THE EFFECT OF THYROID HORMONES ON PANCREATIC B CELL FUNCTION

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Degree of Doctor of Medicine

University of Edinburgh

1979

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ACKNOWLEDGEMENTS

DECLARATION

All the investigations reported in this thesis are the
original work of the author. The thesis has not been previously
submitted to this or any other university for a degree and does not
incorporate any material already submitted for a degree.

ABSTRACT

Hyperthyroidism is known to impair glucose tolerance, but possible direct effects of thyroid hormones on the insulin producing B cell of the islets of Langerhans have not previously been reported. Experiments have been performed to investigate the interactions between thyroid hormones and rat pancreatic islets 'in vitro', using a tissue culture system to maintain the stability of the extracellular environment. The secondary effects of hyperthyroidism on pancreatic hormone secretion were thereby excluded.

Thyroxine had no effect on insulin secretion from isolated islets during one hour incubations. However, after 24 hour tissue culture of islets in the presence of thyroid hormones, both insulin biosynthesis and secretion were significantly reduced in response to higher concentrations of glucose. Theophylline fully restored insulin release to normal levels. The ultrastructure and insulin content of islets were not altered by 24 hour exposure to thyroid hormones, despite a significant inhibition of protein synthesis and a 20% reduction in total islet protein. Thyroid hormones did not affect the activity of islet adenylate cyclase.

Thyroid hormones may thus contribute to the glucose intolerance observed in hyperthyroid states, in part by a direct action on pancreatic B cell metabolism, and a consequent inhibition of insulin biosynthesis and secretion. These effects could be a result of a thyroid hormone-induced increase of $Na^+ K^+$-ATPase activity, with the possible effects on intracellular cyclic AMP concentrations, translocation of cations across the plasma membrane and accumulation of cytosolic calcium.

To overcome the problem of poor islet yield from the pancreas, rats were pretreated with pilocarpine, as described in the Appendix. This significantly enhanced islet yield, but the islets were unsuitable for metabolic studies due to their depleted insulin content. However, long-term islet viability was not altered, and they were used successfully for transplantation experiments.

CHAPTER 1

THE HISTORY OF DIABETES MELLITUS

Diabetes mellitus is a disease of modern civilisation.
Nowadays, it is estimated that 10% of American children will develop
diabetes at some time in their lives (Barker, 1977). It is rarely
encountered in primitive societies and epidemological studies have
shown that the advent of urbanisation and technological development
can dramatically increase the prevalence of diabetes, as demonstrated
in the Pacific Islands, the Pima Indians and the South African Bantus
(Marine, 1969; Bennet, 1971; Zimmet, 1977). The English physician,
Thomas Willis (1621-1675) writes: "The Diabetes was a disease so
rare among the Ancients, that many famous physicians made no mention
of it; and Galen knew only two sick of it".

Ancient Descriptions

One of the oldest medical texts, the Papyrus Ebers, dates
approximately at 1500 B.C., mentioned a disease which was probably
diabetes and provides a medical prescription "to drive away the
passing of too much urine" (Papaspyros, 1952). Perhaps the philosophy
of Hippocrates who asserted that no attempt should be made to treat
an incurable disease, and tended to focus on prognosis rather than
diagnosis, made diabetes an unlikely candidate for study by the
Ancient Greek physicians. Certainly it was ignored by the Greeks,
and the disease was not described until the early Christian era.

Little is known about Aretaeus the Cappadocian who may have
been a contemporary of Galen in the second century A.D., and made the
first accurate observations of diabetes. He concluded that the term

"diabetes" was derived from the Greek "to run through a syphon",
and he gave a vivid account of the course of the disease (Fig. 1).
His ideas on the rôle of curative medicine, so divergent from those
of Hippocrates, contain aphorisms which are still pertinent in our
culture; "it is impossible to make all the sick well ... but the
physician can secure respite from pain and intervals in disease and
can render disease latent".

Diabetes is mentioned in the Chinese and Japanese medical
documents of the third century A.D., and Sustrata gives a good account
in the Sanskrit texts of the Ancient Indians (Papaspyros, 1952).
They were the first to recognise that the urine of diabetics was
sweet, preceding European developments by centuries, and they
regarded the disease as a "malady of rich and greedy persons con-
suming much starchy food".

European Developments

European physicians had little to add to the classical
descriptions of diabetes until Thomas Willis brought a new era of
research to what he named as "the pissing evil", when he confirmed
that the urine of patients with diabetes mellitus was sweet-tasting
and could thus be distinguished from diabetes insipidus.
Matthew Dobson in 1776 noted that the sweetness was due to the
presence of sugar. The aetiology of diabetes mellitus remained
obscure. Aretaeus thought that it was a disease of the kidneys and
Willis claimed that it was a disorder of the blood, although he
observed that it could be aggravated by psychological factors such
as "sadness and long grief" (Papaspyros, 1952). Thomas Sydenham

Figure 1

The Extant Works of Aretaeus, the Cappadocian (R.H. Major, 1948).

ON DIABETES

"Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidneys and the bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease then is chronic, and it takes a long period to form; but the patient is short-lived, if the constitution of the disease be completely established; for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive; which however, is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water."

(Edited and translated by Francis Adams. London Sydenham Society, 1856.)

(1624-1689) attributed the disease to the stomach, while
William Cullen (1710-1790) felt it was due to a disturbance of the
nervous system, Claude Bernard (1813-1878) thought that an
abnormality in the liver led to the disruption of normal nutrition
and hence diabetes. The importance of heredity in the aetiology of
diabetes was demonstrated by Bernard Naunyn (1838-1925), and he also
divided diabetes into three categories, juvenile, senile and organic,
a classification which still stands today in a modified form.
Professor Adolf Kussmaul (1822-1902) who described the classical
air-hunger of diabetic ketosis recognised that diabetes mellitus was
a chemical disturbance and later formulated ideas on the metabolic
acidosis which occurs, but it was not until the late nineteenth
century that the pancreas was implicated as being the cause of
diabetes.

The Pancreas

Some earlier workers did suspect the pancreas. In 1683,
Johan Conrad Brunner showed that the surgical removal of the pancreas
from a dog lead to polydipsia and polyuria, and Sauvages (1706-1767)
mentions that Malphighi had produced diabetes by ligation of the
splenic vein. The proof that the pancreas secretes an essential sub-
stance came in 1889, when Oscar Minkowski (1858-1931) and
J. Von Mering removed the pancreas from dogs and then observed typical
symptoms of diabetes mellitus in the experimental animals, ending
fatally within a few weeks. They write: "as far as the results of
extirpation of the pancreas are concerned, we have already mentioned
the most important: after complete removal of the organ, the dogs

became diabetic. It has not to do simply with a transient glycosuria, but a genuine lasting diabetes mellitus, which in every respect corresponds to the most severe form of this disease in man. The appearance of such diabetes, after complete extirpation of the pancreas, comes without exception, unless the animals have died from the immediate effects of the operation".

The first written description of the pancreas dates back to the fourth century B.C. when it was regarded as a fleshy prop for the stomach and a support for the aorta. Prior to the discovery by Mering and Minkowski, Paul Langerhans had produced a doctoral thesis in 1869 on the structure of the rabbit pancreas, and described discrete groups of cells or islets which could be differentiated histologically from the main tissue. These clusters of cells were named the Islets of Langerhans by E.G. Laguesse in 1893. In the same year, A.S. Dogiel showed that there was no connection between these groups of cells and the pancreatic exocrine tissue and Laguesse and E. Hedon decided that the islets constituted the endocrine portion of the pancreas. In 1916, Sir Edward Sharpey-Schafer suggested that the islets secreted a substance controlling carbohydrate metabolism, and named the hypothetical hormone "insulin" (Papaspyros, 1952).

Treatment of Diabetes and the Discovery of Insulin

Despite the lack of knowledge as to the cause of diabetes mellitus, it was recognised from very early times that nutrition was an important element in disease states, and various dietary regimes have always been imployed in the treatment of diabetes. Even the Papyrus Ebers (R.H. Major, 1948) contain details of a seven-day

diet (Fig. 2). Many prescribed diets were based on a reduced caloric intake, although some physicians claimed that the sugar lost in the urine should be replaced, and prescribed sweet foods for their patients! Thomas Willis was the first to use alkalis by advocating lime water, but his prognosis for diabetes was not encouraging: "as to what belongs to the Cure, it seems a most hard thing in this degree to draw propositions for curing". Prior to the discovery of insulin, a popular remedy was the extracts of Brewer's yeast. Interestingly, this compound was in later years found to have an insulin-potentiating effect (E. Glaser and G. Halpbern,1929) attributable to a dietary agent containing chromium known as the 'glucose tolerance factor' (W. Mertz, 1975).

The period following the great discovery of Mering and Minkowski, until 1920, was filled with avid attempts to extract the essential pancreatic substance which would cure diabetes. Various pancreatic preparations were made, and Zuelzer came close to success in 1908 when his ethanolic extract of pancreas was given by intra-venous injection to eight diabetic patients and resulted in an amelioration of symptoms, but these crude extracts proved to have such toxic effects, that their continued use was untenable.

Discovery of Insulin

The breakthrough was finally made by two Canadians, Frederick Banting and Charles Herbert Best, in the summer of 1921, when they made successful extracts of normal and duct-ligated canine pancreata that produced a rapid amelioration of diabetes when given to depancreatectomised dogs. They originally called their

Figure 2

Papyrus Ebers: "a medicine to drive away the passing of too much urine".

Prescription:

Cakes		Fresh grits	1/8
Wheat grains	1/8	Water	1/3
Green lead earth	1/32		

Let stand moist; strain it; take it for 4 days.

