary to obtain standard curves using different pieces of tissue.

Both rat and mouse diaphragms have been used in the assay. A greater number of tissues can be obtained more easily from mice than

rats. A differential uptake of glucose between the left and right hemidiaphragms have been observed (Bingle, 1963). The osmolarity of the medium with respect to the diaphragm, and inadequate oxygenation can affect the assay (Davidson et al, 1968). Measurement of glycogen synthesis, instead of glucose uptake limits the influence by factors which give insulin-like effects.

Other muscles e.g. minced pigeon breast muscles (Krebs and Eggleston, 1938) can be used.

2. Adipose Tissue Assay.

Insulin enhances glucose uptake, oxidation of glucose and lipid synthesis in isolated epididymal fat tissue. The advantage of using adipose tissue is that the standard solution, and the unknown solution can be assayed on the same tissue. The metabolic effects usually measured are the production of radioactive carbon dioxide after incubation of the tissue with radioactive glucose, uptake of glucose, synthesis of radioactive lipid and the respiratory quotient of the tissue (Renold, 1965).

C. RADIO-IMMUNOLOGICAL METHODS.

All radio-immunological methods used for measurement of protein hormones are derived from that first described for insulin by Yalow and Berson in 1957 (Yalow and Berson, 1968). The principle of the assay system is modification of the hormone by the addition of a suitable radioactive molecule so that the protein gains sufficient intrinsic radioactivity for very small quantities of hormone to be detected. Radioactive I^{131} or I^{125} is used to label insulin.

Specific antibodies e.g. anti-insulin serum can bind the labelled hormone forming a labelled antigin-antibody complex. An equilibrium will exist between the ability of the unlabelled hormone to compete with the labelled hormone for binding sites on the antibody.

The essential requirements for the radioimmunoassay include availability of the individual reactants (labelled antigen and specific antibody) and a technique for separating bound and unbound hormone. Several different separation techniques have been developed: electrophoresis with solvent flow (Yalow and Berson, 1960), fractional precipitation with salts (Grodskey and Forsham, 1960) or alcohol (Heding, 1965), double-antibody precipitation (Morgan and Lazarow, 1962; Hales

and Randle, 1963), coated charcoal (Herbert et al, 1965), ion exchange resin (Lazarus and Young, 1966), gel filtration (Horiuchi et al, 1966), talc and silica (Rosselin et al, 1966). The solid-phase radioimmunossay method developed by Catt and Tregar (1967) has also been used to assay insulin (Ceske et al, 1970).

Sensitivity and precision of the radioimmunoassay do not depend on the same factors, and an assay system must be optimised by investigation. The specificity of the basic reaction is immunochemical and most probably unrelated to the biologic properties of the hormone, as the immuno-reactive and biologically-active sites are unlikely to be in the same positions (Berson and Yalow, 1968).

Radioimmunological methods are considered superior to bioassay, for small concentrations of insulin, because of the greater specificity, reproducibility of results, sensitivity, accuracy, rapidity and smallness of the sample size (Hales, 1965). Studies on reproducibility of results between laboratories have been undertaken (Ashford et al, 1969; Cotes et al, 1969).

For the investigation of insulin levels in sheep pancreas Willes et al (1969b) used the immunoassay system of Grodskey and Forsham (1960) to determine the insulin concentrations in the alcoholic extracts obtained from pancreas glands.

D. CHROMATOGRAPHIC METHODS.

Several chromatographic methods have been developed for insulin assay. Grodskey and Tarver (1956), and Light and Simpson (1956a) developed ascending and descending paper chromatographic methods. The insulin content was measured colorimetrically after eluting the insulin from the paper. Crude insulin samples can be assayed but should be salt free and not contain too much crude protein. Radial paper chromatography gives sharper separation of insulin from other proteins (Bouman and Homan, 1958).

The results must be analysed statistically, and the standard and test samples run at the same time, on the same paper, to reduce errors and variations in results (Fenton, 1959; 1961).

E. FIBRIL FORMATION.

The precipitate formed when a slightly acidic solution of insulin is heated (Blatherwick et al,1927) is composed of radially oriented fibrils

(Waugh, 1948). An assay has been developed in which an insulin solution, (of at least one i.u./ml) is seeded with fibrils. The fibrils formed after mechanical reaction are weighed. Cross-seeding with insulins of different species may not give reproducible results (Grodskey, 1958). The method can be applied to samples containing less than 11 i.u./mg$_n$ or a solution of globulin zinc insulin. Close agreement with bioassays can be obtained (Krylova and Bazarova, 1960; 1962).

F. OTHER METHODS.

Polarographic measurements have been used to assay insulin (Puscura et al, 1958; Marha, 1960; Girad, 1964), as well as paper electropheresis (Voluiskaya, 1963) and starch-gel electrophoresis (Gaudina and Polizzi-Sciaronne, 1963). Disc-gel electrophoresis has been used for an investigation of the heterogeneity of crystalline insulin (Mirsky and Kawamura, 1966) but has not been applied to insulin assay.

G. COMPARISON OF THE ASSAY METHODS.

The sensitivity, precision, quantity of insulin required and sample size differ for the different assays systems (Table III).

In commercial preparation of insulin there are two generally accepted methods of assay of the product - the rabbit assay and the mouse assay, using the International Insulin Standard or the United States Parmacopeia Standard. Both methods have limitations which restrict widespread general application. Since 1925, when the First International Standard for insulin was adopted based on the rabbit assay, all preparations have been assayed, directly or indirectly, in terms of it (Stewart, 1960). Large amount of relatively high concentration of insulin are required e.g. 50 - 100 i.u. of at least one i.u./ml for rabbit assay and five i.u. of 0.1 i.u./ml for the mouse assay. Neither of these methods is suitable for determining insulin in plasma or in extracts produced from very small samples of tissue. Moreover if many samples are to be assayed the expenditure in animals and time becomes considerable.

In vitro and in vivo techniques require smaller amounts of insulin for assay, as do radioimmunoassay techniques, with the latter technique most probably giving the most relevant results (Renold, 1965), although in an investigation of blood insulin levels in sheep no correlation

TABLE III.

COMPARISON OF ASSAY SYSTEMS.

ASSAY METHOD	SENSITIVITY * (gm x 10^9)	ASSAY RANGE (mU/ml)	INDEX OF PRECISION**	UNITS REQ'D FOR ASSAY	QUALITY OF INSULIN (i.u./mgm)	REFERENCE.
Rabbit	40,000	1000-3000	0.1-0.3	50-100	>5	Young & Romans, 1948
Mouse	10,000	100-300	0.2	5-10	>5	Stewart, 1960
ADHA rat	2	0.05-5	0.2	0.1	low conc.	Bornstein, 1950
	5	0.13-5	-	0.2	" "	Anderson et al, 1947
	100	2.5-10	-	0.2	" "	Beigelman et al, 1956
HA mouse	10	0.25-4	0.4-0.8	0.2	" "	Anderson et al, 1957
Rat Diaphragm	5	0.1-1000	0.18-0.74	0.2	" "	Randle, 1956
Minced pigeon breast	400	10 -1000	-	0.5	>17	Krebs & Eggleston, 1938
Mammary gland	400	10 -5000	0.18-0.28	0.5	low conc.	Balmain et al, 1954
Fat Pad	1000	0.031-0.5	-	0.5	" "	Martin et al, 1938
Fibril	10,000	-	-	50	>11	Grodsky, 1958
Paper chromatography	10,000	$(10-40)\times10^{3}$	-	200	>15	Fenton, 1959
Radioimmunoassay	0.01	$(0.25-12)\times10^{-3}$	-	10^{-4}	impure insulin or low conc.	Ceske et al, 1970

* Based on crystalline insulin 25 i.u./mgm

** Standard Deviation / gradient.

between insulin-like activity as measured by the rat epididymal fat pad assay, and the radioimmunoassay results was obtained (Dash and Lindsay, 1967). The insulin-like activity determined by the epididymal fat pad assay may be divided into two parts; one measuring immunologically active insulin and the other an unknown factor (Renold, 1965). In vitro methods tend to give much higher values for insulin-like activity in blood than radioimmunoassay methods.

Chemical methods are less sensitive, probably less specific but more precise and less costly than biological methods. In choosing an assay proceedure for insulin consideration must be made of the accuracy required, purpose for which the test is made, the time and cost of the assay used, and the amount and quality of the insulin to be assayed.

CHAPTER IV.

PANCREAS COMPOSITION AND INSULIN CONTENT OF NEW ZEALAND SHEEP.

A. SHEEP PANCREAS IN NEW ZEALAND.

a. Introduction:

There is little information on the composition and weights of sheep pancreas with age. Willes et al (1969b) and Wallace (1948) reported the pancreatic weights in foetal lambs. Wallace (1948) and Schingoethe et al (1970) reported the pancreatic weights of lambs and mature sheep (Table VI). The insulin content of the pancreas has been found to be related to age, and may be related to other factors such as pancreatic protein or solids content. Further information on the amount of lamb and sheep available in New Zealand and its approximate composition, and insulin content was required.

Details of the number of sheep and lambs slaughtered in New Zealand are available from the Department of Agriculture and the Government Statistican (a lamb being a sheep aged less than one year.). The numbers of lambs and sheep slaughtered in New Zealand yearly have been steadily increasing (Table IVa). During the year there are large variations in the number of sheep and lambs killed (Table IVb). The large number of lambs killed in November and December represent young lambs, 12-16 weeks old, with a light carcass weight (30-36 lb). The numbers of lambs slaughtered gradually decreases in the January to March period, with a peak in April when the heavier carcass weight (40-60 lb) lambs, 9-10 months old, are killed before the winter. The number of lamb killed in the winter months are a small proportion of the annual total.

Sheep slaughter numbers also show two peak periods; December to January when many hoggets (one to two year old animals) and wethers are killed for the export meat market, and March to April when many culled ewes and surplus animals are killed before the winter. Throughout the rest of the year, May to November, the number of sheep killed is fairly constant and represents culled and surplus animals. In this study the weights, approximate composition of pancreas and insulin content from animals typical of the sheep and lambs killed in New Zealand freezing works were determined.

TABLE IV.

Meat Slaughterings in New Zealand Export Works and Abattoirs.

(a) Annual Total Kill.

Year ended:		Head of Stock '000 Cattle	Calves	Sheep	Lambs	Pigs
30th.Sept.	1966	1,153	1,098	5,924	22,219	852
	1967	1,201	1,217	7,699	24,049	770
	1968	1,482	1,233	9,381	26,315	800
	1969	1,679	1,354	8,826	26,750	770
	1970	1,833	1,307	9,072	27,432	808

(b) Monthly Kill of Sheep and Lambs.

Month		Head of Stock '000 Sheep	Lambs	% of Annual Kill Sheep	Lambs
1969	Sept.	332	79		
	Oct.	474	733	5.2	2.7
	Nov.	665	3,905	7.3	14.2
	Dec.	1,185	4,055	13.1	14.8
1970	Jan.	1,294	3,418	14.3	12.5
	Feb.	833	2,854	9.2	10.4
	Mar.	1,054	2,911	11.6	10.6
	April	1,350	3,539	14.9	12.8
	May	844	3,208	9.3	11.7
	June	536	1,943	5.9	7.9
	July	291	531	3.2	1.9
	Aug.	218	233	2.4	0.9
	Sept.	328	102	3.6	0.4

From N.Z. Department of Statistics Monthly Abstracts, December 1970.

b. Source of Glands.

The sheep and lamb pancreas were taken from animals of different age groups, killed at the Ruakura Animal Research Centre, in the course of various experiments. To cover more completely the age range, 42 days to 7 years, pancreas from sheep 1½, 4, 5, and 7 years were obtained from a meat works. Several breeds were included in the investigation: Merino, Romney, Border Leicester x Romney, Southdown, Southdown x Romney, Perendale, Corridale, but full details were not available.

All pancreas glands, except those collected from the meat works, were weighed after being carefully dissected from the digestive tract. The pancreas glands from sheep killed at the Ruakura Animal Research Centre were removed with 10 minutes of slaughter of the animal, weighed and frozen individually on slabs of ice in a forced-circulation freezer (-18°C). Freezing of the glands was begun within 20 minutes of slaughter. The pancreas collected at the meat works were collected from the animals and frozen within 30 minutes of slaughter in a blast freezer.

Pancreas glands collected at the Ruakura Animal Research Centre were air freighted to Massey University packed in dry ice. All glands, on arrival at Massey were stored in a freezer at -18°C until processed.

c. Analysis.

1. Preparation for Analysis. After samples had been taken for insulin extraction (Section D) the pancreas glands were reminced twice through a 10 mm cutting plate. Care was taken to prevent loss or gain of moisture during mincing and sampling. The sample was kept in a sealed plastic bag in a cold-room (7°C) until analysed. If the sample was not to be analysed within 24 hours it was frozen. Analyses were completed in triplicate.

2. Moisture. The minced pancreas sample was mixed with washed sand and dried for 15-18 hours in an air oven at 100°C. No decomposition of the pancreas was observed during this drying period.

3. Protein. The protein was determined by estimating the total nitrogen content; the value obtained being multiplied by 6.25 to give the percentage protein. The non-protein nitrogen content was also analysed by precipitation of the protein with trichloracetic acid in the first seven age groups investigated, but as it did not affect the total

nitrogen content significantly (Protein N = Total N - Non-protein N) it was not determined in the further groups investigated.

The total nitrogen was determined by the Kjeldahl digestion method using 2 gm samples of minced pancreas. A 10 ml sample of the digest was distilled in a micro-kjeldahl distillation apparatus.

4. Fat. The fat content was determined by Soxhlet extraction, with petroleum ether, of a sample of minced pancreas which had been mixed with washed sand and dried.

5. Ash. Ash was calculated by the difference of water, protein and fat content from 100 per cent.

The full methods for protein, moisture and fat determination are given in Appendix 2.

d, Results.

The results are divided into two parts :

1. Weights of individual pancreas glands.
2. Composition of groups of pancreas glands.

1. Weights of Pancreas Glands.

The detailed weights are given in Appendix I, Table A1.I. The individual pancreas weights were obtained from the data for each group of pancreas glands and arranged in chronological order.

From Figure 4, which graphically shows pancreas gland weight with age, six different growth periods can be observed :

42-140 days	-	increase in pancreas with age during the spring.
100-140 days	-	rapid increase in pancreas weight with age during the late spring and early summer period.
197-319 days	-	no significant change in pancreas weight during the winter period.
425-490 days	-	rapid increase of pancreas weight during the spring and early summer.
2-3 years	-	gradual increase of pancreas weight as the animal matures.
5 years	-	decrease in pancreas weight after maturity of the animal.

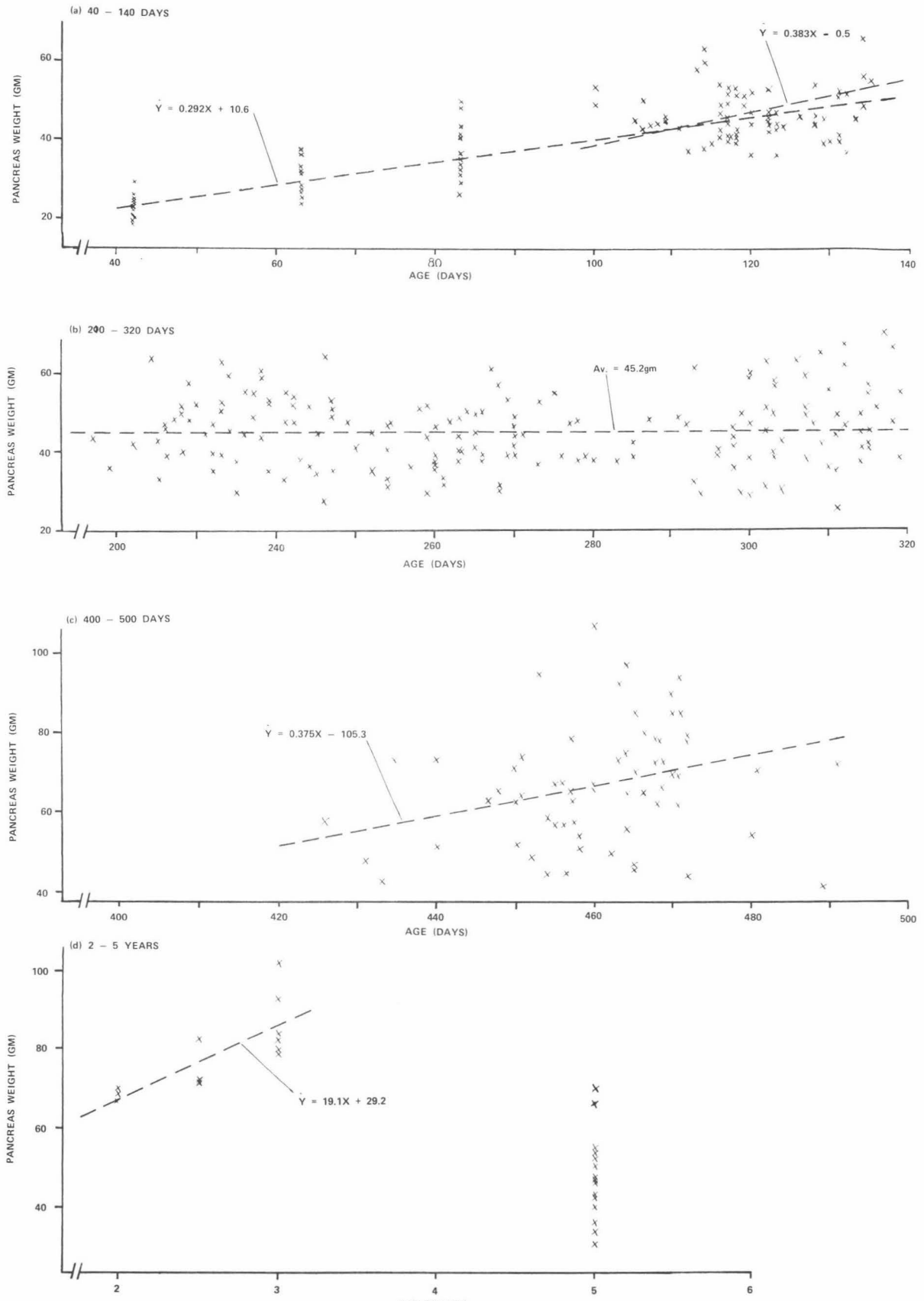


Figure 4: WEIGHT OF SHEEP PANCREAS WITH AGE.

Equations for the regression of pancreatic weight with age were formed by the least squares regression method (Snedecor & Cochran 1967). The data is tabulated in Appendix I, Table A1.I, and shown graphically in Figure 4. The regression data is summarised in Table V and further details can be seen in Appendix 3, Table A3.I.

TABLE V.

Regression Equations : Sheep Pancrease Weight with Age. Probability Value (p) for the Regression Coefficient is also included.