Prescription:

Branches of qaded plant	1/4	Honey	1/4
Grapes	1/8	Berries from uan tree	1/32
Sweet beer	1/6		

Cook; filter and take for 2 days.

Prescription:

Sebesten	1/8	Cakes	1/32
Wheat grains	1/8	Water	1/2
Green lead earth	1/32		

Prescription:

| Cakes | 1/8 | Water | 1/3 |
| Honey | 1/32 | | |

Filter and take 1 day.

extract 'isletin', but it was later named insulin, as suggested
previously by De Meyer (1909) and Sharpey-Schafer (1916). This
decisive point in the history of diabetes cannot be described without
mention of the Roumanian-born physiologist, N. Paulesco, who in fact,
had also managed to extract insulin a few months before Banting and
Best had even met, and they themselves remained ignorant of
Paulesco's 'Pancreine' in the year of their memorable achivement
(Pavel, 1972).

By the end of 1922, with the aid of J.B. Collip's isolation
procedure and the chemical engineering skills of the Eli Lilly
Company, insulin became widely available. The use of the crysal-
line form of insulin, obtained by Abel in 1926, reduced the incidence
of local reactions to the available insulins, and ten years later
a new phase in diabetic treatment was prompted by the advent of
protamine (Hagedorn) and protamine zinc (Scott) insulins, since
the incorporation of basic proteins or heavy metals into the insulin
molecule prolong its action.

The relatively small size of the insulin molecule, a factor
which originally gave rise to controversy over its classification as
a protein or polypeptide, made it ideal for research into detailed
molecular structure. Sanger in 1955 (Ryle *et al.*, 1955) achieved
the first successful elucidation of the amino acid sequence of a
protein using ox insulin. He showed that the intact molecule had
a molecular weight of 6000 and was comprised of a pair of poly-
peptide chains linked by two disulphide bridges. Later it became
clear that insulins from different species of animals had slight
differences in primary structure (Harris, 1956; Smith, 1966). In

1969, Adams *et al.* described the three-dimensional structure of the insulin molecule by X-ray analysis of insulin crystals, which exist in two rhombohedral forms containing a minimum of two or four zinc ions per six insulin molecules (Blundell *et al.*, 1971).

DIABETES MELLITUS

Types of Diabetes

The syndrome of diabetes has been sub-divided into at least
five distinct categories, which differ from each other in clinical
features, hereditary and genetic characteristics and probably in
aetiology and pathogenesis. The five groups are:

1. Insulin-dependent (juvenile onset) diabetes mellitus
    (IDDM).

2. Non-insulin-dependent (maturity onset) diabetes mellitus
    (NIDDM).

3. Maturity-onset type of diabetes in the young, a rare
    dominantly inherited mild type of diabetes mellitus
    (MODY). (Tattersall, 1976).

4. Diabetes mellitus or carbohydrate intolerance associated
    with certain genetic syndromes (Rimoin, 1976).

5. Secondary diabetes.

Some of the characteristics of insulin-dependent diabetes and
non-insulin-dependent diabetes are summarised in Table I, but most
of the following discussion of aetiology will focus on insulin-
dependent diabetes.

Pancreatic Pathology of Diabetes

The non-specific nature of the macroscopical and microscopical
changes in the diabetic pancreas preclude the characterisation of any
definite pathognomonic features. Autopsies of insulin-dependent

Table I

SOME CHARACTERISTICS OF INSULIN-DEPENDENT (IDDM) AND NON-INSULIN

DEPENDENT (NIDDM) DIABETES

(From: Christy, M., Deckert, T. and Nerup, J., 1977)

	IDDM	NIDDM
Clinical Features		
Ketosis	Usual	Rare
Weight	Non-obese	Often obese
Age at onset	Usually <30	Usually >40
Onset	Rapid-gradual	Insidious
Duration at onset of late complications	Usually several years	May be present at diagnosis
Remission	Often occurs	? Absent
Epidemiology		
Incidence	$13-14/10^5$ (<30 years) Peak at 12-14 years	Peak at 65 years
Prevalence	0.5 per cent	2 per cent
Sex	Slight male predominance	Female predominance
Seasonal variation	Present	? Absent
Pathology		
Islet mass	<10 per cent	Only moderate reduction
Beta-cell mass	<10 per cent	
Insulitis at onset	Present in 50-70%	? Absent

/cont.

Table I (Cont.)

	IDDM	NIDDM
Immunology		
Antipancreatic cell-mediated immunity	35-50 per cent at onset	<5 per cent
Antipancreatic humoral immunity	60-85 per cent at onset	5 per cent
Association with other endocrinopathies	Frequent	Infrequent
Genetics		
Concordance in identical twins	<50 per cent	Almost invariable
Association with HLA	Present	Absent

diabetics have yielded general information on pancreatic pathology, but causes of the abnormalities are poorly understood. The main features of the diabetic pancreas in IDDM are insulitis and a selective B cell destruction.

Insulitis

Fifty to seventy per cent of insulin-dependent diabetics show a mononuclear cell islet infiltrate in the first few months from diagnosis, which is not found after one year (Gepts and Pipeleers, 1977). The B cells are degranulated and pyknotic, with a peri- and intrainsular infiltration of cells resembling small lymphocytes (Gepts, 1976). These changes have been confirmed by electron microscopic studies, and may be a manifestation of an autoimmune reaction.

B Cell Destruction

Pancreata from insulin-dependent diabetics have a decreased number of islets and a reduction in the size of the average islets, which is secondary to a selective destruction of B cells. Non-insulin producing cells appear to be preserved (Egeberg, 1976). The number of B cells in the initial phase of IDDM is 10% or less of the normal, as illustrated by the nearly negligible basal and post-stimulatory insulin response in newly diagnosed diabetics (Cerasi and Luft, 1977). Insulin secretion may resume during periods of remission, but studies measuring C peptide levels denote a total absence of secretion after 5 years in most, but not all, patients (Steiner, 1977).

The mechanisms of B cell destruction, the causal agents and the regulation of B cell regeneration are still problematic questions.

Islets and B cells are probably regenerated throughout life, so that
the total B cell mass represents the balance between B cell renewal
and cell death. Factors governing the growth of the endocrine pancreas
are unidentified, but work on experimental animals indicates that the
rate of replication can be influenced by dietary and hormonal ele-
ments (Christy, 1977), and that the capacity for regeneration of B
cells has a strong genetic component (Hellerström, 1976). Thus IDDM
may become apparent only when regeneration of B cells cannot com-
pensate for their destruction, and the potential equilibrium of the
system is lost.

The Inheritance of Diabetes

The importance of inheritance in the pathogenesis of diabetes
has been well recognised, but because diabetes is not a single
disease entity, it has been described as the geneticist's 'nightmare',
with little agreement over its exact mode of genetic expression. It
is too common to be dependent on a single gene: diseases acquired
in this way tend to be selected against unless they confer a par-
ticular biological advantage to the individual, and this has not been
demonstrated for diabetes. Berg (1938) originally noted the "absolute
heredity" of late onset diabetes, and conjugal studies (Tattersall,
1976) have demonstrated a strong genetic component in maturity onset
diabetes (usually non-insulin-dependent). The twin studies of
Tattersall and Pyke (1972) showed that the age of onset of diabetes
was crucial in attempting to understand the characteristics of its
inheritance. All the twin pairs in their survey were concordant for
diabetes if the disease developed after the age of 45, while only 50%

concordance was found for juvenile onset diabetes, and long-term follow-up has not revealed any changes in this pattern. This has confirmed the strong genetic predisposition for late onset diabetes, which differs from the complex and multifactorial inheritance of juvenile onset diabetes (usually insulin-dependent).

Viral Hypothesis

The onset of IDDM has a seasonal pattern, with an increased incidence of diabetes occurring in the autumn and winter months (Gamble and Taylor, 1969). This has led to speculations of an association between the development of diabetes and the viral infections prevalent at these times of the year. Localised islet cell damage and a diabetic state can be produced in mice by infection with the EMC virus (Craighead, 1968 and 1975; Kromann, 1974), and structural damage to mouse islets is observed following infection of animals with Coxsackie B virus (Coleman, 1974). However, the only direct evidence of viral B cell destruction is the electron-microscopic detection of perinuclear inclusions of virus-like particles, in the pancreatic duct cells of both insulin and non-insulin-dependent diabetics (Greider, 1977).

Diabetes and Autoimmunity

Antipancreatic cellular and humoral autoimmunity and concomitant associations with various HLA antigens are established features of insulin-dependent diabetes. Preliminary evidence of cell-mediated immunity against antigens of the endocrine pancreas was gained from the 'in vitro' leucocyte migration inhibition against human pancreatic homogenate. The precise nature of the antigen is unknown, but it

appears to be species and organ specific, and is definitely not insulin. Whether this cell-mediated antipancreatic aggression is persistent (Irvine, 1976a) or declines over a period of time (Nerup, 1974) is not confirmed.

Autoantibodies against the endocrine pancreas have been detected using immunofluorescence techniques (Bottazzo, 1974; MacCuish, 1974). Islet cell antibodies (ICA) are an organ specific class of IgG (Lendrum, 1976a) elaborated against unidentified cytoplasmic antigens (Lendrum, 1976a; Bottazzo, 1976). Human ICA cross-reacts with pancreatic tissue of other species, and also with all types of islet cells so it is unlikely that humoral autoimmunity is the causative agent in the selective B cell destruction apparent in IDDM. Elegant double immunofluorescence experiments with antihormonal antibodies have clearly shown that ICA is not directed against pancreatic hormones (Bottazzo, 1976). The prevalence of detectable ICA in the general population is very low, estimates varying from 0.5% (Irvine, 1976b) to 1.7% (Lendrum, 1976b), compared to one series of newly diagnosed insulin-dependent diabetics; 85% were ICA positive (Lendrum, 1976a). This number declines rapidly in the first few months after diagnosis, indicating that the appearance of ICA is related to the clinical onset of IDDM.

Persistence of ICA for more than 5 years is associated with co-existent organ specific autoimmune disease and certain HLA types (B8, A1, A1+B8, Irvine, 1977). Occasionally, ICA have been detected several years prior to the development of overt diabetes, in patients with autoimmune endocrinopathy, in particular idiopathic Addison's disease (Bottazzo, 1974; Lendrum, 1976a), or first degree relatives of IDDM patients (Nelson and Pyke, 1976), but the prognostic value and

clinical significance of ICA remains to be clarified. No correlation is known to exist between humoral and cellular antipancreatic auto-immunity (Christy, 1976).

The human leucocyte antigens (HLA) represent the major human histocompatibility system, and despite the prevailing lack of insight into the mechanisms whereby susceptibility to HLA-linked diseases is conferred, there are definite associations between IDDM and certain HLA types of the B and D series (Nerup, 1977). The increased frequency of IDDM in a group carrying the relevant antigens when com- pared to a control group without that particular haplotype (relative risk) is illustrated in Table II (Svejgaard, 1977; Nerup, 1977). HLA types B8 and DW3 are shared with several other autoimmune endo- crinopathies, which suggests a common genetic susceptibility, and there may be an increase in intra-HLA recombination in families with more than one juvenile onset diabetic (Rubinstein, 1976).

The above interdependent arguments for a 'nature' or 'nurture' hypothesis of the pathogenesis of IDDM have now been drawn together into a general theoretical framework. In summary, an unknown environmental stimulus (possibly of viral origin) triggers off an autoimmune reaction in predisposed individuals giving rise to B cell destruction. This susceptibility to the development of IDDM may be attributable to a certain genetic configuration located in the HLA region of chromosome 6, which could also influence some of the features of the disease, and the individual's immune response.

Table II

RELATIVE RISKS OF IDDM FOR VARIOUS HLA-B AND D PHENOTYPES

(From: Christy, M., Deckert, T. and Nerup, J., 1977).

HLA Phenotype	Relative Risk
Bw15/x	2.5
BW15	2.1
B8/x	2.5
B8	3.1
B8/Bw15	9.8
Dw3	3.7
Dw4	4.9
Dw3/Dw4	9.4

Data on B-phenotypes compiled by Svejgaard and Ryder (1977).

Data on D-phenotypes compiled by Nerup *et al.* (1977).

x indicates presence of another B-antigen.

HYPERTHYROIDISM AND DIABETES

Hyperthyroidism

Hyperthyroidism is a disease characterised by an increase in
the total daily output of thyroid hormones, a sustained rate in their
plasma level and an increase in tissue concentrations of these
hormones. The disorder was probably first described by Parry in 1825
(Werner, 1978), who recorded a case of heart failure in a patient who
had a swelling in the thyroid area. Graves (1835) collected three
patients with marked palpitations and swelling of the thyroid; he also
recounted a case in which a woman was incapable of closing her eyes
because her eyeballs were apparently so 'enlarged' and showed "the
white sclerotic ... all around the cornea". Von Basedow (1840)
described the gamut of eye changes and most of the other clinical
features of the disease, but he considered it to be an odd manifes-
tation of tuberculosis. Rehn (1884), recognising the association
between hyperthyroidism and an enlarged thyroid gland, performed
the first sub-total thyroidectomy for a toxic goitre; elevation of
the basal metabolic rate in hyperthyroidism was originally noted by
Magnus-Levy (1895). A toxic diffuse goitre (Graves Disease) is the
major cause of hyperthyroidism, and with a few rare exceptions,
most other cases are due to a toxic nodular goitre resulting from
the autonomous functioning of a circumscribed area(s) within the
thyroid gland and unrelated to Graves disease (Werner, 1978).

Thyroid Hormones

Thyroid hormones are derived from thyronine (Fig. 3) by partial or complete substitution in the 3,5,3' and 5' positions with iodine atoms. Thyroxine (tetraiodo-L-thyroxine, T_4) and tri-iodo-L-thyronine (3-5-3' tri-iodo-L-thyronine, T_3) are considered to be the two iodothyronines of physiological importance (Sterling, 1974) and are illustrated in Fig. 3. T_4 was originally isolated by Kendall in 1914 (Kendall, 1929), and circulating iodine was subsequently reported to be principally incorporated into this hormone (Trevorrow, 1939; Taurog, 1948). The total concentration of T_4 in human serum is between 3.3 to 6.3 µg/100 ml, of which only 0.03% exists in the free form (Schussler, 1967; Woeber, 1968) while the rest is bound to three serum protein carriers. The total concentration of T_3 is estimated to be 0.2 µg/100 ml, of which 0.3% is unbound (Woeber, 1970). Two thirds of the thyroid hormones are carried by thyroxine-binding globulin, TBG (Gordon, 1952; Larson, 1952; Sterling, 1964), an acidic glycoprotein which has the maximal affinity for thyroid hormones of the three carriers. Thyroxine-binding pre-albumen, TBPA, binds up to 15% of thyroid hormones (Woeber, 1968), but only occurs in humans, primates and the horse. Serum albumen binds about 10% of thyroid hormones and has the lowest affinity for them. A rapid exchange equilibrium exists between T_4, T_3, bound thyroid hormones and free hormones (Thorson, 1969). Only the free form of the thyroid hormones can enter target tissues and exert their actions at a variety of intracellular sites. In addition to their more obvious function as a means of transporting T_4 and T_3 from the thyroid gland to peripheral organs, the binding proteins also prolong hormone availability by
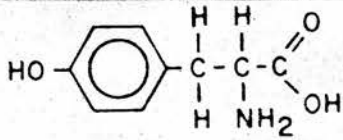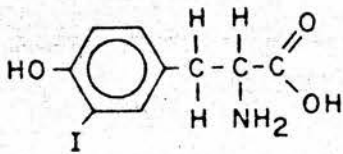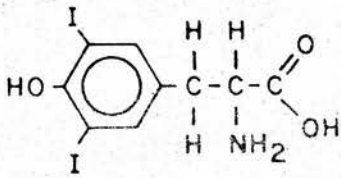
-34-
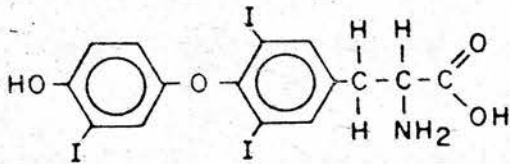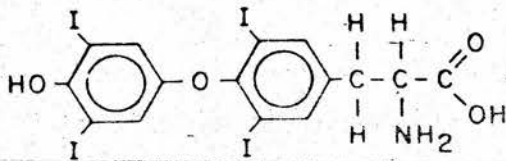
Figure 3 Tyrosine and iodinated thyronines.

retarding the rate of trans-capillary diffusion, provide a
buffering action to dampen the effects of transient pertubations of
thyroid hormones and act as a reservoir for extra-thyroidal hormone
storage.

The Coexistence of Hyperthyroidism and Diabetes

The earliest record of the relationship between an increase in
thyroid hormone secretion and diabetes mellitus was by Dumontpallier
in 1867. He observed the combination of Basedow's disease (1840)
with diabetes in a young woman, and scattered reports of similar
instances followed, until Sattler in 1909 had collected a series of
56 such cases. In the majority of these, the diabetes appeared after
the onset of hyperthyroidism.

Interest in the coexistence of these two diseases was further
aroused by suggestions that surgical or radiotherapeutic manipulation
of the thyroid gland could be useful in the treatment of diabetes.
A reduction of thyroid function had been noted to improve certain
cases of concomitant diabetes (Falta, 1910; Crile, 1915; Rohdenburg,
1920), but Fitz (1921) concluded that the results of thyroidectomy
on the course of diabetes were not encouraging, and the dangers of
the operation did not warrant its advisability as a diabetic cure.
Allen (1922) rightly pointed out that although glucosuria could be
produced in normal people by overdosage with thyroid preparations, in
addition to the association between diabetes and hyperthyroidism, and
the high carbohydrate tolerance in many cases of myxoedema, it
would be unlikely that the superimposition of two hormone
deficiencies could effect an improvement of health.

Hyperthyroidism and diabetes mellitus are both common diseases, and despite initial controversy, it is now clear that the increased incidence of diabetes in thyrotoxic patients is more than a coincidental finding (Lamberg, 1965). Joslin and Lahey (1928) found that 1% of their 4917 diabetics were hyperthyroid, and Andrus (1932) reported 3 diabetics in a smaller series of 200 hyperthyroid patients, but developments in the diagnosis of both diseases have yielded more reliable data. Regan and Wilder (1940) noted the occurence of diabetes in 3.2% of 5353 hyperthyroid patients, Foster and Lowrie (1938) reported 42 patients with hyperthyroidism and diabetes in 1616 diabetics. Andreani (1969) diagnosed a 4.5% incidence of clinical diabetes in 605 hyperthyroid patients and glucose intolerance has been observed in up to 57% of patients with hyperthyroidism (Kreines, 1965). It is interesting that hyperthyroidism due to a toxic nodular goitre is more likely to be associated with diabetes than that due to a diffuse toxic goitre (Abt, 1962; Andreani, 1969), but it may be that the latter group of patients are diagnosed and treated sooner. Regan and Wilder's study showed that 1.7% of the exopthalmic group had diabetes, compared to 5.6% of the toxic adenoma group who were diabetic.

There are at least three possible explanations for the association between diabetes and hyperthyroidism. Firstly, the complex physiological changes resulting from an excess of circulating thyroid hormones may in themselves induce an abnormal carbohydrate tolerance, through metabolic disturbances and their secondary effects on pancreatic insulin secretion. It may be that the two diseases have a common aetiological factor or that certain individuals are genetically predisposed to both pathologies. Finally, thyroid

hormones may exert a direct influence on the insulin secretion from pancreatic B cells.