Period	Equation	p	X	Y
42-140 days	$\hat{Y} = 0.292X + 10.63$	< 0.001	days	gm.
100-140 days	$\hat{Y} = 0.383X - 0.48$	< 0.001	"	"
425-490 days	$\hat{Y} = 0.375X - 105.32$	< 0.025	"	"
2-3 years	$\hat{Y} = 19.1X + 29.2$	< 0.005	years	

i. Comparison of Pancreatic Weight Increases during the Spring.

The age periods 100-140 days and 425-490 days are one year apart, and both occur in a similar Spring - early Summer period when feed is abundant. A comparison of the regression data (Appendix 3, Table A3.II) showed that although the residual variances and elevations of the two groups were significantly different there was no significant difference in the regression coefficients. This indicates that the rate of pancreas weight increase for the two age groups is not significantly different from each other.

ii. Pancreas Weight during Winter.

The data for the pancreas weights of animals killed during the April to July period (197-319 days old) was classified within ten-day age periods. The average pancreas weight and variability of each class was not significantly different from the overall average weight of 45.2 ± 9.3 gm (Appendix 3, Table A3.III), indicating that there is no significant pancreas weight change with age of sheep during the winter. This lack of significance in pancreas weight change is probably due to a reduced growth rate of the animal over the winter period.

iii. Comparison of Pancreas weights with reported values.

Schingoethe et al (1970) reported pancreatic weights of 10.8 g and 44.4 gm for sheep 42 and 365 days old respectively. These weights are not predicted by the equations obtained for the corresponding age

groups in this survey although the average weight in the winter groups (45.2 + 9.3) would include the published figure for the 365 day old sheep. There was not a close agreement between values reported by Wallace (1948) in a study of the growth of Suffolk X Border Leicester-Cheviot Sheep in New Zealand, with values obtained in the present study (Table VI).

TABLE VI.

Comparison of Reported and Calculated Pancreatic Weights.

Reported Weights				Calculated Weights	
Age (days)	Number in Group	Weight (gm)	Source	Basis	$\hat{Y}$(gm)
42	5	10.78	Schingoethe et al, 1970	$\hat{Y}=0.292X + 10.63$	23 ± 1
				$\hat{Y}=0.244X + 7.54$	17.8 ± 2
				Average of 42 days old age group	22 ± 3
365	12	44.5	"	$\hat{Y}=0.375X - 105.32$	32 ± 52
				Av. of 197-319 days age group	45.2 ± 9.3
62 ± 11	4	34	Wallace, 1948	$\hat{Y}=0.292X + 10.63$	28.7 ± 1.4
				$\hat{Y}=0.244X + 7.54$	22.7 ± 2.7
				Av. of 63 day old age group	29.5 ± 4.6
112	8	47	"	$\hat{Y}=0.292X + 10.63$	43.3 ± 2.3
				$\hat{Y}=0.244X + 7.54$	34.9 ± 4.6
				$\hat{Y}=0.383X - 0.48$	42.4 ± 10.7
				112 day old animal	36.0
200 ± 27	6	55	"	Av.of 197-219 age group	46.3 ± 10.0
332 ± 14	2	59	"	Av.of 310-319 age group	45.2 ± 9.3

Earle (unpublished) obtain pancreatic weights from sheep of various ages in a study of the organ weights of Romney, Southdown and Southdown X Romney breeds in New Zealand. The data was obtained from ewes and rams, and each group contained 4-6 animals (Appendix I, Table A1.II).

The regression equations obtained from the data of Appendix I, Table A1.II, are summarised in Table VII. Further details are included in Appendix 3, Table 3.I .

TABLE VII.

Regression Equations : Sheep Pancreas Weight with Age.
The probability value (p) for the regression coefficient is also included.

Period	Equation	p	X	Y
42-112 days	$\hat{Y} = 0.244\ X + 7.54$	< 0.001	days	gm
42-175 "	$\hat{Y} = 0.136\ X + 14.98$	< 0.001	"	"

A comparison of the regression equation obtained from the data of Earle, and data for the 42-120 day age pancreatic weights obtained in this study show that there is no significant difference in the residual variances, or the regression coefficients for the two different samples (Appendix 3, Table A3.IV) although the elevations of the equations obtained are significantly different. Thus, from two different studies similar results were obtained for the pancreas weight increase for New Zealand lambs 40-120 days old.

2. Composition of Pancreas Glands.

The average composition of the sheep pancreas glands in each age group recorded in this study are given in Table VIII. The analyses have been expressed on both a wet and dry basis. As the pancreas was frozen on slabs of ice, pieces of ice adhereed to the glands and were included in the moisture content. Results on a dry basis will give a more accurate comparison of solid content.

Moisture was the largest component of the pancreas, being 75 - 79% during the greater part of the life of the sheep. Protein was approximately 16%, and the most important constituent of the dry solids. Fat content was very variable (3 - 10%) and could reflect the nutritional plane of the group of animals.

There appears to be no set pattern of pancreas composition with age (Figure 5) and no simple relationship between fat, protein and ash content (Figure 6). It should be noted that fat forms 20 - 30% of the solids in the pancreas. No comparative values for gland composition are available, although Schingoethe et al (1970) stated a solids content of 20.9% for 42 day old lambs, which is similar to the value obtained in this study.

TABLE VIII.

Analysis of Pancreas Composition (%).

Age		Wet Basis			Dry Basis		
Av. Days.	S.D.	Water	Protein	Fat	Protein	Fat	Ash
42		79.5	13.5	6.0	65.7	29.4	4.9
63		66.0	22.8	10.2	67.3	30.0	2.7
84		81.0	13.7	5.1	72.3	26.9	0.8
109.4	4.9	76.3	16.9	5.9	71.4	25.1	3.5
117.1	0.8	75.0	15.9	7.0	63.8	27.9	8.3
121.8	1.8	76.8	15.8	4.6	68.2	20.1	11.7
131.2	2.3	76.1	15.6	7.1	65.6	29.9	4.5
218.4	9.9	78.6	15.8	4.1	74.0	19.0	5.0
231.8	2.8	77.3	16.9	4.0	74.5	17.8	7.7
243.0	4.4	77.9	15.3	5.2	69.4	23.4	9.2
253.7	5.9	77.8	14.9	5.3	66.8	24.0	8.5
264.3	4.2	79.0	15.3	3.9	73.0	18.5	8.5
264.9	4.0	78.0	14.0	5.3	63.6	24.3	12.1
276.6	5.4	78.0	14.4	5.2	65.5	23.8	10.7
294.7	5.6	72.6	17.6	7.2	64.1	26.2	9.7
302.2	1.4	74.8	15.9	7.5	63.2	29.6	7.2
308.9	1.9	72.6	18.4	6.5	67.2	23.8	9.0
315.6	3.3	72.4	18.4	7.5	67.4	27.3	5.3
443.0	8.9	73.6	17.8	7.4	67.4	28.0	4.6
456.6	2.2	74.2	15.7	9.2	60.8	35.4	3.8
464.7	1.4	74.4	15.9	8.1	62.3	31.7	6.0
472.7	5.9	73.8	16.1	7.4	61.6	28.2	10.2
Years							
1.5	-	77.0	15.8	3.9	68.8	16.9	14.3
2.2	0.3	79.4	15.5	3.2	75.2	15.7	9.1
3	-	79.5	15.4	3.1	75.1	15.2	9.7
4	-	71.6	17.3	10.4	61.0	36.7	3.3
5	-	76.6	17.9	3.1	76.7	13.3	10.0
5	-	80.3	15.6	2.9	79.6	14.6	5.8
7	-	75.9	18.8	3.8	77.7	15.8	6.5

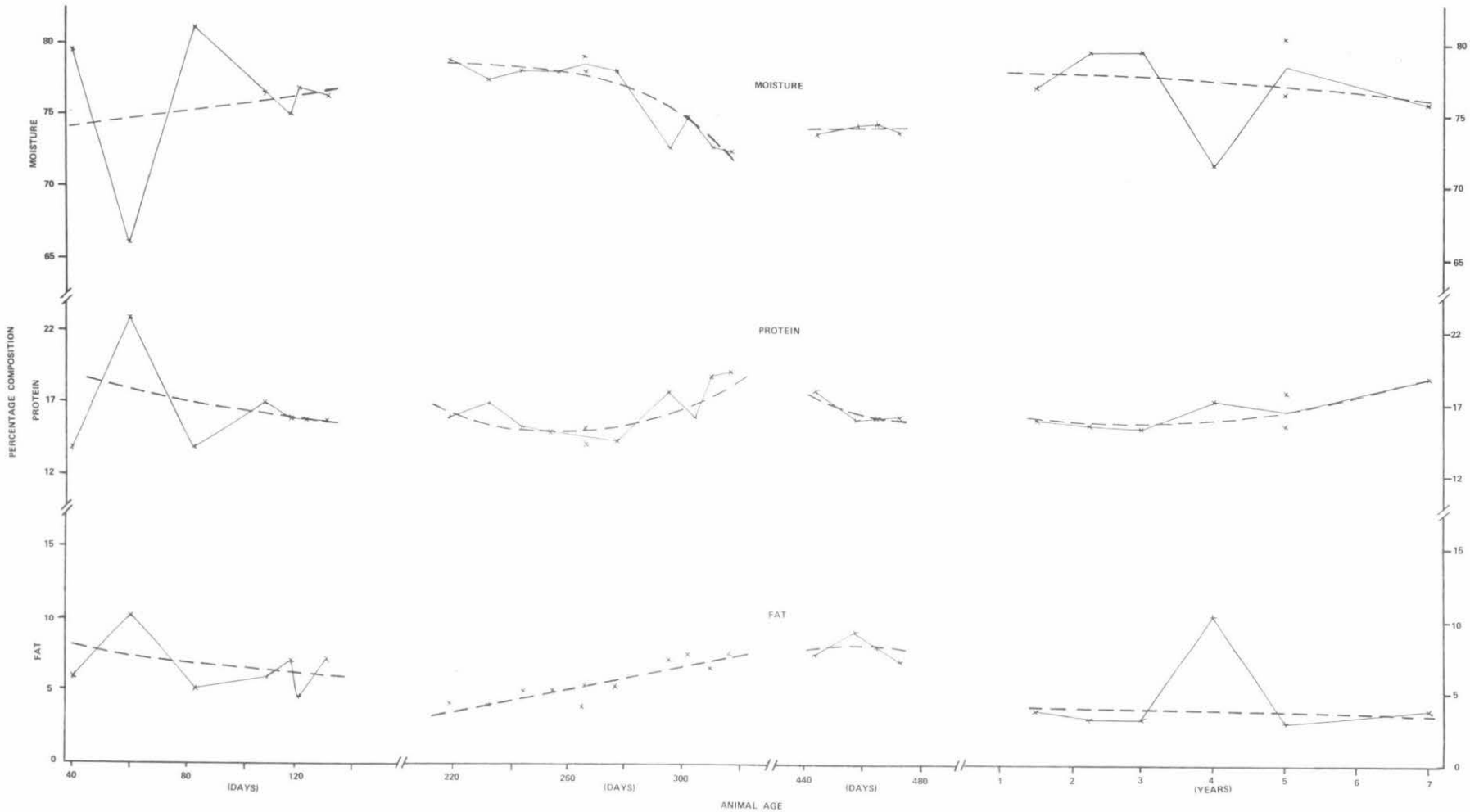


Figure 5: MOISTURE, FAT AND PROTEIN COMPOSITION (wet basis) OF SHEEP PANCREAS WITH AGE.

e. Discussion.

Pancreas glands of New Zealand sheep showed weight increases during the spring and early summer months, in the first two years of age in the sheep. During winter there is no real change in the pancreas weight. Pancreas glands increased in weight until at least the age of three years, the age at which the sheep has reached maturity, but there is a decrease in pancreas weight for five-year-old sheep.

Good agreement of pancreas weight as reported in the literature, and the pancreas weights obtained in this study was not obtained, even for a study of New Zealand animals (Wallace, 1948). However, a good agreement of pancreas weight with age was obtained in a more recent study (Earle, unpublished) of New Zealand sheep organ weights.

For calculating the yields of insulin or enzymes from sheep slaughtered at New Zealand meat works, the following pancreas weight could be used :

Lambs	44 gm
Hogget	64 gm
Sheep	80 gm
Old Sheep	50 gm

No trends for the chemical composition of pancreas glands were obtained, nor was there any simple relationship between the fat, ash and protein content.

B. COMPARISON OF EXTRACTION METHODS

a. Introduction

A suitable method of insulin extraction was required for the investigation of the insulin content of New Zealand sheep pancreas. Several different methods are available both for large and small scale extraction. The study was only over a limited time period of ten months, so detailed investigation of the methods used for insulin extraction could not be carried out, and as the quantity of glands obtained for each age group investigated was small, small scale methods had to be used.

In the evaluation of the methods the following points were considered :

i. The amount of pancreas required for the extraction. As each age group contained 7 - 24 animals representing 300 - 800 gm of glands,

if the insulin extractions were to be in duplicate, and chemical tests were also to be carried out on each group, the method must be suitable for small quantities of tissue.

ii. Quantity and cost of materials, chemicals and equipment. Some chemicals are difficult to obtain in New Zealand at short notice, or unavailable.

iii. Size and type of equipment. If possible, equipment already present in the laboratory had to be utilised or modified for use. Difficulties in obtaining suitable equipment in a short time was experienced.

iv. Time required for the extraction method. The time required for the method must be as short as possible, as twenty nine different age groups had to be investigated. The extraction method must not be too complicated, but still give representative results.

b. Methods Investigated.

Most insulin extraction methods use acidified ethanol, as the greatest yield can be obtained, followed by vacuum distillation of the alcoholic extract. Several investigators have noted that the pH of the extract affects the yield of insulin. Jephcott (1931a) obtained greater insulin yields using 0.4N sulphuric acid than when using 0.1N or 0.2N sulphuric acid. Using hydrochloric acid the greatest yield of insulin was obtained using an acid strength of 0.1N, other factors of time, alcohol concentration and temperature of extraction being constant. During the present investigation orthophosphoric acid was used for acidification of the extract, this being the acid used in the more recent methods. Katkovskii et al (1969a) obtained a 10% increase in insulin yield when using orthophosphoric acid instead of sulphuric acid. Oxalic acid and sulphuric acid was also used in two of the batches of pancreas extracted in the preliminary investigations but the effect on yield was not noted. If the pH of the alcoholic extract from sheep pancreas was less than pH 3.0 a decrease in the insulin produced occurred as seen in Table IX, where the yields of insulin from batches of pancreas for which the pH was accidently less than 3.0, are compared with the corresponding duplicate extractions for which the pH of the extract was greater than 3.0. All samples were extracted by the same method (ether concentration of the extract, Section b,3) and orthophosphoric acid used for acidification of the ethanol.

TABLE IX.

Effect of Extract pH on Insulin Yield.

Sample No.	Age Group days ± S.D.	Extract pH	Crude Insulin Yield mgm/kgm pancreas
1	264.9 ± 4.0	2.6	1190
2	"	3.2	1380
3	"	3.3	1390
4	276.6 ± 5.4	2.7	1080
5	"	3.2	1530
6	"	3.2	1510

In large-scale insulin production the macerate is usually stirred with a paddle, or allowed to stand, for the required time while the insulin is extracted from the tissue. In small scale production the frozen pancreas (50 gm - 1 kilo) is often homogenized in a blender for a short time.(Smith, 1964; Randall, 1964; Kimmel et al, 1968). Mirsky et al (1963) observed no apparent difference in the yields of insulin obtained by homogenization of the macerate for 30 - 60 seconds compared with slow stirring for about six hours.

In this study large quantities of glands (1-5 kilo) were extracted in acidified alcohol in a stainless steel vessel, and a Voss stirrer (Model S30/CB) used to mix the extract. Small quantities (50 gm) were macerated in a Waring blender (Type Aristocrat, Model 2057-701) with a high speed of 13,000 rpm, and a low speed of 2,000 rpm when unloaded.

The three methods investigated were :

1. Conventional method of insulin extraction using acidified alcoholic extraction, followed by vacuum distillation of the extract (Maxwell and Hinkel, 1954a).

2. Alginic acid method (Jorpes, 1960) as an example of an insulin extraction process using ion exchange resins. Several methods have been developed using ion-exchange resins. These methods have the advantage of increased insulin yields, and savings in equipment, power consumption and labour costs. Jorpes (1960) estimated that using alginic acid to obtain the insulin from the alcoholic extract there has a 20 - 30% increase in yield, and Katkovskii et al (1969b) obtained a 40% increase in yield using SDV-3T ion exchange resin.

3. Small scale extraction method, using a two-phase system to concentrate the alcoholic extract. Randall (1964) used diethyl

ether-water-ethanol system to concentrate the alcoholic extract and remove the fat.

1. Vacuum Concentration Method.

i. Extraction.

One kgm of frozen pancreas was minced through a 3 mm plate into two litres 85% v/v ethanol containing acid to give pH3. The material was stirred at room temperature for four hours and the solid removed by centrifugation. The solid was mixed with a further 1.5 litres 75% v/v ethanol at pH3 for two hours, and the solid removed from the extract by centrifugation. Centrifugation was carried out by placing the material in a cotton bag and spinning in a Hoovermatic spin-dryer.

ii. Removal of Inert Protein.

The combined extracts were clarified in an Alfa Laval cream separator, and the pH changed to 8.5 with ammonium hydroxide (10N). The precipitate was removed by centrifugation and 10N sulphuric acid used to bring the pH to 3.

iii. Concentration of ethanol and precipitation of crude insulin.

The extract was concentrated to one-fifth the original volume by three passes through an Alfa Laval Centritherm evaporator. The temperature in the steam jacket was 60°C, but the temperature of the extract could not be measured. Sodium chloride (25%) was used to precipitate the crude insulin.

2. Alginic Acid Method.

i. Extraction as for Method 1.

ii. Removal of Inert Protein. The combined extracts were clarified in an Alfa Laval cream separator and the pH changed to 8.5 with ammonium hydroxide (10N). The precipitate was removed by vacuum filtration and the pH of the filtrate changed to 3.0 ± 2 using Dowex 50-X8. The Dowex 50-X8 was then removed by filtration and recharged by stirring in 0.3N hydrochloric acid.

iii. Heat Treatment. The temperature of the extract was raised to 70-75°C within 5-10 minutes, kept at 70-75°C for 5-10 minutes, and then lowered to 30°C within 15 minutes. For small samples (50 gm pancreas) this was most easily achieved by running hot water (80-85°C) over the outside of a stirred glass flask containing the extract. Cooling was achieved by running cold water over the flask. For larger

quantities of glands the extract was heated in a covered stainless-steel vessel in an autoclave. The temperature was measured using a thermocouple in both methods.

iv. Preparation of Crude Insulin. Wet alginic acid was prepared by washing with 0.3 N hydrochloric acid, and then with water. It was stored slightly moistened at -18°C. New alginic acid was used for each extraction although the capacity of new lots of alginic acid to adsorb insulin is low, and increases after regeneration (Jorpes, 1960). New alginic acid was used for each extraction so that the adsorbtion capacity for insulin would be a constant factor. Wet alginic acid was added to the cooled extract (25 gm wet alginic acid/kg pancreas) and stirred for six hours at room temperature, or sixteen hours at 5°C. The alginic acid was removed by filtration on a Buchner funnel and a second equal amount of wet alginic acid added to the extract. The second batch of alginic acid was removed from the extract by filtration after stirring for one hour at room temperature. The insulin was removed from the combined batches of alginic acid by elution with 0.3N hydrochloric acid (50 ml/kg pancreas). Sodium chloride (25%) was used to precipitate the insulin.