Effect of Hyperthyroidism on Carbohydrate Tolerance

There is ample evidence that the development of hyperthyroidism aggravates an existing carbohydrate disorder (Lakin, 1961) and the severity of diabetes is generally ameliorated by adequate control of excess thyroid hormone production (Fitz, 1921; Wilder, 1926; John, 1932; Eller, 1960). The numerous studies of carbohydrate tolerance in hyperthyroid patients without a previous history of diabetes have been fraught with inconsistencies, possibly because the patients have never been properly grouped according to age, sex or the severity and duration of their thyroid disorders. On the whole, the more recent surveys agree that a significant proportion of hyperthyroid patients have a raised fasting blood glucose (Elrich, 1961; Lamberg, 1965; Andersen, 1977). Hyperthyroidism is also associated with oral glucose tolerance tests of the diabetic type (Hales, 1964; Lamberg, 1965; Doar, 1969) but some authors detected a rapid rise in blood glucose after an oral glucose load, followed by a steep fall and normal 2-3 hour blood glucose levels (Goldberg, 1949; Amatuzio, 1954; Holdsworth, 1968). This would be consistent with the increased absorption rate of glucose from the intestine (Althousen, 1938) or the enhanced gastric emptying rate (Holdsworth, 1968) in hyper-thyroidism, both of which make the evaluation of OGTTs difficult. However, studies attempting to circumvent these problems by using the IVGTT have emerged with equally conflicting data. In hyperthyroidism, the glucose disappearance rate, or K value, after an IVGTT has been

found to be unchanged (Amatuzio, 1954; Elrich, 1961; Andreani,
1970), increased (Lamberg, 1965) or decreased (Jacobsen, 1972;
Andersen, 1977).

More detailed measurements of plasma insulin levels during
glucose tolerance tests have also failed to clarify the issue.
Insulin secretion has been found to be normal (Hales, 1964; Klink,
1964), increased (Field, 1966; Doar, 1969; Andersen, 1977), or
decreased (Levy, 1969; Andreani, 1970). However, Holdsworth and
Besser (1968) pointed out that although insulin release in hyperthyroid
subjects may be comparable to that of euthyroid controls, it may be
insufficient in proportion to the blood glucose stimulus.

The metabolic sequelae of hyperthyroidism include an
increase in hepatic gluconeogenesis (Mirsky, 1936; Levine, 1953;
Danowski, 1962), a reduction of hepatic glycogenesis (Cramer, 1913;
McIver, 1943; Lamberg, 1965), and raised fasting levels of plasma
non-esterified free fatty acids and pyruvate, similar to those
found in diabetes mellitus (Williams, 1943; Hales, 1964; Doar, 1969),
all of which must contribute to the increase in blood glucose. Elgee
(1955) using I^{131}-labelled insulin detected a more rapid degradation
of the hormone in hyperthyroid subjects, but Genuth (1972) concluded
that the metabolic clearance of insulin was normal. The effects of
circulating catecholamines are enhanced in hyperthyroidism (Porte, 1969;
Brewster, 1956; Woeber, 1966), and they are known to inhibit the
insulin response to glucose (Buse, 1970; Cerasi, 1971). This may
explain some of the abnormalities in carbohydrate tolerance described
above, but there again, catecholamine levels in hyperthyroidism have
been found to be reduced (Stoffer, 1973). Andersen (1977) has also
raised the possibility of peripheral insulin resistance, since

his hyperthyroid patients had abnormal OGTTs, despite high plasma insulin levels.

The picture becomes even more confused when patients are re-examined after treatment, once they are euthyroid. Some authors claim that the carbohydrate intolerance is reversible. Levy (1970) reported that 40% of his diabetic patients did not need insulin after treatment with radioiodine. Others failed to find any improvement in carbohydrate tolerance following treatment (Cavagnini, 1974; Kreines, 1965), but again these contradictory results may reflect poor patient selection and grouping.

Aetiological Association Between Hyperthyroidism and Diabetes

The importance of a genetic influence on susceptibility to disease has been discussed in relation to diabetes. Organ specific auto-aggression may be a generalised immune response to an environmental stimulus in predisposed individuals, which is not necessarily confined to one particular tissue. Certainly, there are remarkable similarities in the microscopic appearance of lymphoid infiltration and endocrine target cell destruction of organs affected by auto-immune disease. The prevalence of IDDM in idiopathic Addison's disease and autoimmune thyroid disorders is 30-50 times that of the general population (Nerup, 1977), and although the reverse situation is less clear, there is a higher incidence of non-pancreatic organ specific autoantibodies in diabetics (MacCuish and Irvine, 1975), in addition to the 4 to 5-fold increase in the frequency of autoimmune endocrinopathy in the diabetic population (Christy, 1977). Nevertheless, the identity of the putative initiating factor in these manifestations of immune disease is unknown.

THE MORPHOLOGY OF PANCREATIC ISLETS

Histology

Diamere (1899) and Shulze (1900) both recognised that the islets of Langerhans were composed of more than one cell type, and Lane (1907) submitted the first adequate description of the two varieties of islet cells on the basis of the selective staining of their granules. They were called A cells (originally observed by Ssobolew and Tschassownikow) and B cells. A third category was added to this classification by Bloom (1931) and Thomas (1937), the C cell.

Gomori (1939) confirmed the existence of A and B cells using a chrome alum haematoxylin phloxine technique whereby A cells were identified by red colours and B cells by purple colours, but D cells also stained red. Ferner developed a silver impregnation technique specific to A cells (Ferner, 1938) although other workers had difficulties using this method reliably because of the thick frozen sections which were necessary (Ferner, 1952).

A modified silver stain was developed by Hellerström and Hellman (1960) and they were able to observe two types of A cells; A_1 with cytoplasmic argyrophyllia, and A_2 which did not take up silver. In contradistinction to these findings, the silver impregnation method of Grimelius (1968) demonstrated mainly the A_2 cells, which is perhaps a reflection of the capricious nature of silver staining, an important factor in the controversy that surrounded the classification of islet cell types.

The relationship between A_1 cells and D cells remained unresolved for some time. Many authors claimed that A_1 cells and

D cells were identical (Lazarus and Shapiro, 1971; Fujita, 1964; Petersson, 1962), but Conklin (1962) felt that D cells were a precursor of B cells, while Like (1967) proposed that D cells were altered A cells and did not recognise a third cell type. Electron microscope studies failed to resolve the conflict, with one body of workers reiterating the synonymity of A_1 and D cells (Wellman, 1971) while Deconinck (1971 and 1972) supported the light microscopic findings of Van Asshe (1970) that D cells consituted a heterogeneous group of islet cells, which he labelled types III and IV, in addition to A and B cells.

An agreement on four cell types was eventually established (Solcia, 1973), and they were to be known as A, B, D and D_1, the latter two corresponding to Deconinck's type III and IV respectively. D and D_1 cells are also found in the gastric mucosa (Fujita, 1974; Barrington, 1976) as are A cells in some species (Unger and Orci, 1975; Dobbs, 1975; Larsson, 1975). As far as is known, B cells are exclusive to the pancreas.

Quantitative estimations of islet number, weight and volume have met with considerable problems because of the heterogeneity of islet distribution within the pancreas, and of islet size (Gepts, 1957). The human pancreas contains approximately 1×10^6 islets, but there is a wide variation (Ogilvie, 1937), a higher proportion of islets residing in the pancreatic tail (Laguesse, 1905). It is generally believed that the pancreas is composed of 1 to 2% of endocrine tissue (Laguesse, 1906; De Witt, 1906; Susman, 1942), of which B cells represent about 80%, A cells 15% and D cells about 5% (Pictet and Rutter, 1972; Petersson, 1963; Hellman, 1963).

MacLean and Ogilvie (1955) calculated a mean adult human islet weight of 1.06 g, of which 0.64 g was B cells and 0.22 g was A cells. Gepts's calculations were a little higher with a mean islet weight of 1.36 g (Gepts, 1971). From the developmental viewpoint, a study by Like and Orci (1972) of 20 human embryos between the ages of 8 to 23 weeks showed that A cells were differentiated by 9 weeks, followed by the D cells, and B cells appeared at 10.5 weeks.

Function of Islet Cells and Pancreatic Hormones

Insulin synthesis, storage and secretion by B cells has been extensively studied and will be discussed in more detail later. Early histological studies provided good evidence that insulin is stored in the B cell secretory granule (Baron, 1948; Hartcroft, 1955; Logothetopoulos,1964; Lazarus, 1970) and this was confirmed with the development of fluorescent antibody techniques for the cellular localisation of hormones (Lacy, 1959; Lazarus, 1970; Logothetopoulos,1970; Lange, 1975; Erlandson, 1976).

Likewise, it is also well established that glucagon is the secretory product of the A cells (Logothetopoulos,1960; Volk, 1960; Baum, 1962). With the categorisation of A cells into two groups further experimental evidence conclusively demonstrated that glucagon resides in the A_2 cells. Firstly, protein-bound tryptophan, a prominent component of the glucagon molecule, is only found in A_2 cells (Hellerström, 1964). Administration of glucagon to rats and guinea pigs over a period of time results in degeneration and involution of A_2 cells only (Hellerström, 1962; Petersson, 1963) and histological observations of the uncinate process of the dog pancreas,

in which no measurable glucagon is present (Benscome, 1955), show
that it is devoid of A_2 cells (Hellman, 1962). Definitive proof
came from elegant studies in which A_1, A_2 and B cells were micro-
dissected from sections of equine pancreatic islets and only A_2
cells stained for glucagon (Lundquist, 1970).

D cells belong to the APUD classification (Pearse, 1969), as
do A, B and D_1 cells, and are thought to be of neutoectodermal
origin. Immunochemical localisation of somatostatin to D cells in
several mammals (Pelletier, 1975; Orci, 1975c; Erlandsen, 1976) has
been confirmed by immunofluorescence studies in human and rat islets
(Polak, 1975; Hökfelt, 1975), and ultrastructural localisation of
somatostatin to D cells (Goldsmith, 1975; Refener, 1975). The
belief that gastrin is also a functional product of D cells, at
least in man (Lomský, 1969; Greider, 1971; Erlandsen, 1976), has
not been firmly established, and is at variance with some studies
(Lofstra, 1974; Tobe, 1974), possibly because of differences in the
type of gastrin antisera used in immunocytochemical techniques.
The above-mentioned cells are all microscopically identifiable by
virtue of their characteristic electron-dense secretory granules
(Falkmer, 1977).

Other Pancreatic Hormones

Glucagon

The concept of bihormonal metabolic regulation by a single
organ was expressed by Lane (1907) several years before the discovery

of insulin. He put forward a perceptive theory in his paper on the histology of the pancreatic islets that "one is led to the conviction that the islets of Langerhans are structures which in all probability have a function of producing a two-fold substance, which, when poured into the blood stream, has an important effect on metabolism".

Banting and Best (1921) themselves observed a rise in blood sugar immediately after the injection of their crude pancreatic extract, which persisted for 30 minutes but not surprisingly, their attention was focussed on the elusive hypoglycaemic 'isletin'. Aqueous extracts of the pancreas were prepared by Kimball and Murlin (1923) which also raised blood glucose, and when this glycogenolytic fraction was isolated in 1929 by Collens and Murlin it was called glucagon, or 'glucose-driving'.

During the early stages of the development of insulin preparations, glucagon was regarded as an artefact, and it was not properly evaluated as a discrete hormonal entity until it became possible to measure circulating glucagon levels by radioimmunoassay (Unger, 1959; Unger, 1962; Eisentraut, 1968). Pancreatic glucagon secretion leads to a release of glucose from the liver into the circulation. Glucagon both increases hepatic glycogenolytic activity (Sokal, 1964; Rodbell, 1971) and is the most potent gluconeogenetic agent known (Sokal,1966a). Paradoxically, it also stimulates insulin secretion (Samols, 1965; Crockford, 1966), but although this response may be part of the entero-insulin axis of hormonal metabolic regulation or a means whereby basal insulin secretion is maintained during periods of reduced caloric intake, this aspect of its activity is not understood.

Pancreatic glucagon is a straight chain polypeptide with 29 amino acid residues (which appear to have a similar sequence in most mammals studied so far), and a molecular weight of 3485. Staub (1953 and 1955) was the first to purify and crystallise glucagon, and it was synthesised by Wunsch in 1967. Some antibodies raised to pancreatic glucagon also react with material from the gastro-intestinal tract, which has been termed glucagon-like-immunoreactivity (GLI). The biological significance of this substance is still disputable, but the presence of glucagon-like peptides in the gut has been repeatedly observed (Sutherland, 1948; Unger, 1966, 1968; Buchanan, 1967; Polak, 1971). Attempts at isolation of gut GLI have emphasised the heterogeneity of this extract (Valverde, 1970; Murphy, 1973; Rosselin, 1974; Conlon, 1975), which appears to be composed of at least two separate entitites; a small molecular weight peptide closely resembling pancreatic glucagon, and a larger species with a molecular weight between 10 and 12 thousand, the effect of which is uncertain (Conlon, 1975; Gutman, 1973; Gleeson, 1971). Unfortunately, until GLI is more precisely characterised, the interpretation of glucagon assays and hence hypotheses of its physiological function must be approached with caution.

Somatostatin

Somatostatin, a tetradecapeptide, was originally isolated from the avian hypothalamus (Vale, 1972; Brazeau, 1973) but has since been identified in the mammalian gastrointestinal tract, thyroid gland and pancreas (Hökfelt, 1975). Its presence in the human pancreas has been confirmed by electron microscope immunohisto-chemistry (Luft, 1974; Goldsmith, 1975; Rufener, 1975; Dubois, 1975),

and indirect immunofluorescence (Hökfelt, 1975; Orci, 1975c).
Initially an important discovery because of its ability to inhibit
growth hormone secretion from the pituitary, it also inhibits
insulin release in man and in the isolated perfused pancreas of
rat and dog (Alberti, 1973; Gerich, 1974, 1975a, 1975b; Efendic,
1976), and human glucagon secretion (Gerich, 1974; Dobbs, 1975;
Koerker, 1974). A radioimmunoassay for somatostatin has now been
developed (Arimura, 1975; Elde, 1977).

Pancreatic polypeptide

This hormone-like substance containing 36 amino acids
(Chance, 1975), was first found in the avian pancreas (Kimmel, 1971)
and later in the pancreas of a variety of animal species including
man (Lin, 1972; Kimmel, 1975). It has now been shown to occur in
a small, rare population of islet cells (Larsson 1975, 1976;
Van Assche, 1976) as well as having a scattered distribution in the
pancreatic exocrine parenchyma and duct epithelium (Larsson, 1975).
It has complex effects on the exocrine pancreas, liver, gall
bladder and gastric mucosa (Lin, 1974a, 1974b; Schwartz, 1976),
but its physiological rôle is poorly understood.

The Islet as a Unit

The topographical relationship between the different islet
cells has aroused much interest in recent years because of its
possible implications on the functional regulations of pancreatic
endocrine secretion. Indirect immunofluorescence studies with anti-
insulin, anti-glucagon and anti-somatostatin sera have revealed a

specific distributional pattern of A, B and D cells within islets (Orci and Unger, 1975; Erlandsen, 1976; Orci, 1977). Several animal species demonstrate glucagon-containing cells in the peripheral area of the individual islet, with a sparser, less well demarcated sub-cortical zone of somatostatin and pancreatic polypeptide-containing cells, and a mass of B cells at the centre of the islet, although some A and D cells are also present. A similar pattern exists in normal human islets except that A and D cells border the permeating intra-islet vascular channels in addition to their cortical distribution, as shown diagrammatically in Fig. 4.

This brings into question whether pancreatic endocrine secretion is governed not only by neural impulses and metabolic con-stituents of the extracellular fluid and blood supply surrounding the islets (these aspects will be discussed later), but also by intra-islet regulation of hormone secretion through a syncitial network. The concept is further supported by Orci's finding of gap junctional complexes (low resistance pathways of inter-cellular communication) between the adjacent islet cells (Orci, 1975b).

Since glucagon stimulates insulin secretion (Samols, 1965), and insulin inhibits glucagon secretion (Samols, 1976), while somatostatin is a powerful suppressor of both insulin (Samols, 1976; Alberti, 1973; Koerker, 1974; Mortimer, 1974) and glucagon release (Koerker, 1974; Mortimer, 1974), speculations regarding possible local feedback circuits have arisen (Unger, 1976; Unger and Orci, 1977; Orci, 1977), but until investigations of the microcirculation of islet vasculature are complete, such models of intra-islet hormonal regulation the theories must remain tentative (Hökfelt, 1975).

Glucagon

Somatostatin
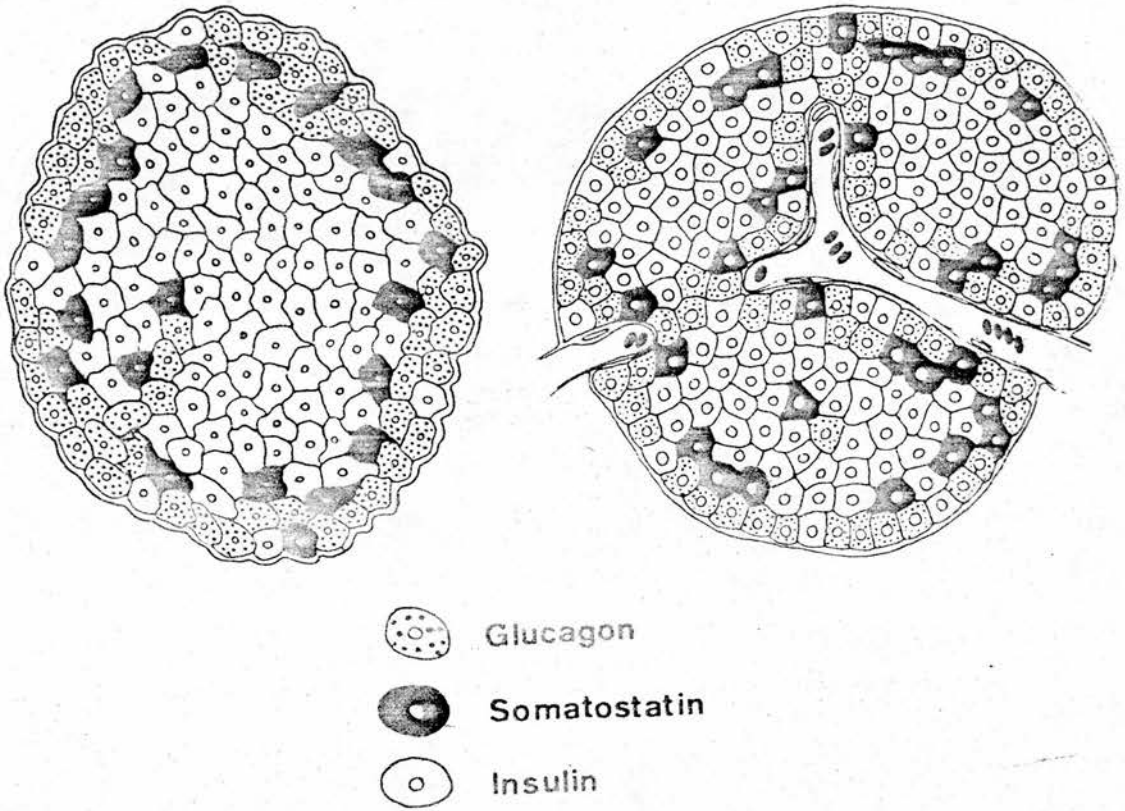
Insulin

Figure 4 Schematic representation of the number and distribution of
insulin, glucagon and somatostatin containing cells in the normal rat
islet (left) and human islet (right). The human islet shows large,
penetrating vascular channels. This pattern divides the total islet
mass into smaller subunits, each of which contains a centre formed
mainly of insulin containing cells and surrounded by glucagon and
somatostatin containing cells. (From L. Orci in "Insulin and Metabolism",
ed. J.S. Bajaj, Excerpta Medica, 1977, pp.1-10.).