3. Ether Concentration Method.

i. Extraction. A sample of pancreas was minced through a 10 mm plate, and 50 gm of material weighed into 175 ml of 76% v/v ethanol containing 1.7 ml 48N orthophosphoric acid, before the material thawed. The material was macerated in a bottom-drive Waring blender at high speed for one minute, and at low speed for two minutes. The solid was removed by centrifugation (2,000 x g for 5 minutes). The solid was re-extracted in 50 ml of 67% v/v ethanol containing 0.2 ml 48N orthophosphoric acid, and centrifuged for the same time as the first extraction.

ii. Removal of Inert Protein. 5N ammonium hydroxide was added to the combined extracts until the final pH was 6.0 ± o.3. The suspension was filtered using a thin layer (2-3 mm) of Celite on a double thickness of filter paper (Whatman No.1) in a Buchner funnel. The precipitate was washed with 25 ml of 67% v/v ethanol and the combined filtrate and wash adjusted to pH 4 ± 0.4 with 5N sulphuric acid.

iii. Removal of ethanol. Three-stage countercurrent extraction with diethyl ether was used to remove the ethanol from the extract.

Three separatory funnels were used. The alcoholic extract was shaken with an equal volume of ether. A small amount of water was added if two phases did not appear. The bottom phase was washed with two further 100 ml portions of ether and collected after filtration using a thin layer of Celite on a double thickness of filter paper (Whatman No.1). The three ether phases were then washed sequentially with two 25 ml portions of water. The aqueous phases were combined and aerated for 30 minutes to remove the residual ether. The ethanol concentration of the extract decreased from 42% w/w to less than 20% w/w (Appendix 7). The ether was distilled for reuse.

iv. Preparation of Crude Insulin. An equal volume of saturated solution of picric acid was added to the filtrate and left overnight. The precipitate was collected by centrifugation (1,600 x g for 10 minutes) and transferred to a 50 ml centrifuge tube using 25 ml saturated picric acid solution, and again separated by centrifugation.

The moist sediment was extracted with 5 ml acetone-water (4:1 v/v) and centrifuged. The sediment was extracted with 5 ml 70% acetone containing 1% sodium chloride. The crude insulin hydrochloride was precipitated from the combined acetone extracts by the addition of 60-80 ml of dry acetone and 0.1 ml 12N hydrochloric acid. The crude hydrochloride was collected by centrifugation in a tared tube, washed with acetone, followed by ether and dried in a vacuum dessicator.

c. Materials.

For the investigation of the different methods a 120 lb. (47 kgm) bulk lot of sheep pancreas was obtained from the meat works. The age of the sheep was estimated to be 14-16 months. The pancreas glands had been collected from the carcass as soon after the slaughter of the animal as possible, frozen in individual moulds, air freighted to Massey University and stored at -18°C until required.

Reagent grade chemicals were used if available, otherwise technical grade chemicals were used.

d. Discussion.

The alginic acid method had been used successfully to obtain insulin from beef and calf by a previous worker in the laboratory. Yields of 2000-6000 i.u./kg pancreas had been obtained from one to two kilogram batches of glands. The first few attempts to use this method

for sheep pancreas extraction were unsuccessful. Reasons for the failures may have included :

i. Not enough skill in using the method for sheep pancreas, although insulin had been obtained from beef pancreas (1200 i.u./kg gland) using the same process.

ii. Low levels of insulin present in the sheep pancreas, and in using small quantities of glands the insulin present may have been 'lost' e.g. on glass surfaces and in transfers of the solutions.

iii. Heat treatment too severe, although the heat treatment is less than the heat treatment for vacuum distillation.

iv. Sheep insulin not bound by alginic acid.

v. Insulin not eluted from the alginic acid.

vi. Acid, alcohol or time for extraction not sufficient.

vii. The method not suitable for extraction of sheep pancreas. Vasavada et al (1964) found extreme difficulty in obtaining crystalline insulin from sheep pancreas, and used mixtures of beef and sheep pancreas to obtain insulin. Katkovskii and Schwartz (1961) also reported that the production of sheep insulin by the same method as for beef (alcohol-sulphuric acid extraction) was not successful.

The successful extraction of sheep pancreas by this method gave a yield of 620 i.u./kg pancreas. (Mouse assay).

Using the conventional method with vacuum concentration of the extract low yields of 100-200 i.u./kg glands was obtained using the same batch of pancreas as for the alginic acid investigation. Further investigations of the method were not carried out because of this low yield. Katkovskii and Schwartz (1961) reported yields of 300 i.u./kg pancreas using alcohol-sulphuric acid extraction.

The ether concentration method was found to be successful for 50 gm pancreas, with yields of 1.5-2.5 mgm crystalline insulin, or approximately 800 i.u./kg gland. As the yields of crystals were small, and difficult to assay, the crude precipitate was used for assay. Eleven of the age groups were investigated in this manner, but when the results of the assays were obtained it was evident that the activity of the crude insulin was much less than the activity detected in the alcoholic extract. Only one-third to one-quarter of the activity present in the alcoholic

extract was found in the crude insulin (Table X).

Three different methods have been used to investigate insulin losses throughout the extraction process :

i. Addition of crystalline insulin at various stages throughout the process. Randall (1964) obtained a good recovery (80-98%) of the added crystalline insulin in all stages of the extraction process except in the maceration stage, where only a 50-55% recovery was obtained.

ii. Addition of crude insulin at various stages throughout the process. Jephcott (1931a) showed that higher recovery rates were obtained if an aqueous extract of crude insulin was used instead of crystalline insulin.

iii. Radioimmunoassay of insulin in samples taken at various stages in the process. The radioimmunoassay can be used on small samples taken at different steps. Fewer extractions are needed as the mass balance of insulin need only be done on one extraction, compared with the several extractions needed if the 'mass balance' for insulin throughout the process is investigated as in the first two methods outlined. Baum et al (1964) used the radioimmunoassay technique for quality control of an insulin extraction process, expressing the results as a percentage of insulin present in the alcoholic extract. 97.3% of the original insulin activity was present in the salt precipitate of insulin.

An investigation of insulin content during the processing by two methods was carried out - the alginic acid and the ether concentration method - using radioimmunoassay of samples taken at different stages. To compare the two methods on the same pancreas material the following method was used:

100 gm of pancreas was extracted as for the ether concentration method (Section b.3.i). The inert protein was removed as in the alginic acid method (Section b.2.ii). The alcoholic extract was sampled, then divided into two equal volumes. One volume was processed using the alginic acid method, the other volume brought to pH 4 $\pm$ 0.4 and processed using the ether concentration method. The investigation was done in triplicate on three different days using pancreas taken from the same batch. The samples were diluted in isotonic saline to a insulin concentration of 30-200 μU/ml for radioimmunoassay. The results were pooled and expressed as a percentage of the initial insulin activity in the alcoholic extract. The results and methods are presented in Figure 7.

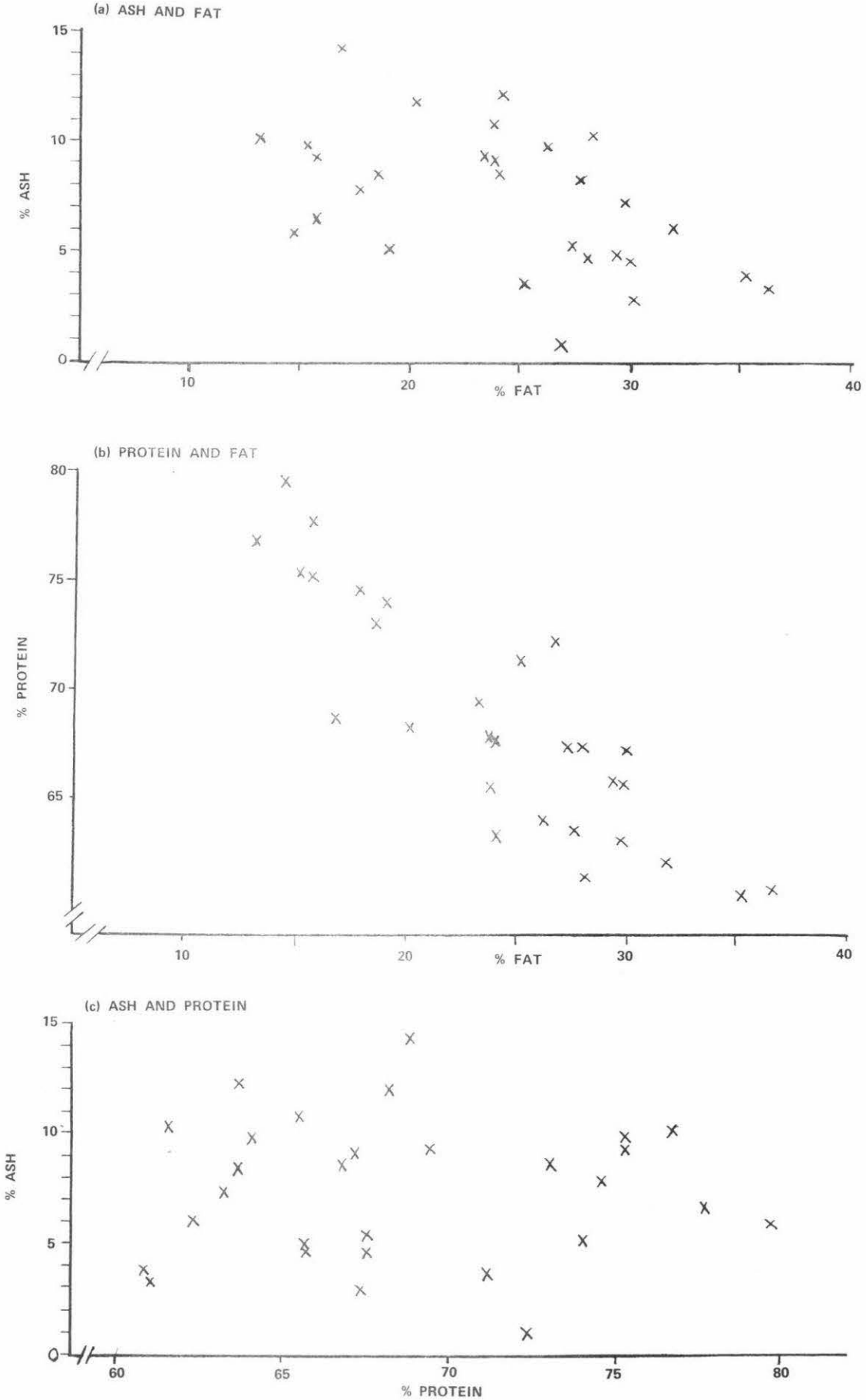


Figure 6: RELATIONSHIPS OF PROTEIN, FAT AND ASH COMPOSITION (dry basis) IN SHEEP PANCREAS.

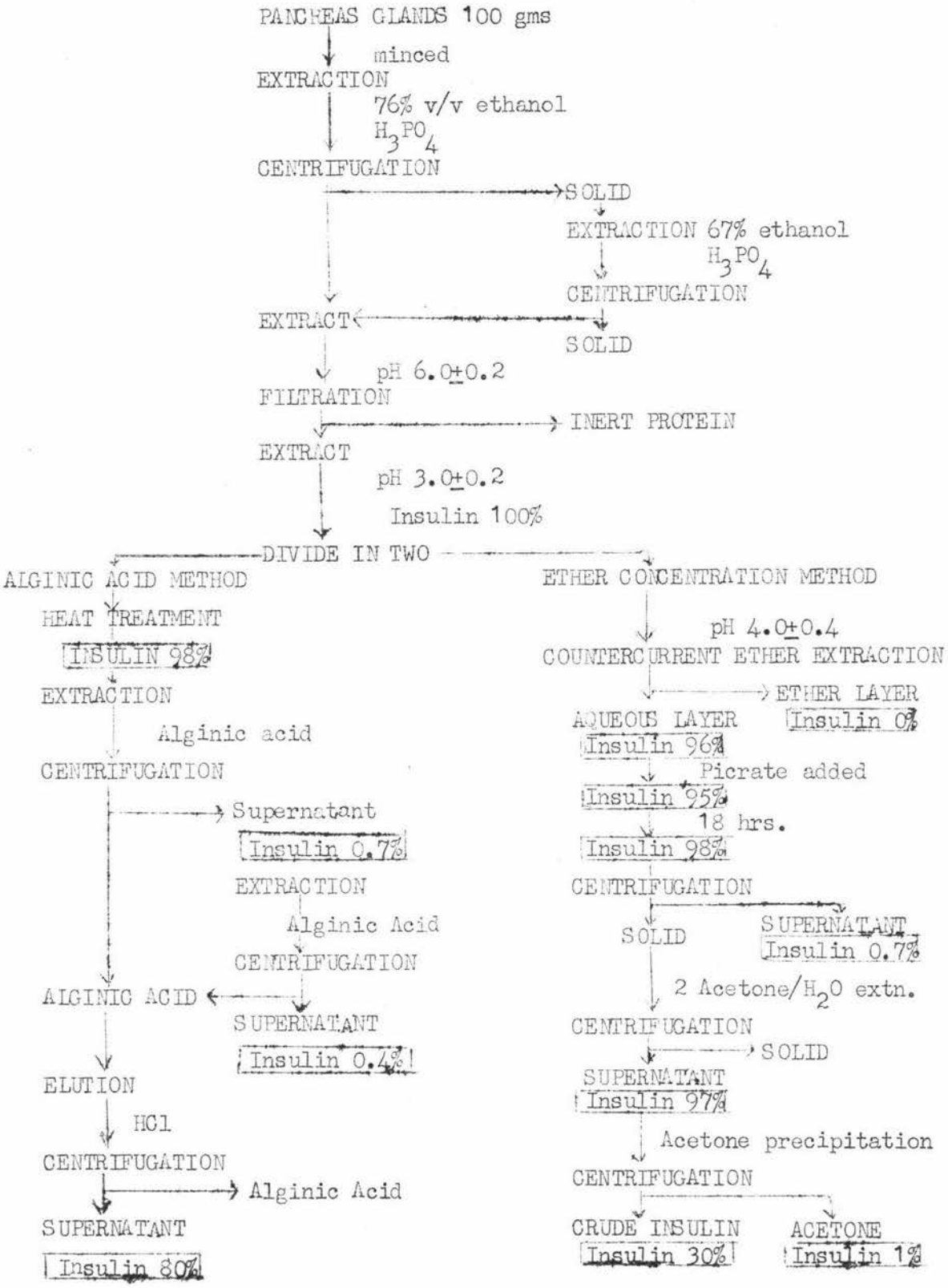


Figure 7.

SCHEMATIC DIAGRAM OF INSULIN YIELDS IN TWO EXTRACTION METHODS.

Insulin is expressed as a percentage of the insulin present in the alcoholic extract.

Nearly all the insulin detected in the alcoholic extract was detected in the final acetone-water extract in the ether concentration method, but only one third of the activity is present in the crude insulin after acetone precipitation. As only a small amount of insulin activity is in the acetone supernatent the crude insulin has been denatured in some way, or insulin-like-factors are present in the extracts, which are not detected when the crude insulin is redissolved for assay.

Only 80% of the initial insulin activity is present in the final solution using the alginic acid method. Most of the activity appears to be still associated with the alginic acid and a second extraction of the alginic acid may have eluted some more of the insulin.

The ether concentration method, although taking a longer period than the alginic acid method involved less man-hours. An extraction from a group of pancreas glands could be completed in triplicate in two days, whereas only one alginic acid extraction could be done per day, using the facilities available.

C. ASSAY METHODS.

a. Introduction.

The methods for insulin assay are outlined in Chapter III. In obtaining an insulin assay in the present study the following points were considered.

i. A large number of insulin samples would be obtained in the study. Twenty-nine different age groups of sheep were investigated, and as extractions were in duplicate or triplicate, this represented sixty to ninety insulin samples. Assays were also to be done on insulin samples obtained in the preliminary studies, representing a further fourty to fifty samples.

ii. Size and purity of insulin needed for the assay. Impurities can affect assay results, and an assay suitable for the small quantities of crude insulin obtained in the investigation was required. A comparison of assay methods is found in Table III.

iii. Techniques involved for the assay system. Most assay systems require a high level of skill, and time is needed to acquire this skill.

iv. Assay Time. As many insulin samples had to be assayed within the short time of the investigations (ten months) the time for each assay must be as short as possible.

v. Materials and Equipment required. Resources available for insulin assay were very limited. Facilities were not available for housing of animals required for bioassay systems, limiting the assay systems investigated to those not requiring animals.

vi. Multiplicity of insulins, pro-insulins, and insulin-like-materials. Any assay system, other than bioassay, for insulin may be affected by other factors and not accurately measure the biologically active insulin.

Insulin assays can be done on the crystalline or crude material, or on alcoholic extracts from the pancreas glands. Jorpes and Rastgeldi (1953) assayed the extracts from human pancreas using the rabbit assay. Willes et al (1969b) in an investigation of the insulin content of sheep pancreas used radioimmunoassay of the alcoholic extracts.

In a comparison of a few of the non-bioassay systems for insulin, Light and Simpson (1956b) noted that the fibril assay method gave erratic quantitative results if crude insulin was used, and other materials such as glucagon were also carried down. The two-phase partition chromatography method was an unsuitable method for running multiple samples as it was a tedious method for even a single sample because purification was needed to remove peptide and protein contaminants. The paper chromatographic method developed by Light and Simpson (1956a) was suitable for small (20 gm) lots of calf pancreas, but as calf pancreas has a high insulin content (10 i.u./gm), compared with pancreas glands of many other domestic animals the quantity used for the chromatogram was much larger than that which could be obtained from an equivalent amount of beef or sheep glands.

b. Methods Investigated.

1. Paper chromatography. Preliminary work was carried out on the assay of insulin samples by paper chromatography using the method of Bouman and Homan (1958). It was found that the insulin samples were too impure to be assayed chromatographically, and the time required to purify the insulin samples would be too long with possible loss of insulin activity during purification so paper chromatography was not investigated further.