Unger has suggested that diabetes mellitus is a bihormonal disorder, implicating glucagon excess as a result of A_2 cell dysfunction in addition to insulin lack (Unger, 1975). Certainly diabetics show an abnormal glucagon secretion (Unger, 1972; Raskin, 1975). Furthermore, Orci and Gepts have noted an increase in the morphometric density of A_2 and D cells in the islets of juvenile onset diabetics (Orci, 1976; Gepts, 1977). Nevertheless, hyper-glucagonaemia does not have deleterious effects on glucose homeostasis if substantial insulin is present (Felig, 1976a) and it seems that insulin deficiency is the primary pathophysiological disturbance in diabetes. The rôle of glucagon in normal subjects may be to maintain euglycaemia after non-carbohydrate stimulation of insulin secretion (for example a protein meal) when hypoglycaemia might otherwise result (Unger, 1969; Felig, 1976b).

B Cell Ultrastructure and Insulin Biosynthesis

The ultrastructural appearance of B cells is similar throughout the mammalian species. Granular endoplasmic reticulum and secretory granules are often concentrated on opposite sides of the cell nucleus, although small ribbons of granular endoplasmic reticulum may be interspersed among secretory granules. Smooth endoplasmic reticulum is scarce; polysomes, ribosomes and mito-chondria occur throughout the cytoplasm. The Golgi complex is made up of parallel. arrays of smooth membranes and small vesicles, usually near the nucleus (Lacy, 1972).

Insulin Precursors

Preparations of pancreatic slices were originally used to
demonstrate that insulin could be synthesised from radioisotopically
labelled amino acids (Pettinga, 1952; Vaughan, 1954; Light, 1956).
Innovative studies on a human islet cell tumour, consisting almost
entirely of B cells, revealed that labelled amino acids were
initially incorporated into an islet protein of higher molecular
weight than insulin, which was nevertheless strongly bound by
insulin antibodies, and could be cleaved by tryptic digestion to
give insulin (Steiner and Oyer, 1967). Further studies on isolated
rat islets, using pulses of labelled amino acids and cycloheximide,
an inhibitor of protein synthesis, confirmed the hypothesis, that
the synthesis of a larger protein (pro-insulin) precluded the
formation of insulin (Steiner, 1967).

Pro-insulin is a single polypeptide chain, composed of 78
(dog) to 86 (human, horse, rat) amino acid residues (Chance, 1968;
Steiner, 1973). Conversion of pro-insulin to insulin involves the
cleavage of the molecule in two areas, giving rise to insulin and a
connecting peptide (C-peptide) fragment. These events take place
within the secretory granules of the B cell, as will be described
below, so that C-peptide accumulates with insulin in equimolar
amounts, and is secreted with the hormone by exocytosis of the
granule contents (Steiner, 1976a).

There is no definitive evidence of the exact mechanism of
conversion of pro-insulin to insulin. Approximate combinations of
pancreatic trypsin and carboxypeptidase B can convert pro-insulin
to insulin *in vitro* (Kemmler, 1971), but any disruption of B cells

by sonication or freezing destroys their converting ability (Steiner, 1974). In an attempt to differentiate between possible converting enzyme ability or dependence of the process on an intact cellular environment, crude secretory granule fractions were prepared (Kemmler, 1970) under conditions of maximal granule stability (Howell, 1969b). Such granules could convert endogenously labelled pro-insulin to insulin (Kemmler, 1970), and conversion was effectively inhibited by granule disruption, suggesting that conversion must occur within intact secretory granules or closely associated intra-cellular organelles. The possible relationship of the pro-insulin transforming enzymes to exocrine pancreatic proteases has not been established, but poses an interesting teleological suggestion that the two groups of enzymes may be co-ordinated during development.

Modern progress in the use of cell-free systems to translate messenger RNA from rat islets or islet-cell tumours has identified an insulin precursor larger than pro-insulin (Steiner, 1976b). The rapidity with which this molecule is transformed to pro-insulin made it impossible to trace with whole-cell labelling techniques. Now known as pre-pro-insulin, it consists of pro-insulin with an amino terminal extension of approximately 2,500 daltons. The postulated flow of information from the gene for insulin in the chromosomal DNA to the assembly of the insulin molecule is summarised in Fig. 5.

Insulin Synthesis

Autoradiographs of pulse-chase experiments on B cells, with tritiated leucine, accompanied by gel chromatographic identification of the newly-synthesised radio-labelled proteins, have confirmed
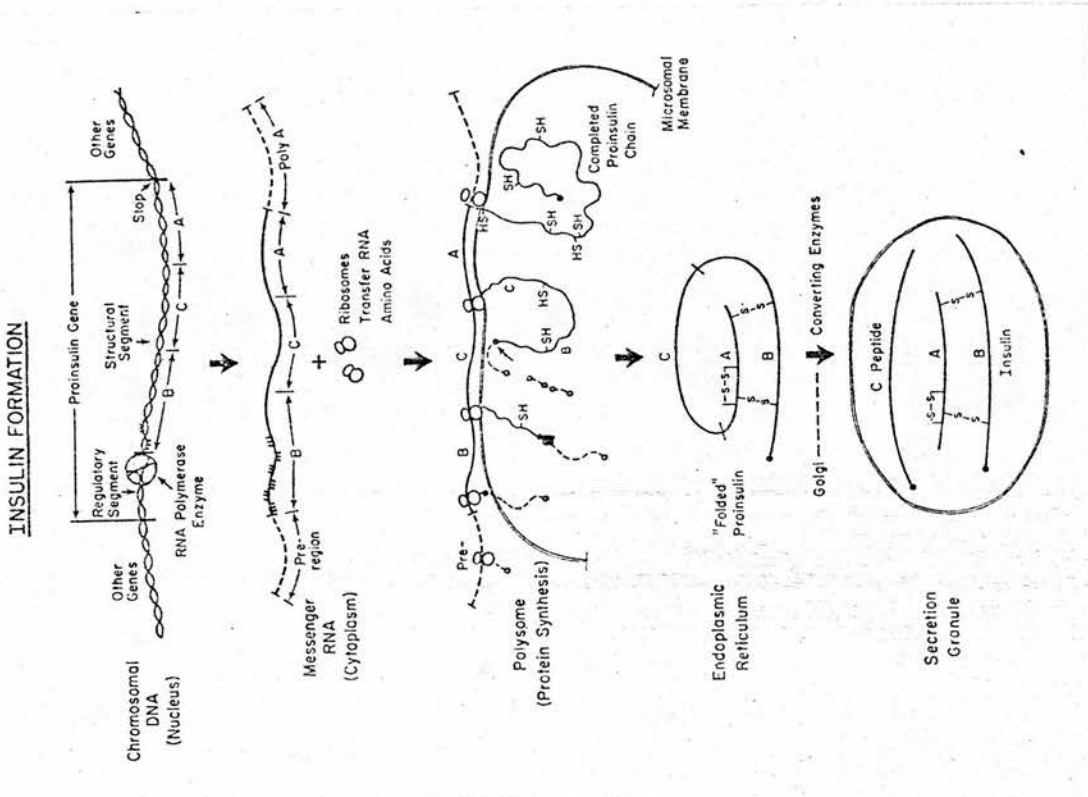
Figure 5 (From Steiner, D.F., 1976). Scheme of the molecular biology of insulin formation. The proinsulin gene is represented in the upper section. RNA polymerase is necessary for the transcription of messenger-RNA (mRNA) from the gene, and this then serves to guide the formation of polypeptide chains on the polyribosomes. The initial translation product, *preproinsulin*, is believed to promote the association of ribosomes with the microsomal membrane, thereby leading to the vectorial discharge of the peptide chain into the cisternal space of the endoplasmic reticulum. The N-terminal *"pre"* region is then rapidly cleaved away from the pro-insulin in the microsomes (3rd section from top). After folding and formation of disulfide bonds the proinsulin is transferred to the Golgi apparatus and converted to insulin and C-peptide which are stored in the secretory granules pending release.

that pro-insulin is manufactured in the rough endoplasmic reticulum (Howell, 1969a; Orci, 1973). Synthesis of insulin precursors can be induced by glucose (Steiner and Oyer, 1967).which also stimulates translation of pro-insulin messenger RNA in short-term experiments (Permutt and Kipnis, 1972).