2. Mouse Bioassay. Although facilities were not available for the bioassay in the laboratories, Tasman Vaccine Laboratories carried

out mice bioassays on the preliminary insulin samples obtained. As only a small number of animals were available only a few assays could be done. The results for the assay were usually obtained in 1-2 weeks. As the crude insulin samples from the age groups were small (50 mgm) the bioassay could not be used , and the impurities may have influenced the results obtained.

3. Radioimmunoassay. This system can be used for small amounts of insulin, and the purity of the sample is not important. Development of the antiserum takes a period of two - three months. As crystalline sheep insulin was not available beef anti-insulin serum was produced by Professor Flux, Massey University.

The equipment needed for the assay is not complex but was not readily available and special arrangements to obtain radioactive iodine or iodated-insulin were necessary. Dr. Ferguson (personal communication) advised that the time of the investigation was too short to acquire the necessary skill for the technique.

The Radiotherapy Department, Palmerston North Hospital, agreed to assay the sheep insulin samples. Sheep and human insulins behaved in a similar manner over the concentration range 0-100 /uU/ml (Appendix 4, Figure A4.1). The radioimmunoassay method used by the Radiotherapy Department is given in Appendix 4. Although a bulk lot of crude insulin was prepared, a bioassay to compare the radioimmunoassay results with the biological activity could not be done due to difficulty in obtaining consistent results in the bioassay using the mice available.

Crude insulin samples were prepared for assay by dilution in isotonic saline, pH <5.0, to an estimated concentration of 30-200 /uU/ml. Samples of the alcoholic pancreatic extract samples were diluted in the same manner. Willes et al (1969b) in the study of sheep pancreatic insulin assayed the immunoreactive insulin present in pancreatic extracts using the method of Grodsky and Forsham (1960).

4. Disc Electrophoresis. Disc electrophoresis was used to investigate the qualitative properties of the insulin obtained but not used for assay of insulin.

D. YIELDS OF INSULIN FROM NEW ZEALAND SHEEP PANCREAS.

a. Method.

1. Crude Insulin Samples. The method used for the first eleven age groups investigated was :

Eight to twelve frozen pancreas glands from each age group were minced through a 10 mm plate and three 50 gm samples taken randomly from the minced material before thawing occurred. Each 50 gm sample was placed in 175 ml 76% v/v ethanol. The samples were stored at -18°C until processed, the maximum storage time being three hours. The minced pancreas glands remaining after taking the three samples were reminced twice and stored in a sealed plastic bag at -18°C until required for chemical analyses (Section A, c.).

The three 50 gm minced pancreas samples were extracted by the ether concentration method (Section B,b,3). The acetone precipitate of crude insulin was collected and dried under vacuum in a tared 5-ml centrifuge tube. After weighing, the crude extract was transferred to a numbered tube and stored at -18°C until assayed.

For assay, two to three milligrams of the crude insulin sample were dissolved in isotonic saline and diluted to an estimated insulin concentration of 30 - 200 /uU/ml. The samples were then assayed by the method described in Appendix 4, and the results presented as insulin yield per gm tissue, per gm solid-not-ash, per gm solid, per gm protein and insulin content per pancreas (average pancreas weight X insulin yield), for each age group (Table X).

The eleven age groups investigated were the pancreas collected during the winter months. No significant trends in the insulin yields with age were observed, although a slightly lower insulin yield was obtained from the older age groups in the series.

2. Alcoholic Extracts. Radioimmunoassay of the alcoholic extract from the age group 315.6 ± 1.9 days gave an insulin yield six times as great as the yield obtained by assay of the crude insulin. After investigation of the method and consideration of the results (Section B,d) it was decided that it would be better to work from the alcoholic extract rather than the crude insulin for the assays. Consequently subsequent immunoassays were therefore carried out on samples from the alcoholic extracts. Thus the maximum yield of

TABLE X.

Insulin Yields from New Zealand Sheep Pancreas.
(assayed from crude insulin samples)

Each result is expressed as an average from radioimmunoassay of three different crude insulin samples from each age group.

Sheep Age Group(days)				Pancreas Weight(gm)			Insulin yield i.u. per				
Av.	S.D.	Range	No. in Group.	Av.	S.D.	Range.	gm gland	gm solid-not-ash	gm solid	gm protein	pancreas
218.4	9.9	197-226	12	45.3	9.6	31.2-64.0	0.19	0.98	0.93	1.29	8.62
231.8	2.8	227-236	21	47.1	8.4	29.2-62.5	0.18	0.88	0.88	1.09	8.46
243.0	4.4	237-252	24	48.2	8.2	35.0-64.2	0.18	0.72	0.67	0.96	8.68
253.7	5.9	241-260	14	38.8	7.8	27.3-51.9	0.13	0.68	0.67	0.94	5.05
264.3	4.2	260-273	15	38.9	7.9	29.8-57.1	0.16	0.81	0.74	1.00	6.38
264.9	4.0	254-270	14	46.3	6.3	37.7-60.3	0.23	1.19	1.05	1.64	10.63
276.6	5.4	270-285	14	43.3	5.5	37.8-54.5	0.22	1.14	0.98	1.49	9.52
294.7	5.6	278-300	17	43.1	9.1	29.0-61.0	0.19	0.77	0.69	1.08	8.19
302.2	1.4	300-304	14	45.1	11.5	28.5-62.9	0.16	0.77	0.65	1.03	7.22
308.9	1.9	306-312	14	49.0	11.4	25.5-66.9	0.17	0.67	0.61	0.90	8.33
315.6	1.9	312-319	14	50.1	9.8	37.2-70.0	0.13	0.52	0.49	0.70	6.51
315.6	1.9	312-319	assay of alcoholic extract				0.88	3.36	3.19	4.73	44.1

insulin obtainable for the extraction method used would be known, and this maximum yield of insulin from the extract could be obtained by further investigation of the factors affecting the precipitation of the insulin from the extract. Willes et al (1969b) used radioimmunoassay of the alcoholic extract to investigate the insulin concentration in lamb and sheep pancreas.

For the investigation of the concentration of extractable insulin in lamb and sheep pancreas using radioimmunoassay of a sample from the alcoholic extract, the method was :

Duplicate 50 gm samples were obtained from eight to twelve pancreas glands which had been minced, while frozen, through a 100 mm plate. The samples were each placed in 175 ml of 76% v/v ethanol and stored at -18°C for a maximum time of one hour until processed. The minced pancreas glands remaining after taking the two samples were reminced twice and stored in a sealed plastic bag at -18°C for chemical analysis.

Each of the duplicate samples was extracted using the ether concentration method (Section B,b, 3) and a sample for radioimmunoassay was taken from the alcoholic extract at pH 4.0 ± 0.4 after the inert protein had been removed from the extract. The sample was diluted with isotonic saline to an estimated insulin concentration of 30 - 200 /uU/ml and stored at -18°C for a maximum of seven days until sent to Palmerston North Hospital for assay.

The results obtained from the radioimmunoassays were averaged for the two duplicate samples from each age group. The yields of insulin were expressed in the following ways :

i. international units per gm tissue
ii. i.u. per gm ash-free solid
iii. i.u. per gm solid
iv. i.u. per gm protein
v. i.u. per pancreas

Methods ii to iv were obtained by calculation using the experimentally determined composition of the pancreas glands (Table VIII) and method v by using the yield per gm of tissue with the average pancreas gland weight for each age group.

b. Discussion.

For the reasons given the results based on direct assay of the

alcoholic extract were considered to give the most accurate indication of insulin yields. These yields from New Zealand sheep pancreas glands are presented in Table XI and Fig. 8. Analysis of the insulin yields indicates that there is a decrease in insulin yield with increasing age. This decrease is very rapid in the 40 - 120 day age period and may be due to the changing of the lambs' diet from milk to increasing amounts of grass. The insulin content of rat pancreas glands has been found to decrease if there was a decrease in carbohydrate-forming substances in the diet (Best et al, 1939b) and so the decrease in insulin obtained from the lamb pancreas may also be due to the changes in diet.

The yield of insulin from winter-killed sheep (215 - 393 days old) was constant at 0.45 ± 0.2 i.u./gm tissue and slightly greater than the yields obtained from spring-killed animals (120 - 140 days and 440 - 460 days). A similar effect of higher yields in winter-killed animals has been observed in pigs and other young domestic animals (Ostling, 1926) and beef (Fisher and Scott, 1934).

An increase in insulin yield was observed in the 440 - 480 day age groups but as only a small time period is covered this apparent trend may not be real. Insulin yields from mature sheep (3 - 7 years) increased with age, and were greater than the yields obtained from older lambs and hoggets.

The insulin yields obtained in this study were lower than those obtained by Willes et al (1969b) which are presented in Table XII.

TABLE XII.

Pancreas Insulin Concentration ± standard error of Young Lambs and Adult Sheep.

Age	No. of Animals	Insulin Concentration i.u./gm.
1 day	8	8.2 ± 1.2
1 week	8	6.2 ± 0.9
7 weeks	9	5.6 ± 0.6
14 weeks	9	3.0 ± 0.4
2-6 years	10	1.2 ± 0.1

From Willes et al, 1969b.

The low yields obtained in the present study could have been due to several factors :

i. The freezing method may not have been suitable and storage of

TABLE XI.

Insulin Yields From New Zealand Sheep Pancreas.
(assayed from alcoholic extracts)
Each result is expressed as the average of radioimmunoassays obtained for two separate samples of pancreas glands.

Sheep Age Groups				Pancreas Weight			Insulin yield i.u. per				
Av. days.	S.D.	Range	No. in Group.	Av.	S.D.	Range	gm gland	gm solid	gm solid not-ash	gm protein	pancreas
42			12	22.0	3.0	18.0-28.2	1.76	8.6	9.0	13.1	38.8
63			10	29.5	4.6	23.6-36.3	1.65	4.9	3.8	7.2	48.6
84			12	36.5	7.5	25.6-48.8	1.58	8.3	8.4	11.5	57.6
109.4	4.9	100-116	17	46.5	7.4	36.0-62.0	0.76	2.2	3.5	3.4	35.3
117.1	0.8	116-118	18	44.9	5.3	38.5-53.2	0.45	1.8	2.0	2.8	20.2
121.8	1.8	119-126	18	44.4	4.5	35.0-52.0	0.31	1.3	1.5	2.0	13.8
131.2	2.3	128-135	17	46.9	7.7	36.0-65.0	0.36	1.5	1.6	2.3	16.9
218.4	9.9	197-226	12	45.3	9.6	31.2-64.0	0.45	2.1	2.3	2.8	20.4
231.4	2.8	227-236	21	47.1	8.4	29.2-62.5	0.42	1.6	2.0	2.5	19.8
243.0	4.4	237-252	24	48.2	8.2	35.0-64.2	0.49	2.2	2.4	3.2	23.6
253.7	5.9	241-260	14	38.8	7.8	27.3-51.9	0.39	1.7	1.9	2.6	15.1
264.3	4.0	254-270	14	46.3	6.3	37.7-60.3	0.34	1.6	1.8	2.3	15.7
264.9	4.2	260-273	15	38.9	7.9	29.8-57.1	0.57	2.6	2.9	4.1	22.3
276.6	5.4	270-285	14	43.3	5.5	37.8-54.5	0.55	2.5	2.8	3.8	23.8
294.7	5.6	278-300	17	43.1	9.1	29.0-61.0	0.63	2.3	2.5	3.6	27.6

Sheep Age Groups			No. in Group	Pancreas Weight			Insulin yield i.u. per				
Av.	S.D.	Range		Av.	S.D.	Range	gm gland	gm solid	gm solid not ash	gm protein	pancreas
days.											
302.2	1.4	300-304	14	45.1	11.5	28.5-62.9	0.56	2.2	2.6	3.5	25.3
308.9	1.9	306-312	14	49.0	11.4	25.5-66.9	0.45	1.6	1.8	2.2	22.1
315.6	1.9	312-319	14	50.0	9.8	37.2-70.0	0.41	1.5	1.6	2.2	20.5
443.0	8.9	426-453	14	63.5	12.2	48.0-90.2	0.21	0.8	1.0	1.2	13.3
456.6	2.2	452-460	17	62.1	14.6	44.5-107.1	0.33	1.3	1.3	2.0	20.5
464.7	1.4	462-467	15	70.0	15.7	46.0-97.0	0.46	1.8	1.9	2.9	32.2
472.7	5.9	468-491	17	72.6	12.8	44.5-94.5	0.53	2.0	2.3	1.7	38.4
years.											
1.5			7				0.50	0.9	1.0	1.3	
2.3	0.3	2-2.5	7	72.5	5.2	63.3-83.0	<0.01	<0.1	<0.1	<0.1	<0.7
3			6	87.2	9.2	78.8-102.5	0.35	1.7	1.9	2.3	30.6
4			-				0.85	3.0	3.1	4.9	
5			-				1.03	4.4	4.7	5.7	
5			15	47.8	11.1	30.9-70.3	0.72	3.7	3.9	4.6	34.4
7			-				0.69	2.9	3.1	3.7	

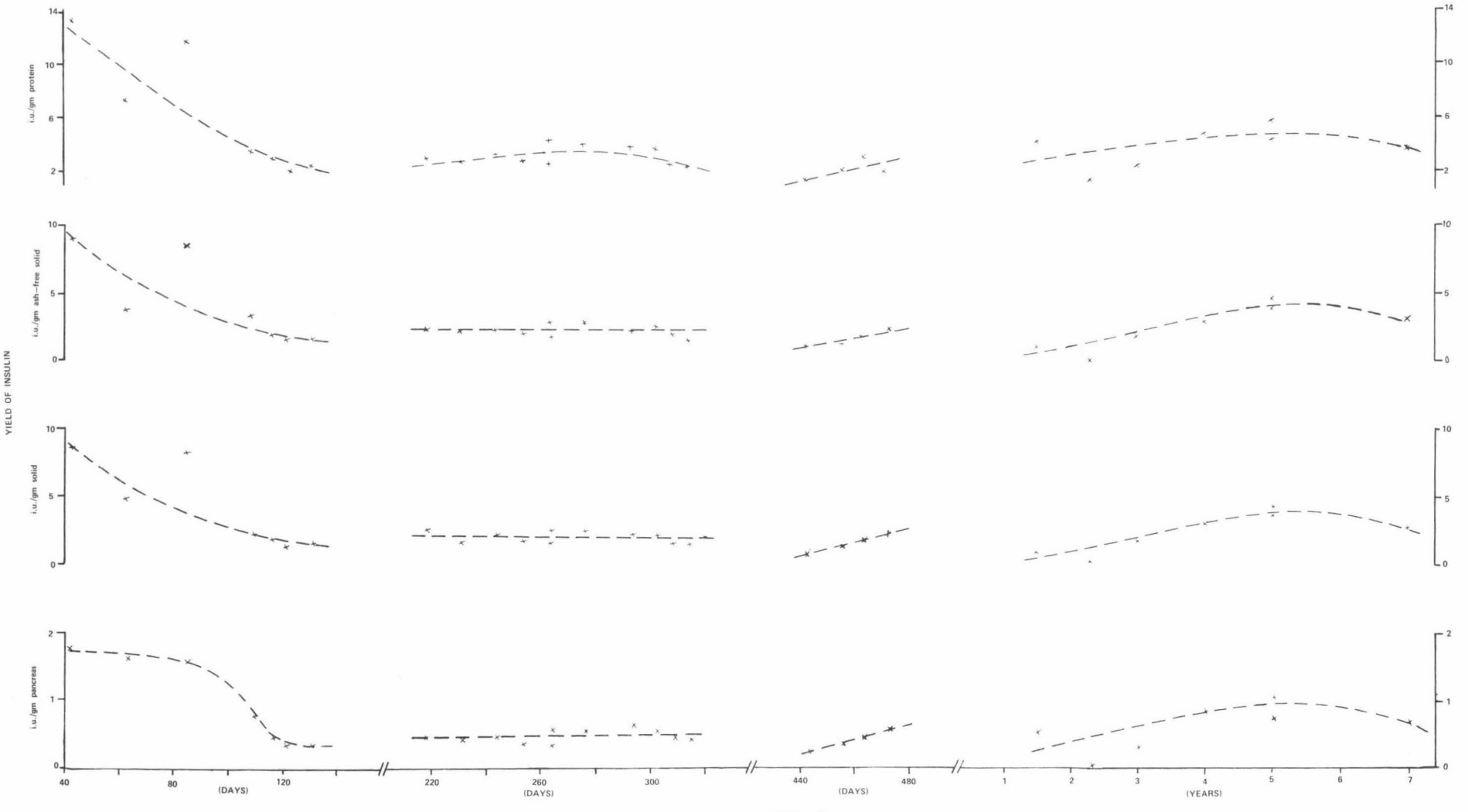


Figure 8: INSULIN YIELDS FROM SHEEP PANCREAS WITH ANIMAL AGE.

YIELDS EXPRESSED PER GM TISSUE, PER GM SOLID, PER GM ASH-FREE SOLID AND PER GM PROTEIN.

the glands too long to obtain maximum insulin yields. Some of the glands had been stored at -18°C for up to eight months. Willes et al (1969b) used fresh pancreas glands.

ii. New Zealand sheep are grass-fed throughout the whole year with supplementary feeds of hay, silage, root crops and chou moullier in the winter. The levels of digestable carbohydrates in this diet are much lower than the diet of milk for a calf, the pancreas of which has a high concentration of extractable insulin (up to 10 i.u./gm).

iii. The possibility that the assay method used gave only a partial indication of the insulin levels present in the samples taken.

iv. Losses during the extraction procedure and time of storage of the sample for assay.

v. The possibility that the extraction method is unsuitable for the extraction of insulin from sheep pancreas. The method of Grodsky and Peng (1959) used by Willes et al (1969b) includes alcohol-HCl extraction of the protein obtained by homogenisation of a 350 to 400 milligram wet weight of pancreas with chilled saline - 10% trichloro-acetic acid solution.

The insulin yields obtained in this study, although lower than those of Willes et al (1969b), are comparable to yields obtained in other investigations (Table II). Insulin yields per pancreas did not show any particular trend with age (Table XI), the average yield per pancreas being 20 i.u. The total insulin content per pancreas was higher in adult sheep, mainly because of the greater pancreas weight in these age groups.

The yields of insulin which can be extracted from New Zealand sheep pancreas have been found to be 300 - 700 i.u./kg glands from lambs, 500 i.u./kg hogget pancreas and up to 1000 i.u./kg pancreas in adult sheep. These values are much lower than the reported commercial yields obtained from pig, beef, and calf pancreas of 1,500 - 10,000 i.u./kg pancreas (Table II). Thus the New Zealand sheep pancreas appears to be relatively unpromising as the raw material for the commercial extraction of insulin, unless a different process can be developed with which higher yields can be obtained. Therapeutic importance could arise if crystalline sheep insul has properties, such as prolonged action time or reduced antibody build-up which make it advantageous in the treatment of diabetics.

E. CRYSTALLINE SHEEP INSULIN.

Crystalline or non-crystalline insulin can be used for the treatment of diabetes mellitus, but the prolonged action time of crystalline insulin gives it an advantage in the therapy of diabetics. Many processes have been published describing the production of crystalline insulin since Abel (1926) published a suitable method. Biologically active insulin crystals in which none of the metal ions zinc, nickel, cobalt or cadmium are present have not yet been prepared, and the crystals are more correctly described by terms such as zinc-insulin crystals.

The shape of the crystals depends on the species of insulin. Single, sharp and perfect rhombohedral bodies can be obtained from recrystallized pig and insulin using citrate, phosphate, ammonium and acetate buffers, but the crystals from sheep and cattle insulin are often twinned with curved faces (Schlichtkrull, 1956). If urea or a halogenide was present in the mother liquor during recrystallisation perfectly shaped rhombohedral crystals were obtained from all three species.

The difficulty of obtaining crystalline sheep insulin has been noted in the literature. Vasavada et al (1964) could only obtain crystalline insulin from alcoholic extracts of mixtures of sheep and beef pancreas, and could not obtain crystals from sheep pancreas extracts. Katkovskii and Schvartz (1961) obtained crystalline insulin from pig and sheep pancreas extracts using phosphate and citrate buffers, but only low yields were obtained if acetate buffers were used.

Three different methods were used to obtain crystalline sheep insulin (Appendix 5). The precipitate which had formed in the bulked alcoholic extracts from the investigation of the insulin yields from sheep pancreas, stored at $5^{\circ}C$ for 15 to 25 days was collected by centrifugation. The remaining alcoholic extract was concentrated to one-fifth the original volume and the crude insulin precipitated with sodium chloride. Each precipitate was processed separately but only a few crystals, with imperfect faces, were obtained using the method of Romans et al (1940). The poor yields may be because sheep insulin will not crystallise readily in the acetate buffers which were used. Yields from the method of Baker (personal communication) were also low. This

method has been used successfully for crystallisation of pig insulin.

One to two millegram yields of crystalline sheep insulin from 50 gm of pancreas were obtained using the method of Randall (1964). The citrate-borate-acetone solution used for the crystallisation has a unique and useful property of changing pH with time. The acetone is not essential for the reaction and sodium hydroxide or ammonium hydroxide can be used to make the initial pH adjustment (Randall, 1964).

The crude insulin obtained by the ether concentraction method was impure. A disc polyacrylamide gel electrophoretogram of the crude insulin (Plate 1, No.5) indicates that large amounts of glucagon and proinsulins are present. The dosage rate was 0.5 mgm per tube and the electrophoretograms were performed at Nordisk Insulinlaboratorium, Denmark. From the crude insulin the crystalline insulin was obtained. The crystals were small (5 - 10 μ) but rhombohedral shaped bodies were obtained (Plates 3 and 4). The crystals were not recrystallised because of the small quantity available.

In a disc polyacrylamide gel electrophoretogram of crude sheep insulin, crystalline sheep insulin, and crystalline beef insulin (Calbiochem bovine insulin, Lot 901709), the crystalline sheep and beef insulins moved at the same rate under the conditions used (Plate 2). Three bands appeared in the crude sheep insulin but there was not much movement from the origin. Multiplicity of bands was not observed in the crystalline insulins investigated but this may have been due to the low dose applied or the system used (Appendix 6). Several major and minor bands have been obtained in a disc electrophoretic study of the heterogeneity of crystalline insulins (Mirsky and Kawamura, 1966), but the system used was very different to that used in this investigation.

The results of the disc electrophoretogram (Plate 2) together with the evidence of rhombohedral crystals (Plates 2 and 3) indicate that crystalline sheep insulin was obtained although, because of the small quantities, the biological activity could not be tested.

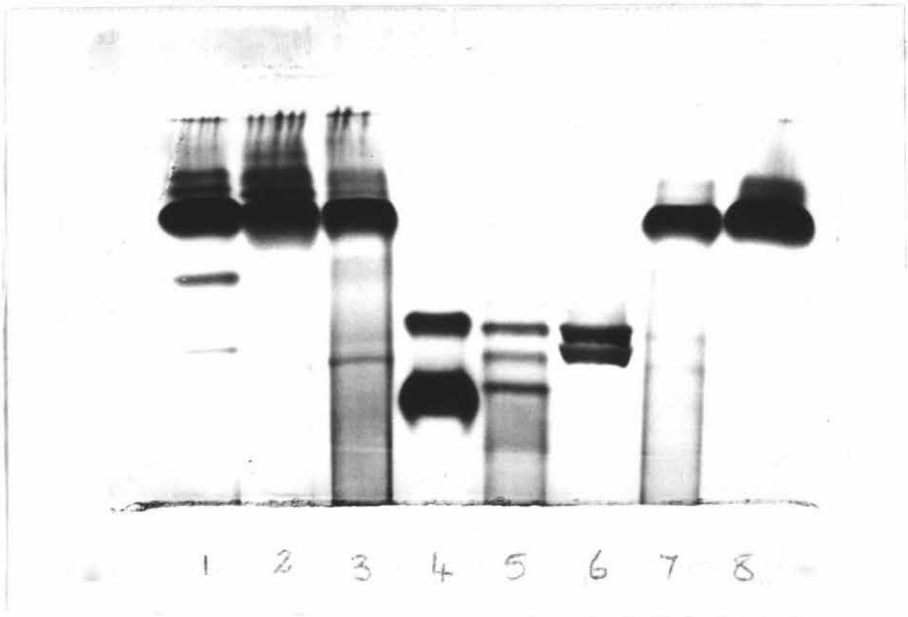


Plate 1

Disc-gel-electrophoresis of Insulin, Pro-Insulin and Glucagon.

No.	Description
1	Partly refined pig insulin
2 & 8	Partly refined sheep insulin, Denmark.
3 & 7	Partly refined sheep insulin, New Zealand.
4	Glucagon (LILLY).
5	Crude sheep insulin from present study.
6	Proinsulin and desamido insulin.

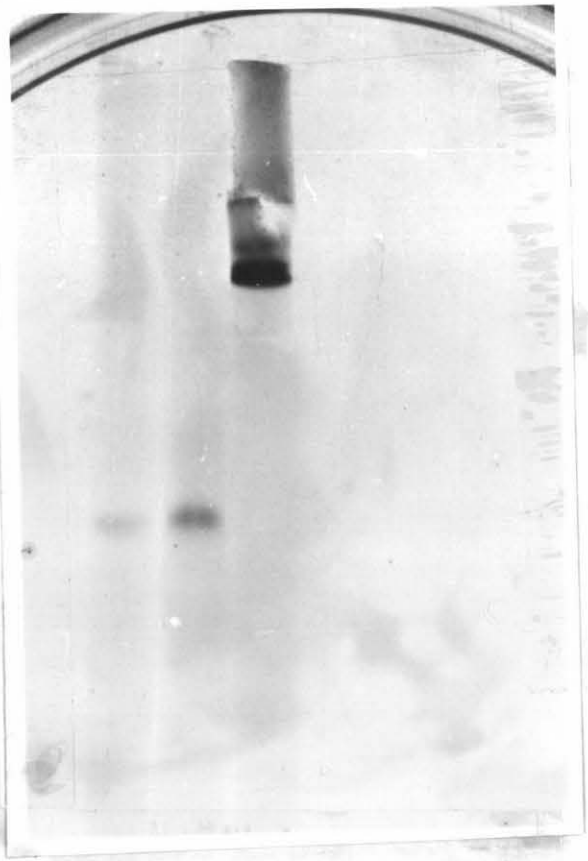


Plate 2

Disc-gel-electrophoresis of Insulin.

Left	Crystalline sheep insulin from present study.
Middle	Crystalline bovine insulin (CALBIOCHEM).
Right	Crude sheep insulin from present study.

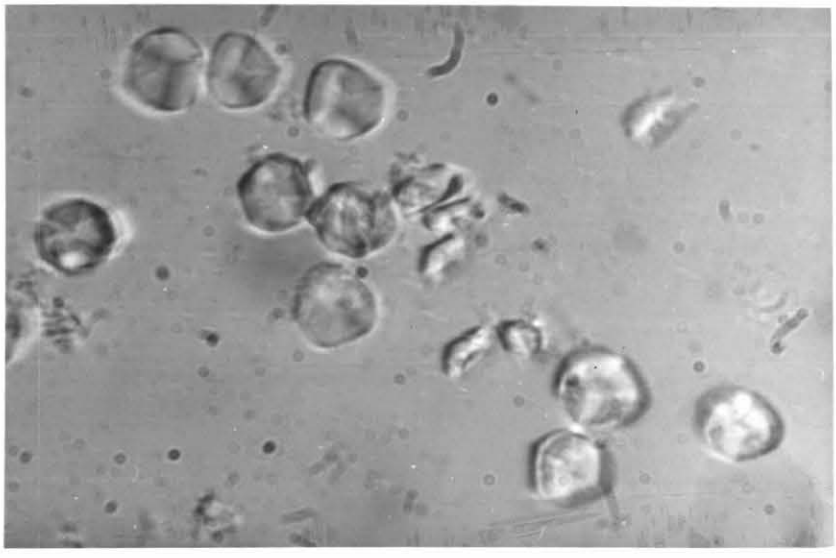


Plate 3

Crystalline Sheep Insulin. (X 1000)

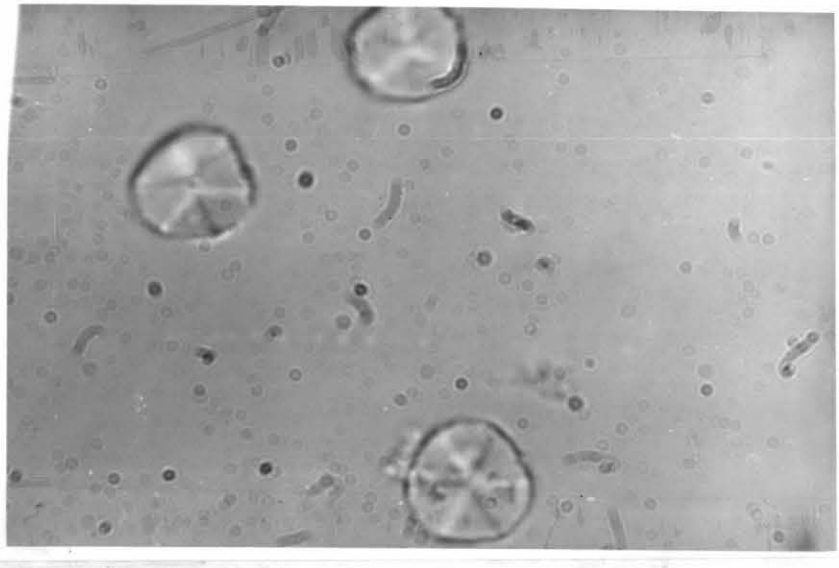


Plate 4

Crystalline Sheep Insulin. (X 1500)

CONCLUSIONS.

Yields of insulin from alcoholic extraction of pancreas glands from New Zealand sheep varied between 0.2 and 2.0 international units per gm pancreas, and were dependent on the age of the animal. Higher yields were obtained from young lambs and mature sheep than from hoggets or older lambs. These yields were low when compared with the extractable insulin levels from beef, calf and pig pancreas, used in commercial production of insulin, and lower than the values reported in another recent study of insulin yields from American lamb and sheep pancreas.

The low yields may be due to diet, breed and climatic effects on New Zealand sheep, or because the extraction methods used are not optimal for extraction of sheep pancreas. If the low yields obtained truely indicate the total available insulin extractable from sheep pancreas, it appears that sheep pancreas is unlikely to be a suitable raw material for commercial extraction of insulin unless sheep insulin is found to have particular properties which make it desirable for treatment of diabetics.

Crystallisation of sheep insulin was difficult, but small, rhombohedral crystals were obtained and the crystalline sheep insulin had similar electropheretic properties to crystalline beef insulin.

In the three different methods investigated, comparable yields were obtained from the two methods; the alginic acid method and the method using two-phase concentration of the alcoholic extract. The yields obtained using either of these methods were much higher than that obtained using vacuum concentration of the alcoholic extract. The short heat treatment, or absence of any heat treatment in these two methods, compared with vacuum concentration, may have accounted for the higher yields.

The pancreas weight of New Zealand sheep showed definite trends with the age of the animal. An increase in the average weight with age was observed, from young lambs to mature sheep three years old. A decrease in the pancreas of five-year old animals was observed. The growth rate of pancreas glands during the spring-early summer was similar for lambs and one year old sheep in the first two years. No significant change of pancreas weight with age was observed in animals killed during the winter.

POSSIBILITIES FOR FURTHER INVESTIGATIONS.

1. Investigations of different methods of insulin extraction, and optimisation of the yields which can be obtained from sheep pancreas from New Zealand meat works. Temperature, extraction time, solvent effects, and pH effects should be investigated.

2. Optimisation of the collection and storage methods including the determination of the time for which sheep pancreas can be stored before yields of insulin are affected. Work on fresh pancreas glands may indicate the maximum yields of insulin which can be obtained.

3. Development ofquantitative and qualitative methods of insulin assay so that the loss of yields of insulin at different steps in the process can be investigated. A comparison of the different methods such as radioimmunoassay, disc gel electrophoresis, bioassay and chromatography may indicate the relation between biologically active insulin and insulin-like-activity measured in each method.

4. Investigations on the different factors which influence the crystallisation of sheep insulin.

5. Exploration of the therapeutic properties of sheep insulin to determine any particular advantages or disadvantages it may have over the available insulins.

APPENDIX I.

PANCREAS WEIGHTS

TABLE A1.I.

Sheep Pancreas Weight with Age.

AGE (DAYS)	WEIGHT (gm)
42	18.8, 22.2, 18.0
	18.5, 19.8, 22.3
	21.7, 28.2, 24.0
	25.3, 23.8, 20.8
63	26.4, 23.6, 32.1
	30.9, 26.4, 31.8
	36.3, 35.8, 23.9
	27.9
84	33.2, 40.3, 30.0
	40.3, 47.8, 35.9
	48.8, 25.6, 42.4
	31.5, 34.2, 28.1
100	52.6, 48.2
5	44.0
6	49.0, 42.0
7	43.0
8	43.9
9	45.0, 45.0
111	42.1
12	36.0
13	57.0
14	62.0, 58.5, 37.0
15	38.0
16	46.0, 41.9, 48.0
	53.2, 39.8
17	51.0, 39.5, 48.5
	40.0, 45.0, 44.5
	40.5, 52.5
18	41.5, 50.5, 52.2
	40.4, 38.5, 39.9
19	50.1, 48.0
120	35.5, 43.1, 51.0
	46.0
22	44.1, 44.0, 41.1
	52.0, 43.5, 46.0
123	42.1, 35.0, 43.0
	46.5
24	43.0
26	45.6
28	43.0, 53.0, 45.5
29	44.5, 38.1
130	38.3
31	50.0, 51.9, 39.0
	40.0
32	36.0, 50.9
33	44.5
34	65.0, 55.0, 48.0
35	54.0
197	38.2
203	55.1
211	58.3
217	43.7
219	36.1
222	42.0
24	64.0
25	43.5, 31.2
26	46.0, 39.0, 46.0
27	48.0
28	39.2, 50.0, 51.8
29	48.0, 57.0
230	52.2
31	44.2
32	39.5, 47.2, 35.2
33	50.2, 52.5, 39.5
	62.5
34	45.5, 59.2
135	29.9, 37.7
36	55.6, 44.5
37	55.3, 49.0
38	58.8, 43.7
	60.0
39	52.2, 35.0
	52.5
241	55.0, 47.6
	33.0
42	47.9, 54.0
43	37.8
44	36.2, 51.8
45	34.0, 45.3
46	64.2, 27.3
47	35.9, 50.8
	49.0, 53.0
49	47.8
250	41.0
52	45.0, 34.6
54	47.1, 40.3
	31.9, 33.0
	47.0
57	36.0
58	50.5
59	51.9, 43.3
	29.8
260	39.0, 36.2
	46.5, 35.7
	36.5
61	29.8, 30.9
62	47.8
63	40.0, 44.0
	40.0, 37.8
	48.2

AGE (DAYS)	WEIGHT (gm)
264	50.2
65	45.5, 49.8, 40.2
66	50.2, 37.7, 38.9
67	60.3
68	31.1, 30.7, 57.1
69	52.7, 39.1
270	48.8, 44.2, 41.9 38.7, 46.3
71	44.3
73	34.8, 52.2
75	54.5
76	38.5
77	47.0
78	47.8, 38.0
79	39.0
280	37.8
83	37.8
85	38.9, 42.0
87	48.0
291	48.5
92	45.0
93	32.0, 61.0
94	29.0
96	40.0, 39.0
98	41.1, 35.8, 43.9 46.0
99	49.9, 29.5
300	58.8, 46.9, 28.5 38.0, 59.0
2	30.5, 62.9, 51.9 45.0
3	49.9, 39.5, 57.8 39.0, 57.0
4	43.0, 30.0
6	63.0
7	50.5, 59.2, 49.9 38.0
308	46.9
9	41.8, 65.0
310	55.5, 35.9
11	25.5, 44.2, 49.0 35.0
12	61.8, 66.9, 46.5
14	45.5, 49.0, 40.9 37.2
15	45.0, 40.3, 41.1 57.2, 54.2
16	50.9
17	70.0
18	47.2, 66.5
19	55.2, 38.2
426	58.0
431	48.0
33	43.0
34	73.0
440	52.2, 72.1
6	63.0
7	65.1
450	62.5, 71.5, 52.0
51	64.2, 74.0
52	49.1
53	90.2
54	44.5, 58.1
55	57.0, 67.0
56	57.5, 67.9, 44.9
57	78.5, 65.0, 58.0 63.0
58	54.5, 51.2
460	107.1, 66.1, 66.0
62	50.0
63	70.3, 93.2
64	75.0, 64.8, 56.0 97.0
65	85.0, 70.0, 46.0 47.0
466	65.5, 80.0
67	78.1, 72.5
68	62.0, 78.1
69	66.0, 73.0
470	90.0, 85.2 69.5
71	94.5, 69.1 62.1, 85.5
72	78.5, 44.5 79.0
480	55.0
81	70.1
491	72.0
2 yrs.	67.3, 68.8 69.6
2½ yrs.	72.6, 72.7 73.3, 83.0
3 yrs.	78.8, 84.1 80.0, 83.8 93.7, 102.5
5 yrs.	47.0, 47.4 46.3, 54.0 36.0, 40.0 42.8, 34.1 50.7, 43.2 30.9, 66.8 70.3, 55.5 53.2

TABLE A1. II.

Average Pancreatic Weights of Sheep with Age.

Average Pancreas Weight(gm.)