The newly-formed proteins are channelled from the rough endoplasmic reticulum to the Golgi apparatus (Howell, 1969a and 1974; Orci, 1971), by an energy-requiring process (Howell, 1972a) possibly involving the budding-off of microvesicles (Orci, 1973). Conversion of pro-insulin is initiated in the Golgi cisternae (Kemmler, 1970) prior to the formation of mature β granules (Steiner, 1969), and new secretory granules emerge from the Golgi apparatus to integrate with the pre-existing granule pool. It is not clear whether new and mature granules mix at random (Sando, 1972 and 1973), accumulate in different cellular compartments (Grodsky, 1970) or become aligned to intracellular structures such as microtubules (Orci, 1973). The newly-synthesised proteins packaged in the granule core mature over the next 45-150 minutes (Howell, 1969a; Orci, 1973), by continued conversion of pro-insulin to insulin, with granular accumulation of zinc to form microcrystals of insulin (Kemmler, 1972). Fig. 6 summarises diagrammatically the time course and series of events which occur from the production of pro-insulin to the extrusion of insulin-containing granules by the B cell.

Diagrammatic summary of insulin biosynthesis in the pancreatic 'B' cell.
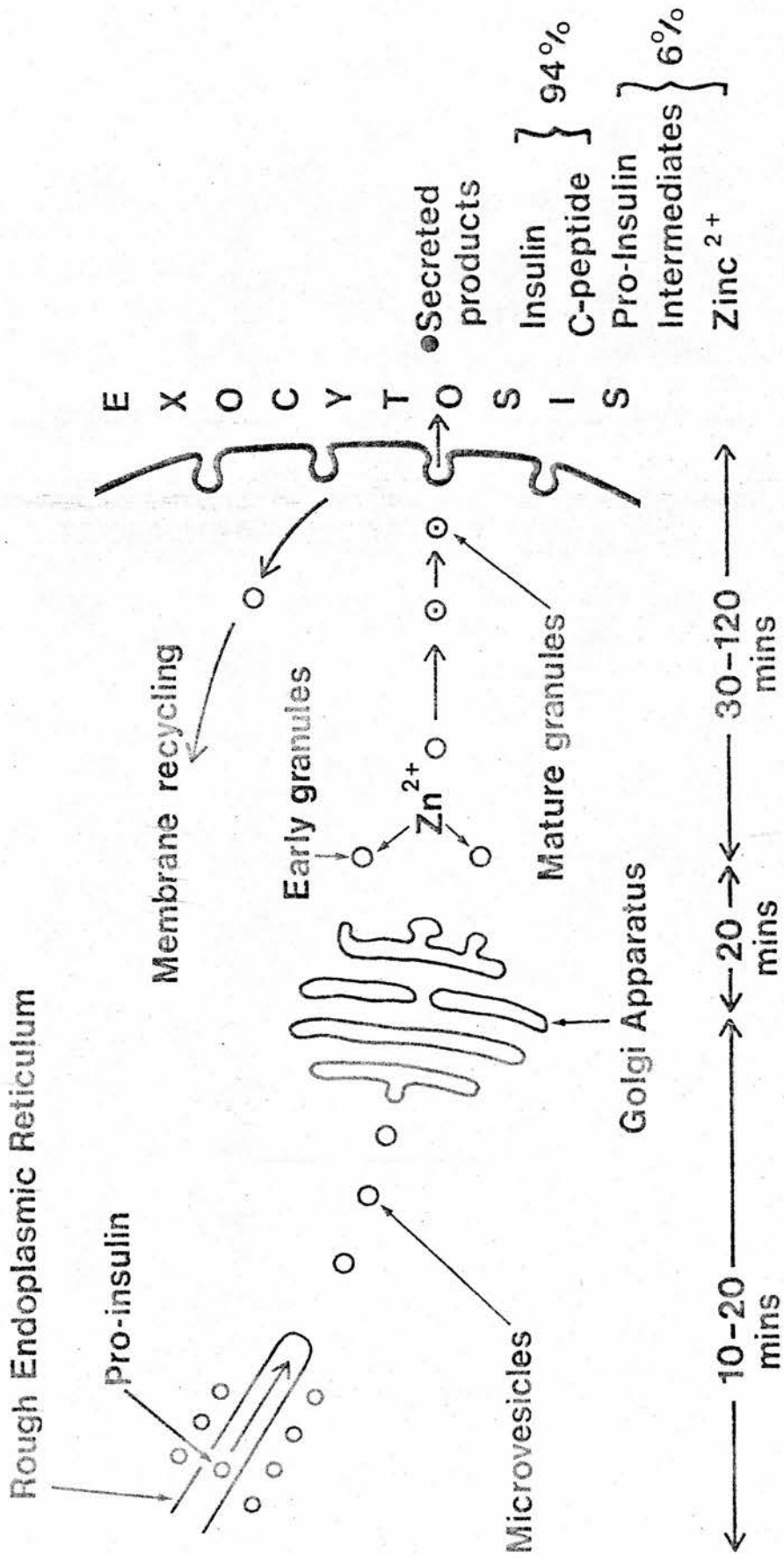
Figure 6 (Adapted from Steiner and Rubenstein, 1973)

THE MECHANISM OF INSULIN SECRETION

The Physiological Control and Modification of Pancreatic Endocrine
Hormones

The maintenance of blood glucose concentrations at appropriate
levels for the adequate nutrition of the central nervous system, by
the endocrine pancreas, implies far more than a simple stimulatory/
inhibitory "feedback" sensitivity to extracellular glucose. Not
only are the fluxes in glucose utilisation during exercise, or
absorption during feeding, rapid and comparatively vast, but at times
of severe stress the actual glucose homeostasis may have to be sub-
jugated to the requirements of the brain. Thus hypoperfusion of the
central nervous system is accompained by compensatory hyperglycaemia.
Not surprisingly, the apportioning of control to the various factors
which influence islet activity is an unrealistic task when
describing integrated systems of response, but they can be classi-
fied into four separate groups:-

   1. Central and autonomic nervous system

   2. The entero-insular axis

   3. Circulating hormones

   4. Circulating nutrients and other agents.

The Nervous System

Evidence for central nervous system modulation of islet
hormone secretion arises from experiments where insulin release can
be evoked by behavioural conditioning (Woods, 1972), visual,
olfactory, gustatory or hypnotic stimuli. These responses are

effected through the parasympathetic innervation of the gut and
pancreas, and can be abolished by atropine (Goldfine, 1970;
Parra-Cavarrubias, 1971). Furthermore, destruction of the ventero-
medial hypothalamus (VMH) in rats results in a chronic increase in
circulating insulin levels, which precedes the B cell hypertrophy
and hyperplasia, and obesity following such lesions (Han, 1970;
Martin, 1974). Stimulation of the VMH increases glucagon secretion
and is accompanied by a reduced insulin release, but the latter is
probably due to the raised adreno-medullary catecholamines
(Frohman, 1971).

The pancreas has both a splanchnic sympathetic innervation and
a parasympathetic vagal supply. The adrenergic and cholinergic
nerve terminals have been identified by light and electron micro-
scopy, more commonly at the islet periphery. The sympathetic
nervous system also affects insulin secretion through circulating
catecholamines. Adrenaline inhibits glucose induced insulin
release (Malaisse, 1967 ; Karam, 1966), while augmenting glucagon
secretion (Karam, 1966), and noradrenaline has similar effects
(Porte, 1966). There is some evidence for sympathetic regulation
of basal insulin secretion, since α-blocking drugs, such as
phentolamine, increase circulating insulin and glucagon levels
(Robertson, 1973). No consistent alteration in basal insulin
secretion is demonstrable after vagotomy or anti-cholinergic drugs
(Goodner, 1972), and it is probable that parasympathetic innervation
to the pancreas co-ordinates responses to meals, either directly or
through other gastro-intestinal hormones, whereas the sympathetic
supply modulates the autonomic response to stress (Bloom, 1973).

The Entero-insular Axis

The "entero-insular axis" denotes the multiple neural and hormonal factors which mediate the insulin response to absorption of nutrients from the gut. The insulin response to an oral glucose load is greater than that to intravenous glucose (McIntyre, 1964; Elrick, 1964), and it has been estimated that approximately half of the insulin secreted during glucose ingestion is released under the influence of gastrointestinal factors, other than the direct effect of the substrate on B cells (Perley, 1967). It appears that there are three distinct elements in the control of the endocrine pancreas during absorption of food. Firstly, there are the nutrients themselves, which provide the main stimulus for islet hormone release. Secondly, there is accumulating evidence that the various peptides which have been identified in the gut, such as bombesin, cholecystokinin, enkephalin, gastrin, neurotensin, somatostatin, substance P and vaso-active intestinal peptide (VIP), act as local neurotransmitters (Daniel, 1978), and several of these are known to directly affect insulin secretion (Fig. 7). Finally, it seems likely that there is an additional endocrine transmitter ("incretin"), which is released from the gut in response to the absorption of nutrients, particularly carbohydrates, and which augments insulin secretion in the presence of glucose. The identity of incretin is not yet established, but the most favourable gut hormone which could be identified with such a rôle is gastric inhibitory peptide (GIP) (Creutzfeldt, 1979).