Age. weeks	Romney(R)		Southdown(S)		S X R	
	Ram	Ewe	Ram	Ewe	Ram	Ewe
6	15.0	18.7	13.6	14.0	19.2	14.3
11	32.3	36.9	23.1	25.9	31.1	32.9
16	34.5	34.8	30.9	25.7	36.9	34.5
25	40.0	37.5	33.8	31.5	37.7	36.0
41	45.5	44.0	39.4	32.0	47.1	40.4
80	56.7	55.1	42.0	45.4	51.7	47.1
mature	79	67	89	56	-	60

From : Earle, Unpublished.

APPENDIX 2.

METHODS OF ANALYSIS.

Analyses were completed in triplicate.

1. MOISTURE.

Approximately 10 gm of washed sand and a short stirring rod was placed in an aluminium moisture dish 2.5" diameter and 1" high. The dish was dried in an air oven at 100°C for a minimum of two hours

Approximately 10 gm of prepared pancreas was weighed into the cooled, tared moisture dish, plus lid. The sand was carefully mixed into the pancreas.

The sample was dried for 16-18 hours in an air oven at 100°C, then transferred to a dessicator. When cool the dish was reweighed.

2. TOTAL NITROGEN.

Approximately 2 gm of pancreas was weighed accurately on Whatman No.1 filter paper. The sample was digested with 0.7 gm of nitrogen-free mercuric oxide, 5 gm potassium sulphate and 25 ml nitrogen-free sulphuric acid. After the solution had cleared it was boiled for a further one hour.

The cooled digest was diluted to 200 ml with distilled water. Duplicate ten ml samples were distilled in a micro-Kjeldahl distillation apparatus into five ml 2% boric acid solution. After distillation the distillate was titrated with 0.05N hydrochloric acid.

A blank determination was carried out, and the total nitrogen content calculated from the titre.

3. FAT.

Approximately 5 gm of pancreas was weighed into a tared soxhlet thimble containing washed sand. The pancreas was mixed into the sand and dried in an air oven at 100°C for four hours. The sample was extracted with petroleum ether, boiling point 40-60°C, for 20-24 hours. After evaporating the excess ether the ether extract was dried for 30 minutes in an oven at 100°C, cooled and weighed.

4. NON-PROTEIN NITROGEN.

Approximately 2.5 gm of pancreas was accurately weighed into a glass vessel. Ten ml of distilled water was added and mixed at 7,000

rpm for one minute. The contents were transferred to a 50 ml centrifuge tube with 20 ml of distilled water.

Five ml of 50% trichloracetic acid was added and the solution centrifuged at 1,000 g for five minutes. Five ml of the supernatant were distilled as in the total nitrogen method.

APPENDIX 3.

STATISTICAL ANALYSIS OF SHEEP PANCREAS WEIGHTS.

TABLE A3.I.

Regression Data : Sheep Pancreatic Weight with Age.

Period	b	s_b	p	r^2	Equation
Present Study					
42-140 days	0.292	0.021	<0.001	0.64	$\hat{Y} = 0.292X + 10.63$
100-140 days	0.383	0.10	<0.001	0.18	$\hat{Y} = 0.338X - 0.48$
425-490 days	0.375	0.14	<0.025	0.10	$\hat{Y} = 0.375X - 105.3$
2-3 years	19.1	4.75	<0.005	0.60	$\hat{Y} = 19.1X + 29.2$
Earle, Unpublished.					
42-112 days	0.244	0.04	<0.001	0.70	$\hat{Y} = 0.244X + 7.54$
42-175 days	0.136	0.02	<0.001	0.62	$\hat{Y} = 0.36X + 14.98$

b Sample regression coefficient

s_b Sample Standard deviation of b

p Probability that $b \neq 0$

r^2 Square correlation coefficient, indicating the amount of variation explained by b in the relationship between Y and X.

Y Weight of pancreas

X Age of animal

TABLE A3.II.

Comparison of Regression Coefficients for Pancreatic Weights of Lambs 100-140 days old and Sheep 425-490 days old.

	Reg.Coef.	Deviations from Regression d.f.	M S
Within (W)	0.375	61	188
	0.383	68	48
		129	114
Pooled	0.378	130	113
	Differences between slopes	1	0
	Adjusted between means	1	1357

Differences in residual variances $F = \frac{188}{48} = 3.9$ $p < 0.05$

Comparison of slopes $F = \frac{0}{114} = 0$ N S

Comparison of elevations $F = \frac{1357}{113} = 12.0$ $p < 0.001$

TABLE A3.III.

Analysis of Variance of Pancreas Weights from Sheep during the Winter Period.

n	Range (Days)	Av.	S.D.
5	197-219	46.3	10.0
13	220-229	46.6	8.3
23	230-239	47.9	8.9
17	240-249	45.3	9.8
13	250-259	40.9	7.3
26	260-269	42.2	8.2
14	270-279	44.0	5.8
5	280-289	40.9	4.3
13	290-299	41.6	9.0
24	300-309	48.0	10.9
24	310-319	48.3	11.1
Totals 177	197-319	45.2	9.3

Variation	d.f.	M S	
Between	10	139	N S
Within	166	84	
Total	176		

TABLE A3.IV.

Comparison of Regression Coefficient for Pancreas Weights of Sheep 42-140 days old from Earle and this study.

		Deviations from Regression	
	Reg.Coeff.	d.f.	M S
Within (W)	0.291	102	41
	0.244	16	23.3
		118	38.6
Pooled	0.284	119	38.6
	Differences between slopes	1	29
	Adjusted between means	1	677

Comparison of residual variances $F = \frac{41}{23} = 1.8$ N S

Comparison of slopes $F = \frac{29}{38.6} = 0.7$ N S

Comparison of elevations $F = \frac{677}{38.6} = 17.5$ $p < 0.001$

APPENDIX 4.

RADIOIMMUNOASSAY METHOD USED BY PALMERSTON NORTH HOSPITAL.

The Insulin Immunoassay kit produced by The Radiochemical Centre, Amersham, England, is used for the radioimmunoassay of human insulin. The Insulin Immunoassay kit is intended for use in the Double Antibody method which closely resembles that described by Hales and Randle (1963).

Method Outline:

0.1 ml insulin solution, standard or unknown is mixed with 0.1 ml of antiserum and left at 2° - 4°C for six hours. 250 picograms of radioactive insulin (125-I) is added to all tubes and incubated at 2° - 4°C for 18 hours.

The precipitate from each sample is collected by microfiltration. The filter disc is wrapped in aluminium foil and the radioactivity counted in a well-type crystal scintillation counter. Blank and zero determinations are also carried out.

The results are expressed as counts/min and the insulin activity of the unknown solutions obtained from a standard graph (Figure A4.1).

For assay of the crude insulin samples two to three milligrams were dissolved in isotonic saline and diluted to an estimated insulin concentration of 30-200/uU/ml. The alcoholic extracts for assay were also diluted in isotonic saline to an estimated insulin concentration of 30-200/uU/ml.

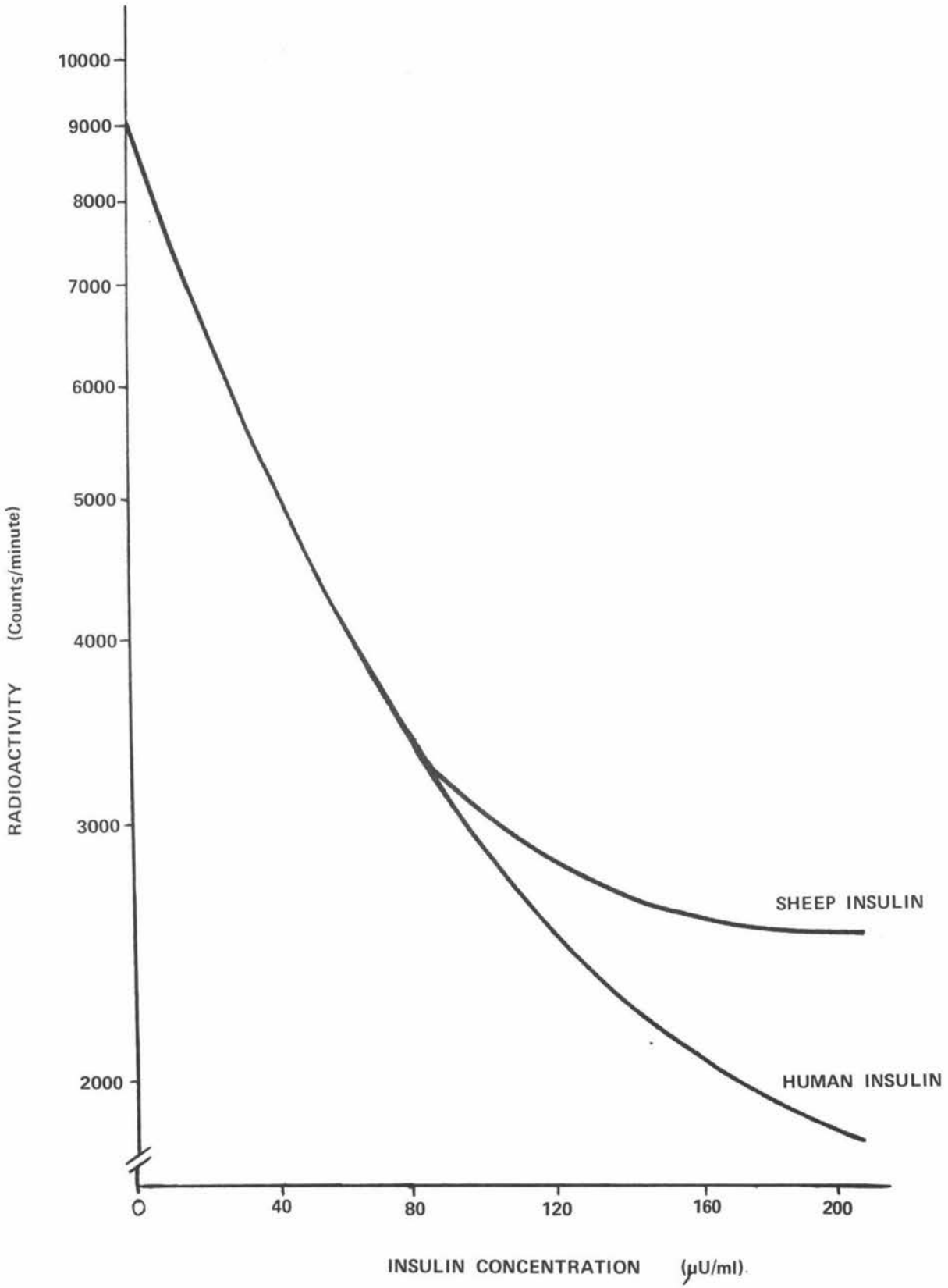


Figure A4.1: STANDARD CURVES FOR RADIOACTIVITY (counts/min) WITH CONCENTRATION (μU/ml) OF SHEEP AND HUMAN INSULINS FOR RADIOIMMUNOASSAY OF INSULIN USING HUMAN INSULIN ANTISERUM.

From Radiotherapy Department, Palmerston North Hospital.

APPENDIX 5.

METHODS INVESTIGATED FOR THE CRYSTALLISATION OF SHEEP INSULIN.

1. Randall, 1964.

The dry solid obtained by acetone precipitation is dissolved in 2 ml water. The solution is adjusted to about pH 5 by the addition of 0.25N NaOH. The precipitate is collected by centrifugation at 1600 g for 10 minutes and the supernatant tested for completeness of precipitation by the cautious addition of further NaOH or 0.25N HCl. Any further precipitate which forms at a pH blow 6 is collected and added to the main precipitate.

The isoelectric precipitate is suspended in 2 ml water and dissolved by the addition of 0.25N NaOH to pH 7.4±0.2. 1% $ZnCl_2$ solution is added dropwise until precipitation is complete. The zinc-complex is collected by centrifugation (1600 X g for 10 min) and dissolved in 2 ml citrate-borate-acetone solution (2.5gm citric acid, 1.0gm $Na_2B_4O_7 \cdot 10H_2O$ and 10 ml acetone per 100 ml solution, allowed to react for at least 1 hour) freshly adjusted to pH 7.4±0.2 with 5N aq. NH_4OH. The insoluble residue is immediately removed by centrifugation at 1600 X g for 2 minutes and washed by resuspension in 0.5 ml of the citrate-borate-acetone solution (again freshly adjusted to pH 7.4) and centrifugation.

The supernatants are transferred to a tared 5-ml centrifuge tube and shaken until crystallisation occurs. The tube is set aside for 2 hours, or overnight, at 2-3°C and the insulin crystals collected by centrifugation, washed twice with 1 ml water, then acetone and finally ether. The crystals are dried in a desiccator over $CaCl_2$.

2. Romans et al, 1940.

The precipitate which had formed in the bulked alcoholic extracts after storage at 5°C for 15-25 days was collected by centrifugation (1600 X g for 10 min). The precipitate is dissolved in water and the solution adjusted to about pH 5 with 5N NaOH. The precipitate formed after 2 days storage of the solution at 5°C is collected by filtration. The precipitate is dissolved in 1% aq. acetic acid and made to the required volume with acetone. The pH is adjusted to 5.9-6.0 with 2% NH_4OH. The flocculent precipitate formed after about 2 hours at room

temperature is removed by filtration. The acetone filtrate is diluted with ammonium acetate buffer at pH 5.9-6.0 (10 ml glacial acetic acid, 11 ml .88 NH_4OH, made to 100 ml with water). 2.5% zinc acetate solution is added and the solution kept for 24 hours at room temperature, followed by 24 hours in a refrigerator. The precipitate is collected and dissolved in 1.75% aq. acetic acid. After filtration through hardened filter paper, 2.5% zinc acetate solution is added. The acidity is adjusted to pH 6.1-6.6 and diluted to the required volume with dilute NH_4OH. The acidity is then adjusted to pH 5.9 with glacial acetic acid. The crystals form in about 2 hours and settle to the bottom. After removing the supernatant liquor the precipitate is washed with distilled water and collected by centrifugation.

3. Baker, personal communication.

50 mgm of insulin is dissolved in 5 ml 0.02M HCl. Then 0.5 ml of 0.012M zinc sulphate, 2.5 ml of 0.2M sodium citrate, 1.5 ml acetone and 0.5 ml water are added. If the solution is not clear the pH is raised to 7 using 1M NaOH. 1M HCl is added dropwise until a slight permanent cloudiness is seen (pH 6.2-6.3). The test-tube is placed in a Dewar flask containing water at 55°C and the solution allowed to cool slowly over several days. The crystals are collected by centrifugation.

APPENDIX 6.

DISC POLYACRYLAMIDE GEL ELECTROPHORESIS.

The technique of Davis (1964) was used with the gel and buffer system of Thompson et al (1964) which employs Trisborate buffer at pH 8.8, with ethylenediaminetetra-acetic acid in the gel and buffer and 4.6M urea in the gel. The spacer gel (large pore) contained 5% polyacrylamide and the small pore gel 7%.

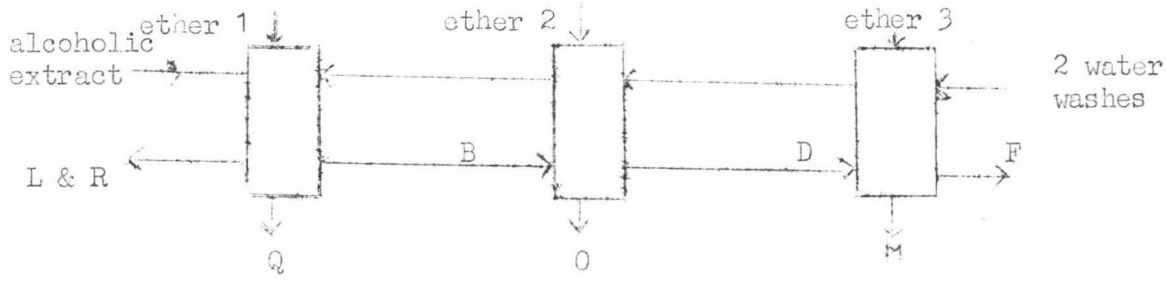
Electrophoretic conditions:	2mA per tube for the first ten minutes, increased to 3mA per tube for the next fifty minutes.	
Staining:	2% amido black/7% acetic acid.	
Destaining:	Washing with 7% acetic acid.	
Dosage:	Crystalline beef insulin	0.44 i.u. per tube
	Crystalline sheep insulin	0.2 i.u. per tube
	Crude sheep insulin	0.5 mgm per tube

APPENDIX 7.

TWO-PHASE COUNTERCURRENT EXTRACTION OF THE ALCOHOLIC EXTRACT.

Data for the phase diagram of ethanol-ether-water combinations from "International Critical Tables", Vol 3, p405, Table 6.

Block Diagram of Process:



Calculations of alcoholic content in extract obtained from 50gm pancreas.

				Composition % w/w	
			ether	water	ethanol
1. Extractions of alcoholic extract with ether.					
1st extraction.					
Input	extract	190gm	-	58	42
	ether 1	150gm	100	-	-
Output	A	210gm	67	12	21
	B	130gm	12	64	24
2nd extraction.					
Input	B	130gm	12	64	24
	ether 2	75gm	100	-	-
Output	C	105gm	82	6	12
	D	100gm	9	73	18
3rd extraction					
Input	D	100gm	9	73	18
	ether 3	75gm	100	-	-
Output	E	90gm	89	4	7
	F	85gm	8	79	13
2. 1st water wash.					
1st extraction.					
Input	water	25gm	-	100	6
	E	90gm	89	4	7
Output	G	84gm	92	3	5
	H	31gm	6	84	10

2nd extraction.					
Input	H	31gm	6	84	10
	C	105gm	82	6	12
Output	I	102gm	86	5	9
	J	34gm	8	76	16
3rd extraction					
Input	J	34gm	8	76	16
	A	210gm	67	12	21
Output	K	204gm	69	11	20
	L	40gm	12	66	22

3. 2nd water wash.

1st extraction.					
Input	water	25gm	-	100	-
	G	84gm	92	3	5
Output	M	79gm	95	2	3
	N	30gm	6	86	8
2nd extraction					
Input	N	30gm	6	86	8
	I	102gm	86	5	9
Output	O	101gm	89	5	6
	P	31gm	7	79	14
3rd extraction.					
Input	P	31gm	7	79	14
	K	204gm	69	11	20
Output	Q	191gm	72	9	19
	R	44gm	11	67	22

Alcoholic extract containing insulin = F + L + R

F	85gm	8	79	13
L	40gm	12	66	22
R	44gm	11	67	22

Total= 169gm containing 17.5% w/w ethanol

REFERENCES CITED.

Abel, J.J. (1926)
"Crystalline insulin."
Proc. natn. Acad. Sci. U.S.A., 12, 132-36.

Adams, M.J., Blundell, T.L., Dodson, E.J., Dodson,G.G., Vijahan, M., Baker, E.N., Harding, M.M., Hodgkin, D.C., Rimmer, B & Sheats, S.
"Structure of rhombohedral two zinc insulin crystals." (1969).
Nature, Lond., 224, 491-95.

Allan, B.J., Zager, N.I. & Keller, P.J. (1970)
"Human pancreatic proteins: Amylase, proelastase & trypsinogen."
Archs Biochem. Biophys., 136, 529-40.

Anderson, E., Lindner, E. & Sutton, V. (1947)
"A sensitive method for assay of insulin in blood."
Am. J. Physiol., 149, 350-54.

Anderson, E., Wherry, F., Bates, R.W. & Cornfield, J. (1957)
"A method for assay of insulin using the A.D.H. mouse."
Proc. Soc. exp. Biol. Med., 94, 321-35.

Ashford, W.R., Campbell, J., Davidson, J.K., Fisher, A.M., Haist, R.E., Lacy, A.H., Lin, B., Martin, J.M., Morley, N.N., Rastogi, S. & Storvick, W.D. (1969)
"A consideration of methods of insulin assay."
Diabetes, 18, 828-33.

Avrameas, S. (1966)
"Pancreatic enzymes studied using immunologic methods."
Rev. Atherscler. Suppl., Arch Mal. Coeur, 8, 119-21. Chem.Abstr., 67:70694

Baker, E. (personal communication)
Chemistry Department, Massey University, Palmerston North.

Baker, S.L., Dickens, F. & Dobbs, E.C. (1924)
"Distribution of insulin in tissues & a micromethod for estimation."
Br. J. exp. Path., 5, 327-31. Chem.Abstr., 20:2168.

Balmain, J.H. Cox, C.P., Folley, S.J. & McNaught, N.L. (1954)
"The bioassay of insulin 'in vitro' by manometric measurements on slices of mammary glands."
J. Endocr., 11, 269-76.

Bangham, D.R. & Mussett, M.V. (1959)
"The fourth international standard for insulin."
Bull. Wld Hlth Org., 20, 1209-20.

Banting, F.G. & Best, C.H. (1922)
"The internal secretion of the pancreas."
J. Lab. clin. Med., 7, 251-66.

Beigelman, P.M. (1960)
"Bioassay for insulin-like activity utilizing glucose uptake by adipose tissue."
Metabolism, 9, 580-86. Chem. Abstr.,54:22812d.

Barollier, J. (1954)
"Preservation of pancreas glands for preparation of crystalline insulin." Chem.Abstr.,52:14988d.
Ger., 902, 429.

Baum, W.E., Brown, W.F. & Crabtree, R.E. (1964)
"Immunoassay for extracted insulin."
J. Am. pharm. Ass., 53, 738-45.

Beigelman, P.M. & Goetz, F.C., Antonaides, H.N. & Thorn, G.W. (1956)
"Insulin-like activity of human plasma constituents; I, Description & evaluation of biologic assay for insulin-like activity."
Metabolism, 5, 35-43. Chem.Abstr.,50:13239g.

Berson, S.A. & Yalow, R.S. (1968)
"General principles of radioimmunoassay."
Clinica chim. Acta, 22, 51-69.

Best, C.H. (1960)
"Fourty years of interest in insulin".
Br. med. Bull., 16, 179-82.

Best, C.H., Campbell, J. & Haist, R.E. (1939a).
"The effect of anterior pituitary extracts on the insulin content of the pancreas."
J. Physiol., Lond., 97, 200-206.

Best, C.H., Haist, R.E. & Ridout, J.H. (1939b)
"Diet and the insulin content of pancreas."
J. Physiol., Lond., 97, 107-19.

Best, C.H. & Macleod, J.J.R. (1923)
"Chemical reactions of insulin."
Am. J. Physiol., 63, 390. Chem. Abstr., 18:1133.

Bingle, J. (1963)
"A significant variable in the rat hemidiaphragm assay of insulin."
Nature, Lond., 197, 595.

Blatherwick, N.R., Bischoff, F., Maxwell, L.C., Berger, J. & Sahyun, M. (1927)
"Studies on insulin."
J. Biol. Chem., 72, 57-89.

Bloodworth, J.M.B. (1968)
"Diabetes mellitus."
In: Endocrine Pathology.
J.M.B. Bloodworth, ed. Williams & Wilkens, Baltimore, 1968.

Bornstein, J. (1950)
"A technique for the assay of small quantities of insulin using A.D.H.A. rats."
Aust. J. exp. Biol. med. Sci., 28, 87-91.

Bouman, J. & Homan, J.D.H. (1958)
"A method for the determination of insulin by paper chromatography."
Biochim. biophys. Acta. 29, 417-22.

Boquist, L. (1970)
"Intracellular digestion & structural variations of secretory granules in pancreatic islet beta-cells."
Horm. Metab. Res., 2, 166-71.

Brown, H., Sanger, F. & Kitai, R. (1955)
"The structure of shep and pig insulins."
Biochem. J., 60, 556-65.

Buchanan, K.D., Vance, J.E. & Williams, R.H. (1969)
"Insulin and glucagon release from isolated islets of Langerhans."
Diabetes, 18, 381-86.

Carpenter, F.H. (1966)
"Relationship of structure to biological activity of insulin as revealed by degredative studies."
J. Am. med. Ass., 40, 750-58.

Catt, K. & Tregear, G.W. (1967)
"Solid-phase radioimmunoassay in antibody-coated tubes."
Science, N.Y., 158, 1570-72.

Ceska, M., Grossmuller, F. & Lundvist, U. (1970)
"Solid-phase radioimmunoassay of insulin."
Acta endocr., Copenh., 64, 111-25.

Chance, R.E., Ellis, R.M. & Bromer, W.W. (1968)
"Porcine proinsulin: Characterization and amino acid sequence."
Science, N.Y., 161, 165-67.

Chibnall, A.C. (1942)
"Amino acid analysis and the structure of proteins."
Proc. R. Soc., B131, 136-60.

Clark, J.L. & Steiner, D.F. (1969)
"Insulin biosynthesis in the rat: Demonstration of two proinsulins."
Proc. natn. Acad. Sci. U.S.A., 62, 278-85.

Clough, H.D. & Murlin, J.R. (1923)
"Extraction of insulin by perfusion."
Proc. Soc. exp. Biol. Med., 20, 417. Chem.Abstr., 18:3676.

Clough, H.D., Allen, R.S. & Murlin, J.R. (1924)
"Aqueous extracts of insulin."
Am. J. Physiol., 68, 213-38.

Cotes, P.M., Mussett, M.V., Berryman, I., Ekins, R., Glover, S., Hales, N., Hunter, W.M., Lowry, C., Neville, R.W.J., Samols, E. & Woodward, P.M. (1969).
"Collaborative study of estimates by radioimmunoassay of insulin concentrations in plasma samples examined in groups of five or six laboratories."
J. Endocr., 45, 557-69.

Crockford, P.M., Hazzard, W.R. & Williams, R.H. (1969)
"Insulin response to glucagon."
Diabetes, 18, 216-24.

Dash, J.A. & Lindsay, D. B. (1967)
"Comparison of an immunoassay and bioassay of insulin in sheep plasma."
J. Endocr., 37, 119-28.

Davidson, J.K., Coddling, J.A. & Haist, R.E. (1968)
"Glycogen content of mouse hemidiaphragm as a biologic indicator of insulin action."
Can. J. Physiol. Pharmac., 46, 361-71.

Davidson, J.K. & Haist, R.E. (1964)
"A study of the levels of extractable insulin of guinea pig pancreas."
Can. J. Physiol. Pharmac., 42, 315-17.

Davis, B.J. (1964)
"Disc electroporesis."
Ann. N.Y. Acad. Sci., 121, 404-27.

Davoren, P.R. (1962)
"Isolation of insulin from a single cat pancreas."
Biochim. biophys. Acta, 63, 150-53.

Dixit, P.K., Lowe, I. & Lazarow, A. (1962)
"Effect of alloxan on the insulin content of microdissected mammalian pancreatic islets."
Nature, Lond., 195, 388-89.

Dixit, P.K., Lowe, I.P., Heggestad, C.B. & Lazarow, A. (1964)
"Insulin content of microdissected foetal islets obtained from diabetic and normal rats."
Diabetes, 13, 71-77.

Dodds, E.C. & Dickens, F. (1924a)
"Preparation of insulin by aqueious extractions."
Lancet, I, 330-31. Chem.Abstr., 18:1683.

Dodds, F.C. & Dickens, F. (1924b)
"Properties and preparation of insulin."
Br. J. exp. Path., 5, 115-22. Chem.Abstr., 18:8351.

Domba, E. & Tarsai Ceg, T.G. (1949)
"Extractions of hormones, viruses, toxins and anti-toxins."
Hung., 139, 965. Chem.Abstr., 44:2710b.

Dudley, H.W. (1924)
"Insulin from the cod fish."
Biochem. J., 18, 665-68.

Dudley, H.W. & Starling, W.W. (1924)
"Improvement in the preparation of insulin: Alkaline extraction of pancreas."
Biochem. J., 18, 147-50.

Earle, M.D. (unpublished)
Food Technology Department, Massey University, Palmerston North.
Study of sheep organ weights.

Eingorn, G.L. (1946)
"Extraction of Insulin."
USSR, 66, 677. Chem. Abstr.,41:1814h.

Epple, A. (1969)
"Existance of a third hormone in the pancreas."
Umschau, 69, 698-99. Chem. Abstr.,72:52838.

Falkmer, S. & Wilson, S. (1967)
"Comparative aspects of the immunology and biology of insulin."
Diabetalogia, 3, 519-28.

Fenger, F. & Wilson, R.S. (1924)
"Amount of available insulin in domestic animals."
J. biol. Chem., 59, 83-90.

Fenton, E.L. (1959)
"A method for the assay of insulin by paper chromatography."
Biochem. J., 71, 507-13.

Fenton, E.L. (1961)
"Assay of insulin solutions by paper chromatogrpahy."
Biochem. J., 81, 570-75.

Ferguson, J.K.W. (1970)
Director, Connaught Medical Research Laboratories, Toronto, Canada.
Personal communication.

Fisher, A.M. & Scott, D.A. (1934)
"Insulin content of the pancreas in cattle of various ages."
J. biol. Chem., 106, 305-10.

Fisher, N.F. (1923)
"Preparation of insulin."
Am. J. physiol., 67, 57-63.

Frederiksen, S.E. (1961)
"Hot aqueious extraction of enzymes from insulin-free pancreas."
U.S., 2,571, 126.

Frohman, L.A. (1969)
"The endocrine function of the pancreas."
A. Rev. Physiol., 31, 353-82.

Gaudiano, A. & Polizzi-Sciarrone, M. (1963)
"Electrophoresis on starch gel of insulin and its analytical application."
Chimica ind. appl., 45, 211-12.

Girard, M.L. (1964)
"The polarograph in pharmeceutical investigations."
Prod. Probl. Pharm., 19, 47-57. Chem.Abstr., 60:14334b.

Gorill, A.D.L. & Thomas, J.W. (1967)
"Bodyweight changes, pancreas size and enzyme activity, and proteolytic enzyme activity and protein digestion in intestinal contents from calves fed soybean and milk protein diets."
J. Nutr., 92, 215-23.

Griffiths, M. (1941)
"Influence of anterior pituitary extracts on the insulin content of the pancrease of the hypophysectomized rat."
J. Physiol., Lond., 100, 104-11.

Griffiths, M. (1942)
"The effects of derivitives of stilbene on the insulin content of the pancreas of the rabbit."
Proc. Linn. Soc. N.S.W., 67, 279-81.

Grillo, T.A.I. (1961)
"The ontogeny of insulin secretion in the chick embryo."
J. Endocr., 22, 285-92.

Grillo, T.A.I. (1964)
"Occurrance of insulin in the pancreas of foetuses of some rodents."
J. Endocr., 31, 67-73.

Grillo, T.S.I. & Shima, K. (1966)
"Insulin content and enzyme histochemistry of the human foetal pancreatic islet."
J. Endocr., 36, 151-58.

Grodsky, G.M. (1958)
"An assay of insulin by fibril formation from small samples of pancreas."
Biochem. J., 68, 142-45.

Grodsky, G.M. & Forsham, P.H. (1960)
"An immunochemical assay of total extractable insulin in man."
J. clin. Invest., 39, 1070-79.

Grodsky, G.M. & Peng, C.T. (1959)
"Extractable insulin measured by immunochemical assay."
Proc. Soc. exp. Biol. Med., 101, 100-103

Grodsky, G.M. & Tarver, H. (1956)
"Paper chromatography of insulin."
Nature, Lond., 177, 223-25.

Haist, R.E. (1944)
"Factors affecting the insulin content of the pancreas."
Physiol. Rev., 24, 409-44.

Hales, C.N. & Randle, P.J. (1963)
"Immunoassay of insulin with insulin-antibody precipitate."
Biochem. J., 88, 137-46.

Hales, N. (1965)
"Radioimmunological methods for hormone determination in blood."
Bull. Schweiz. Akad. med. Wiss., 21, 249-59. Chem. Abstr.,64:968f.

Hama, H., Chitani, K., Sakaki, S. & Narita, K. (1964)
"Amino acid sequence in fin-whale insulin."
J. Biochem., Tokyo, 56, 285-93. Chem. Abstr.,62:2992f.

Harris, J.I., Sanger, F. & Naughton, M.A. (1956)
"Species differences in insulin."
Archs Biochem. Biophys., 65, 427-38.

Hazelwood, R.L., Kimmel, J.R. & Pollock, H.G. (1968)
"Biological characterization of chicken insulin activity in rats and domestic fowl."
Endocrinology, 83, 1331-36.

Heding, L. (1965)
"Ethanol precipitation as a substitute for the double antibody reaction in a simplified immunoassay method."
First Meet.Eur.Ass. for the study of Diabetics, Montecatini,Terme,1965.

Herbert, V., Lau, K., Gottlieb, C.W. & Bleicher, S.J. (1965)
"Coated charcoal immunoassay of insulin."
J. clin. Endocr. Metab., 25, 1375-84.

Homan, J.D.H. (1952)
"Insulin."
Dutch, 71, 303. Chem. Abstr.,47:7169f.

Horiuchi, Y., Kakinuma, M. & Iriyama, R. (1966)
"Use of gel filtration for insulin immunoassay."
Endocr. jap., 13, 7-13. Chem. Abstr.,65:9273g.

Houssay, B.A. (1958)
"Comparative physiology of the endocrine pancreas."
Proc. Columbia. Univ. Symp. Comp. Endocr., 1958.
In: Comparative Endocrinology. A. Gorbman, Ed. Wiley & Sons, 1959.

Huber, J.T., Jacobson, N.L., Allen, R.S. & Hartman, P.A. (1961)
"Digestive enzyme activities in the young calf."
J. Dairy Sci., 44, 1494-1501.

Hudman, D.P., Friend, D.W., Hartman, P.A., Ashton, G.C. & Catron, D.V.
"Digestive enzymes of the baby pig: Pancreatic & salivary (1957)
amylase."
J. agric. Fd Chem., 5, 691-93.

Indian Institute of Science. (1952)
"Insulin."
Indian, 43,403. Chem. Abstr.,47:835e.

Isihara, Y., Saito, T., Ito, Y. & Fujino, M. (1958)
"Structure of sperm & sei-whale insulins & their breakdown by whale pepsin."
Nature, Lond., 181, 1468-69.

Janholm, K.M. & Holm, P. (1962)
"Extraction of proteolytic enzymes, amylase and insulin."
Dan., 102, 821. Chem. Abstr.,64:10028h.

Jeffrey, P.D. & Coates, J.H. (1966)
"An equilibrium ultracentrifuge study of the self-association of bovine insulin."
Biochemistry, N.Y., 5, 489-98.

Jensen, H. & Evans, E.A. (1935)
"Studies on crystalline insulin."
J. biol. Chem., 108, 1-9.

Jephcott, C.M. (1931a)
"Extraction of insulin and stability of various preparations."
Trans. R. Soc. Can., Sect V, 25, 183-86.

Jephcott, C.M. (1931b)
Cited in Haist, 1944.

Jeske, J., Stochla, K. & Hofman, A. (1966)
"A comparison of biological insulin standardization by determining blood sugar levels in rabbits & by producing convulsions in mice."
Acta Pol. pharm., 23, 189-90. Chem. Abstr.,65:18425f.

Jorpes, J.E. (1950)
"Insulin content of whale pancreas."
Hvalrad. Skr., 35, 5-15. Chem. Abstr.,47:3523b.

Jorpes, J.E. & Rastgeldi, S. (1953)
"Insulin content of human pancreas."
Acta physiol. scand., 29, 163-9.

Jorpes, J.E. & Rastgeldi, S. (1960)
"A new principle for large scale production of insulin."
Acta chem. scand., 14, 1777-80.

Katsoyannis, P.G. & Tometsko, A. (1966)
"Insulin synthesis by recombination of A & B chains."
Proc. natn. Acad. Sci. U.S.A., 55, 1554-61.

Katsoyannis, P.G., Tometsko, A. & Fukuda, K. (1963)
"Insulin peptides, IX."
J. Am. chem. Soc., 85, 2863-65.

Katkovskii, S.B. & Schvarts, S.I. (1961)
"Production of crystalline insulin."
Myas. Ind. SSSR, 32 (1), 52-55.

Katkovskii, S.B., Shvarts, S.I., Sarkisova, I.G., Nedobora, A.F., Morozova, T.I., Rukina, N.M., Frolova, G.K. & Leonova, T.M. (1962)
"Insulin."
USSR, 150, 582. Chem. Abstr.,58:8229f.

Katkovskii, S.B., Shvarts, S.I., Shubinskaya, E., Rubina, N.M., Zaretskaya, A. & Grinkevitz, T. (1969a)
"Methods of increasing the production of crystalline insulin."
Myas. Ind. SSSR, 40(6), 12-13.

Katkovskii, S.B., Shvarts, S.I., Shubinskay , E., Turetskaya, G.E., Samsonov, G.V. & Dmitrenko, L.V. (1969b)
"Comparative characteristics of adsorption methods for obtaining crystalline insulin."
Khim.-farm. Zh., 3(9), 57-59. Chem. Abstr.,72:15714.

Katkovskii, S.B., Zvereva, L.A., Anufriev, V.V. & Zemlyannikov, K.F. (1946)
"Insulin & adrenaline."
USSR, 67,571.

Kaulbersz, G. (1930)
"Preparation of insulin from alkaline aqueous extracts."
Bull. Soc. Chim. biol., 12, 464-69. Chem. Abstr.,24:5934.

Keller, P.J. & Allan, B.J. (1967)
"Protein composition of human pancreatic juice."
J. biol. Chem., 242, 281-87.

Keller, P.J. & Cohen, E. (1961)
"Enzymic composition of some cell fractions of bovine pancreas."
J. Biol. Chem., 236, 1407-13.

Keller, P.J., Cohen, E. & Neurath, H. (1958)
"The proteins of bovine pancreatic juice."
J. biol. Chem., 233, 344-349.

Kimmel, J.R. & Pollock, H.G. (1967)
"Studies of human insulin from non-diabetic & diabetic pancreas."
Diabetes, 16, 687-94.

Kimmel, J.R., Pollock, H.G. & Hazelwood, R.L. (1968)
"Isolation & Characterization of chicken insulin."
Endocrinology, 83, 1323-30.

Kimotsuki, K., Shibata, T. & Itani, A. (1959)
"Insulin manufacture without condensing in vacuum."
Japan, 5448('59). Chem. Abstr.,53:22762i.

Krebs, H.A. & Eggleston, L.V. (1938)
"The effect of insulin on oxidations in isolated muscle tissue."
Biochem. J., 32, 913-25.

Krylova, N.N. & Bazarova, K.I. (1960)
"Determination of the activity of insulin."
Myas. Ind. SSSR, 31(1), 53-54. Chem. Abstr.,55:15604h.

Krylova, N.N. & Bazarova, K.I. (1962)
"Determination of the activity of powdered insulin."
Trudy vses. nauchno-issled. Inst. myas. Prom., No.13, 112-29.
Chem. Abstr.,62:2668a.

Kung, F'-T. et al. (1965)
"Total synthesis of crystalline bovine insulin."
Scientia Sin., 14, 1710-16. Chem. Abstr.,64:9817f.

Kupke, D.W. & Linderstrom-Lang, K. (1954)
"On the size of the monomer of insulin."
Biochim. biophys. Acta, 13, 153-53.

Lacey, A.H. (1941)
"The rabbit method of insulin assay."
Endocrinology, 29, 866-76.

Langecker, H. & Wiechowski, W. (1925)
"Pancreas Hormone."
Klin. Wschr., 4, 1339-43. Chem. Abstr.,19:2975.

Lazarus, L. & Young, J.D. (1966)
"Radioimmunoassay of human growth hormone using ion exchange resin."
J. clin. Endocr. Metab., 26, 213-18.

Levin, E. (1970)
"Solid storable pancreas product containing insulin."
Ger. Offen., 1,810,991. Chem. Abstr.,73:38537.

Lewis, C.J., Hartman, P.A., Liu, C.H., Baker, R.O. & Catron, D.V.(1957)
"Digestive enzymes of the baby pig: Pepsin & trypsin."
J. agric. Fd Chem., 5, 687-90.

Light, A. & Simpson, M.V. (1956a)
"Paper chromatography of insulin."
Nature, Lond., 177, 225.

Light, A. & Simpson, M.V. (1956b)
"Studies on the Biosynthesis of insulin."
Biochim. biophys. Acta, 20, 251-61.

Low, B.A. & Einstien, J.R. (1960)
"Symmetry of insulin dimers & hexamers."
Nature, Lond., 186, 470.

Malins, J.M. (1961)
cited in: Recent Advances in Pharmocology, 3rd. ed.
J.M. Robson & R.S. Stacey. Churchill, London, 1962.

Marcker, K. (1960)
"Association of zinc-free insulin."
Acta chem. scand., 14, 194-96. Chem. Abstr.,56:6578b.

Marcker, K. & Graae, J. (1961)
"Dimerization of insulin."
Acta chem. scand., 15, 565-69. Chem. Abstr.,56:10276d.

Marcker, K. & Graae, J. (1962)
"A proposed structure for crystalline zinc insulin."
Acta chem. scand., 16, 41-45. Chem. Abstr.,57:1246d.

Marglin, A. & Merrifield, R.B. (1966)
"Synthesis of bovine insulin by the solid-phase method."
J. Am chem. Soc., 88, 5051-52.

Marha, K. (1960)
"Polarographic determination of the activity of insulin in insulin preparations."
Czech., 97,551. Chem. Abstr.,55:26800f.

Marks, H.P. (1925)
cited in Stewart, 1960.

Marks, H.P. & Young, F.C. (1940)
"Species variation in the insulin content of pancreas."
Nature, Lond., 146, 31-32.

Martin, D.B., Renold, A.E. & Dagenais, Y.M. (1958)
cited in Stewart, 1960.

Massaglia, A., Pennisi, F., Rosa, U., Ronca-Testoni, S. & Rossi, C.A. (1968)
"Effect of chemical modification induced in insulin on the reactivity of the interchain disulphide bonds towards sodium sulphite."
Biochem. J., 108, 247-55.

Maxwell, L.C. & Hinkel, W.P. (1952)
"Preparation of insulin from pancreas glands."
U.S. 2,595,278.

Maxwell, L.C. & Hinkel, W.P. (1954a)
"Preparation of insulin from pancreas glands."
U.S. 2,674,560.

Maxwell, L.C. & Hinkel, W.P. (1954b)
"Preparation of insulin."
U.S. 2,695,861.

Milner, R.D.G. & Hales, C.N. (1969)
"The interaction of various inhibitors & stimuli of insulin release studied with rabbit pancreas 'in vitro'."
Biochem. J., 113, 473-79.

Mirsky, A.I., Jinks, R. & Persutti, G. (1963)
"Isolation & crystallization of human insulin."
J. clin. Invest., 42, 1869-72.

Mirsky, I.A. & Kawamura, K. (1966)
"Heterogeneity of crystalline insulin."
Endocrinology, 78, 1115-19.

Moloney, P.J. & Findlay, D.M. (1924)
"Purification of insulin by sorption on charcoal."
J. phys. chem., 28, 402-406. Chem. Abstr.,18:1730.

Morgan, C.R. & Lazarow, A. (1962)
"Immunoassay of insulin using a two-antibody system."
Proc. Soc. exp. Biol. Med., 110, 29-32.

Murray, D.W.G. & Waters, E.T. (1932)
"Effect of infection on the insulin content of the pancreas."
Trans. R. Soc. Can., 26, Sect V, 169-72. Chem. Abstr.,27:1677.

Nabarro, J.D.N. (1960)
"Chemical effectiveness of insulin substitutes."
Br. med. Bull., 16, 255-59.

Nagasawa, K. (1968)
"Use of fish and whale insulin as drugs in Japan."
J. Ass. off. anal. Chem., 51, 326-29.

Netter, F.H. comp. (1965)
Endocrine System & Selected Metabolic Diseases, Ciba, 1965.

New Zealand Department of Statistics. Monthly Abstract of Statistics, December, 1970, p.29, Table 27, "Meat Slaughterings."

Nicol, D.S.H.W. & Smith, L.F. (1960)
"Amino acid sequence of human insulin."
Nature, Lond., 187, 483-85.

Nothmann, M. (1925)
"Distribution of insulin in the normal & pancreatic dog."
Arch. exp. Path. Pharmak., 108, 1-63. Chem. Abstr.,20:441.

Ogilvie, R.F. (1937)
cited in Haist, 1944.

Ostling, G.J. (1928)
"Insulin & its manufacture."
Meddn finska KemSamf., 35, 50-58. Chem. Abstr.,23:236.

Peterson, G. (1953)
"Extraction of insulin & enzymes from pancreas."
U.S., 2,637,680. Chem. Abstr.,47:8972g.

Pettinger, C.W. (1958)
"Insulin."
Biochem. Prep., 6, 28-31.

Phillips, D.M.P. & Mercer, E.H. (1953)
"Fibrous Insulin."
Biochim. biophys. Acta, 12, 592-93.

Piper, H.A., Allen, R.S. & Murlin, J.R. (1924)
"Aqueous extracts of pancreas. II: Physical & chemical behaviour of insulin."
J. biol. Chem., 58, 321-66.

Pollak, L. (1926)
"Amount of insulin in the pancreas of diabetes."
Arch. exp. Path. Pharmak., 116, 15-34. Chem. Abstr.,21:609.

Preston, F.W. & Kukral, J.C. (1962)
"Surgical physiology of the pancreas."
Surg. Clins N. Am., 42, 103-18. Chem. Abstr.,58:14509b.

Puscaru, E., Russu, C., Bercovici, M. & Sisman, T. (1958)
"The physico-chemical study of insulin solutions & its application to the analysis & purity of products manufactured in Romania."
Lucr. pres. conf. natl. farm., 210-20. Chem. Abstr.,53:4653e.

Randall, S.S. (1964)
"Small-scale preparation of crystalline insulin."
Biochim. biophys. Acta, 90, 472-76.

Randle, P.J. (1956)
"The assay of insulin 'in vitro' by means of the glucose uptake of the isolated rat diaphragm."
J. Endocr., 14, 82-86.

Redenbaugh, H.E., Ivy, A.C. & Koppanyi, T. (1926)
"The presence of insulin in chicken tissues."
Proc. Soc. exp. Biol. Med., 23, 756-57.

Renold, A.E. (1965)
"Methods of detecting insulin levels in blood."
Triangle, 7, 26-31.

Renold, A.E. (1970)
"Insulin biosynthesis & secretion - a still unsettled topic."
New Engl. J. Med., 282, 173-82.

Renold, A.E., Martin, D.B., Dagenais, Y.M. & Steinke, J., Nickerson, R.J. & Sheps, M.C. (1960)
"Measurement of small quantities of insulin-like activity using rat adipose tissue."
J. clin. Invest., 39, 1487-98.

Richardson, K.C. & Young, F.G. (1937)
"The 'pancreatropic' action of anterior pituitary extracts."
J. Physiol., Lond., 91, 352-64.

Romans, R.G. (1954)
"Preparation & chemistry of crystalline insulin."
Recent Progr. Horm. Res., 10, 241-63.

Romans, R.G., Scott, D.A. & Fisher, A.M. (1940)
"Preparation of crystalline insulin."
Ind. Engng Chem., 32, 908-10.

Rosselin, G., Assan, R., Yalow, R.S. & Berson, S.A. (1966)
"Separation of antibody-bound and unbound peptide hormones labelled with I-131 by talcum powder & precipitated silica."
Nature, Lond., 212, 355-58.

Ryle, A.P. & Sanger, F. (1955)
"Disulphide interchange reactions."
Biochem. J., 60, 535-40.

Ryle, A.P., Sanger, F., Smith, L.F. & Kitai, R. (1955)
"Disulphide bonds of insulin."
Biochem. J., 60, 541-56

Sanger, F. & Thompson, E.O.P. (1953)
"The amino acid sequence in the glycyl chain of insulin."
Biochem. J., 53, 353-74.

Sanger, F. & Tuppy, H. (1951)
"Amino acid sequence in the phenylalanine chain of insulin."
Biochem. J., 49, 463-81.

Santenoise, J.P.D. (1961)
"Extraction of pancreatic hormone."
Fr., 1,267,101. Chem. Abstr.,56:11720i.

Schingoethe, D.J., Gorrill, A.D.L., Thomas, J.W., & Yang, M.G. (1970)
"The size & proteolytic enzyme activity of the pancreas of several species of vertebrate animals."
Can. J. Physiol. Pharmac., 48, 43-49.

Schlichtkrull, J. (1956)
"Insulin crystals, parts I - VII."
Acta chem. scand., 10, 1455-86, 11, 291-302, 439-60, 484-86, 1248-56. Chem. Abstr.,52:11512e-i.

Schlichtkrull, J. (1960)
"Insulin growth."
Svenks kem. Tidskr., 72, 166-77. Chem. Abstr.,54:19078i.

Scott, D.A. (1934)
"Crystalline insulin."
Biochem. J., 28, 1592-1602.

Scott, D.A. & Best, C.H. (1925)
"Preparation of insulin."
Ind. Engng Chem., 17, 238-40. Chem. Abstr.,19:1176.

Scott, D.A. & Fisher, A.M. (1938a)
"Pancreas & liver of normal & zinc-fed cats."
Am. J. Physiol., 121, 253-60.

Scott, D.A. & Fisher, A.M. (1938b)
"The insulin & Zinc content of normal & diabetic pancreas."
J. clin. Invest., 17, 725-28.

Sellar, R. F. & Smart, J.V. (1959)
cited in Stewart, 1960.

Shangraw, R.F. & Lamy, P.P. (1969)
"Pharmaceutical aspects of the treatment of diabetes mellitus."
J. Am. pharm. Ass., 9, 117-26.

Shibata, T. (1953)
"Insulin from whale pancreas."
Japan, 1997('53) Chem. Abstr.,48:4781a.

Smith, K.L. (1962)
"Insulin assay."
In: Methods in Hormone Research,
R.I.Dorfman, ed. Academic Press, 1962.

Smith, L.F. (1964)
"Isolation of insulin from pancreatic extracts using carboxymethly & diethylaminoethyl celluloses."
Biochim. biophys. Acta, 82, 231-36.

Smith, L.F. (1966)
"Species variation in the amino acid sequence of insulin."
J. Am. med. Ass., 40, 662-66.

Snedecor, G.W. & Cochran, W.G. (1967)
Statistical Methods, 6th. ed., Iowa State Univ.Press, Iowa, 1967.

Sneyd, J.G.T. (1964)
"Pancreatic & serum insulin in the New Zealand strain of obese mice."
J. Endocr., 28, 163-72.

Sober, H.A. ed. (1968)
Handbook of Biochemistry, Chemical Rubber Co., 1968.

Somogyi, M., Doisy, E.A. & Shaffer, P.A. (1924)
"On the preparation of insulin."
J. biol. Chem., 60, 31-58.

Soong, H.Y. (1940)
cited in Haist, 1944.

Sordelli, A. (1927)
"Preparation of insulin."
An. Asoc. quim. argent., 25, 57-63. Chem. Abstr.,21:3252.

Steiner, D.F. (1969)
"Proinsulin & the biosynthesis of insulin."
New Engl. J. Med., 280, 1106-13.

Steiner, D.F. & Clark, J.L. (1968)
"Spontaneous reoxidation of reduced beef & rat proinsulins."
Proc. natn. Acad. Sci. U.S.A., 60, 622-29.

Steiner, D.F. & Cunningham, D., Spigelman, L. & Aten, B. (1967)
"Insulin biosynthesis: Evidence for a precursor."
Science, N.Y., 157, 694-700.

Steiner, D.F., Hallund, O., Rubenstein, A., Cho, S. & Bayliss, C. (1968)
"Isolation & properties of proinsulin, intermediate forms, & other minor components from crystalline bovine insulin."
Diabetes, 17, 725-36.

Stewart, G.A. (1960)
"Methods of insulin assay."
Br. med. Bull., 16, 196-201.

Takeuchi, S. (1928)
"Amount of hormone in pancreas of several species of animals."
Tohuku J. exp. Med., 12, 62-74. Chem. Abstr.,23:3494.

Tartenc, S. (1948)
"Preparation of fish insulin".
Nogaku, 2, 115-21. Chem. Abstr.,45:10511c.

Thompson, M.P., Kiddy, C.A., Johnston, J.O. & Winberg, R.M. (1964)
J. Dairy Sci., 47, 378-81.

Thompson, W.A. (1954)
"Proteolytic enzymes from pancreas residues."
U.S., 2,686,148. Chem. Abstr.,48:14132a.

Toyama, Y., Tetsumoto, S., Fukaya, S. & Yamada, S. (1941)
"Insulin from fishes."
J. Agric. chem. Soc. Japan, 17, 901-12 Chem. Abstr.,45;443f.

Vasavada, S.A., Makik, P.A., Anantanarayanan, K.G. & Keliwala, C.V.
"Insulin & other clinical products from slaughter house (1964)
wastes."
Indian J. Technol., 2, 139-41.

Volini, M. & Mitz, M.A. (1962)
"Insulin recovery using cellulosic anion exchangers."
U.S., 3,069,323. Chem. Abstr.,58:6652g.

Voluiskaya, E.N. (1963)
"Quantitative paper elestrophoresis for the determination of purity and activity of insulin."
Probemy Endokr. Gormonoter., 9(2), 51-55. Chem. Abstr.,59:7833g.

Walker, D.M. (1959)
"Development of the digestive system of the young animal.
III. Carbohydrase enzyme development in the young lamb."
J. agric. Sci., Camb., 53, 374080.
"IV. Proteolytic enzyme development in the young lamb." Ibid,p.381-86.

Wallace, L.R. (1948)
"The growth of lambs before & after birth in relation to the level of nutrition."
J. agric. Sci., Camb., 38, 243-302.

Waugh, D.F. (1948)
"Regerneration of insulin from insulin fibrils by action of alkali."
J. Am chem. Soc., 70, 1850-57.

Wellcome Foundation Ltd. (1966)
"Insulin isolation."
Neth. appl. 6,602,259. Chem.Abstr.,66:31965.

Wernicke, R. (1924)
"Preparation of insulin."
c.r. Seanc. Soc. Biol., 91, 320-21. Chem.Abstr.,18:3681.

Wessells, N.K. & Cohen, J.H. (1967)
"Early pancreas organogenesis: Morphogenesis, tissue interactions & mass effects."
Devl Biol.,15, 237-70.

Willes, R.F., Boda, J.M. & Manns, J.G. (1969a)
"Insulin secretion by the ovine foetus 'in utero'."
Endocrinology, 84, 520-27.

Willes, R.F., Boda, J.M. & Stokes, H. (1969b)
"Cytological localization of insulin & insulin concentration in the foetal ovine pancreas."
Endocrinology, 84, 671-75.

Wilson, S. & Dixon, G.H. (1961)
"A comparison of cod & bovine insulins."
Nature, Lond., 191, 876-79.

Wrenshall, G.A., Best, C.H. & Hartroft, W.S. (1957a)
"Factors influencing the amount of insulin extractable from beef pancreas. I. Effects of aging fresh pancreas at room temperature."
Can. J. Biochem. Physiol., 35, 527-535.

Wrenshall, G.A., Casselman, W.G.B. & Best, C.H. (1957b)
"Factors influencing the amount of insulin extractable from beef pancreas. III. Effects of temperature & of freezing & thawing on changes accompanying the ageing of fresh pancreas."
Can. J. Biochem. Physiol., 35, 545-48.

Wrenshall, G.A. & Bogoch, A. (1955)
"The weight & extractable insulin of the pancreas in adult non-diabetic man".
J. clin. Endocr. Metab., 15, 435-41.

Yalow, R.S. & Berson, S.A. (1960)
"Immunoassay of endogenous plasma insulin in man."
J. clin. Invest., 39, 1157-75.

Yalow, R.S. & Berson, S.A. (1968)
"Topics on radioimmunoassay of peptide hormones."
Proc. Int. symp. Protein & Polypet. Horm., Belgium, 1968.

Young, D.M. & Romans, R.G. (1948)
"Assays of insulin with one blood sample per rabbit per test day.
Biometrics, 4, 123-31.

Young, F.G. (1960)
"Insulin & diabetes."
Br. med. Bull., 16, 175-78.

Zahn, H. (1966)
"Synthesis of insulin."
Annals Endocr., 27, 575-89.

Zahn, H. & Zabel, R. (1962)
cited in Zahn, 1966.