Diagrammatic summary of main factors modulating pancreatic endocrine secretion
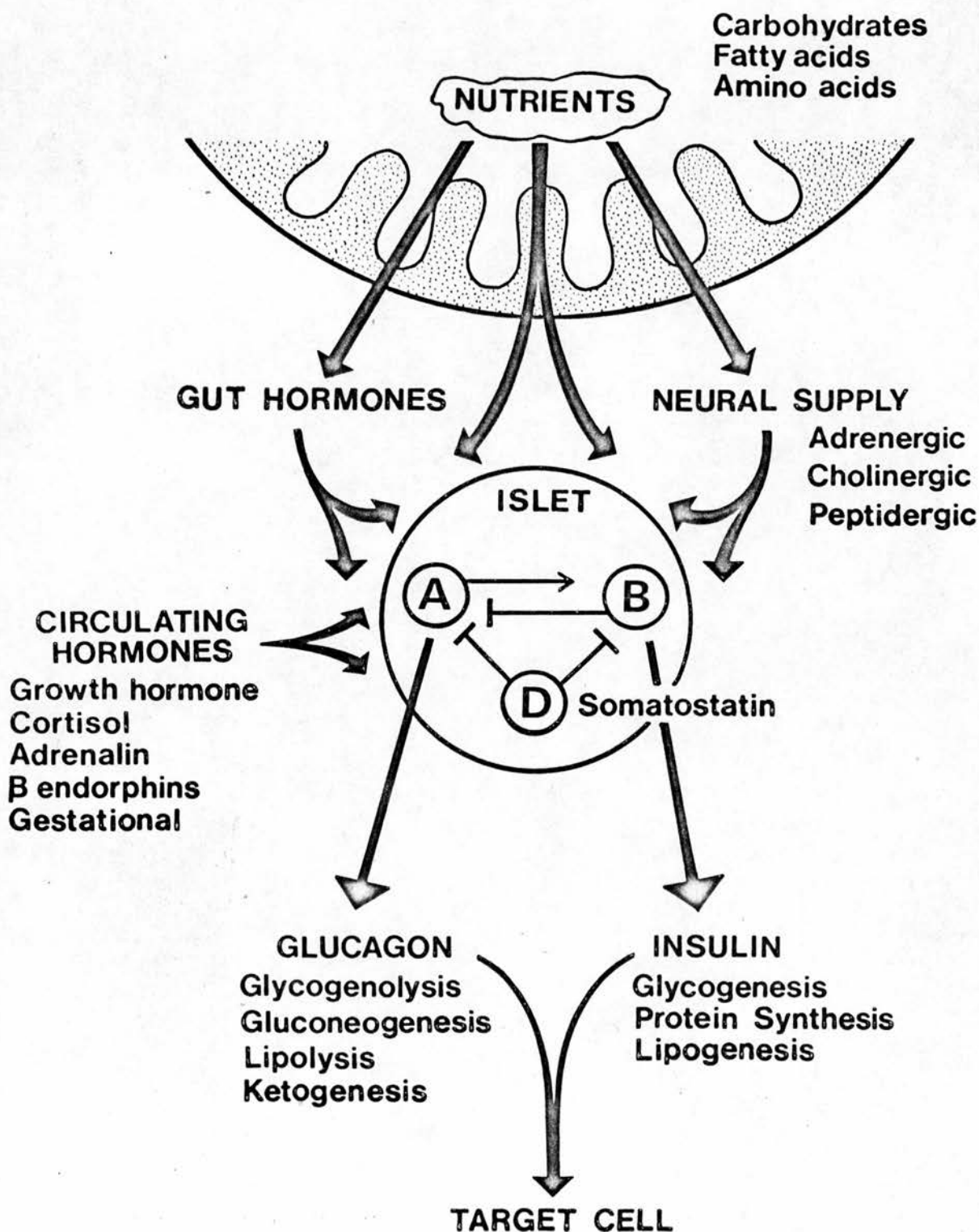
Figure 7 (From Creutzfeldt, 1979)

Hormones and Other Substances

The hormones which are known to affect insulin secretion are summarised in Table III, with the exception of thyroid hormones, which will be discussed in more detail later. The influence of glucose and other agents on insulin secretion is elaborated below.

Table III

HORMONES EFFECTING AN INCREASE IN INSULIN SECRETION

Hormone	Direct/Indirect Effect	Reference
Pituitary		
Growth hormone	Indirect	Malaisse, 1972
	Direct	Whittaker, 1977
ACTH	Indirect	Schatz, 1973
	Direct	Malaisse, 1972
TSH	Direct	Malaisse, 1972
Gastro-intestinal		
Secretin	Direct	Fahrenkrug, 1978; Dupré, 1969
Gastrin	Direct	Rehfeld, 1973; Dupré, 1969
Pancreozymin	Direct	Dupré, 1969
Enteroglucagon	Direct	Sasaki, 1974
Vaso-active-intestinal peptide (VIP)	?	Said, 1974; Ohneda, 1977; Schebalin, 1977
Gastric-inhibitory-peptide (GIP)	Direct	Dupré, 1973; Brown, 1974
Incretin	Direct	Creutzfeldt, 1974, 1979
Adrenal corticosteroids	Indirect	Perley, 1966; Wise, 1973
Gonadal hormones		
Progesterone	Indirect	Hager, 1972
Oestradiol	Direct	Howell, 1977
Pregnancy	?	Malaisse, 1969; Costrini, 1971; Green, 1972

Regulation of Insulin Secretion

Prior to the existence of methods for direct insulin measurements, the cross-circulation experiments of Gayet and Guillaumine (1928) had demonstrated that a rise in blood glucose leads to a release of insulin. Anderson and Long (1947) provided good evidence that insulin is released in response to hyper-glycaemia, using an insulin bioassay. The advent of radioimmunoassay techniques (Yalow and Berson, 1960) finally paved the way for the detailed qualitative and quantitative studies of insulin release which have been the focus of modern diabetic research.

Any hypothesis concerning the aetiology of diabetes can only accrue from a fundamental appreciation of B cell function. Ideally, only 'in vivo' observations give a full understanding of the co-ordinated chemical, neural and hormonal stimuli which enable the individual organism to maintain glucose homeostasis. However, pancreatic islets lend themselves well to 'in vitro' studies. Transplantation experiments show that a normal blood sugar can be maintained by denervated islets (Minkowski, 1908; Kemp, 1973) and the perfused pancreas, pancreatic pieces or isolated islets can be investigated 'in vitro' with relative ease. Bearing in mind the limitations of these models, essential information about basic cellular mechanisms can be gained from such preparations, and they have been studied extensively to formulate ideas on the regulation of insulin secretion.

Factors Influencing Insulin Secretion

(a)   Glucose

Glucose is the main physiological stimulus for insulin secretion. Basal insulin output is modifed by extracellular glucose concentrations above 3.5 mmol/l and the increase in insulin secretion is proportional to the rise in glucose levels (Coore and Randle, 1964). The rate curve for glucose induced insulin secretion is sigmoidal, with a stimulant threshold of 4-6 mmol/l, a large and rapid stimulation of insulin release over glucose concentrations of 7-10 mmol/l, and a maximal secretion at about 16 mmol/l (Malaisse, 1967a). One of the main features of insulin secretion is the rapidity with which the B cell can respond to hyperglycaemia: Portal venous samples *'in vivo'* (Blackard, 1970), and *'in vitro'* preparations (Grodsky, 1968a) show that insulin secretion can achieve near maximal rates within a few seconds to a few minutes of raising the extracellular glucose concentration. The system demonstrates saturation kinetics which suggest that the B cell "glucose recognition unit" is based on enzyme controlled mechanisms (Randle, 1968). Another aspect of insulin release is the marked changes which occur in time if the glucose stimulation is maintained, in that the insulin secretory response is biphasic. An abrupt initial insulin output lasting 5-6 minutes falls as quickly to near basal values and is followed by a second gradual increase in insulin secretion which persists while extracellular glucose levels are high (Grodsky, 1968a). Three explanations have been proposed for this biphasic pattern:

(1)   The existence of intracellular insulin in two distinct

functional units: a small labile compartment responsible for the rapid release of insulin, and a larger, more stable compartment, supplying the former unit and perhaps discharging insulin itself, although at a slower rate (Grodsky, 1969). This model was further adapted by suggesting that the smaller compartment consists of heterogeneous insulin 'packets' with different stimulant threshold levels (Grodsky, 1972a and 1972b).

(2) The above theory was modified in the light of subsequent observations of B cell morphology, namely the microtubular skeleton and the microfilamentous cell web. Possibly the initial secretory phase results from extrusion by emiocytosis of the B granules in close relationship to the microfilamentous cell web, while the later phase corresponds to the mobilisation of secretory granules along the microtubules (Malaisse, 1974b).

(3) Attractive as the above hypotheses are, they both assume different dose response kinetics between the two postulated compartments. In fact, the dose response curves of the initial insulin secretory phase and that of the second phase are remarkably alike, both achieving saturation at high concentrations of glucose (Cerasi, 1974), in which case the first and limited response cannot be explained by the exhaustion of insulin in a small pool or adjacent to the cell membrane. A different model, which assumes the generation of three simultaneous events when glucose reaches the B cell, has been proposed. It is suggested that glucose recognition encompasses both a positive initiating input with a rapid time course, and a separate potentiating effect which amplifies the former. The third input, with an intermediate time course, is a negative feedback inhibition of insulin release (Cerasi, 1975).

(b)   Stimulus recognition

The means whereby the B cell recognises glucose is still unresolved. There is continuing controversy between the "substrate-site" hypothesis (Randle, 1968) which suggests that glucose may be recognised as an insulin secretagogue by its metabolism in the B cells (Grodsky, 1963; Coore, 1964), and the "regulatory-site" hypothesis (Randle, 1972), maintaining that the glucose molecule interacts with a specific receptor on the B cell membrane, and that this interaction initiates the cellular events leading to insulin secretion.

(c)   The "substrate-site" hypothesis

The following indirect evidence supports the theory that changes in extracellular glucose concentrations lead to changes in the rate of metabolism of glucose to a key metabolic intermediate acting as an intracellular modifier of the rate of insulin secretion. A diagram of the metabolic pathways of the B cell is shown in Fig. 8.

(1)   Metabolisable sugars such as glucose, mannose and fructose stimulate insulin release, while non-metabolisable sugars (galactose, 2-deoxyglucose) are poorly active (Grodsky, 1963; Coore, 1964; Ashcroft, 1973a; Hellman, 1974).

(2)   Chemical energy in the form of ATP is a prerequisite for insulin release. Without energy sources the islet ATP content declines within 5 minutes, but is maintained by glucose and meta-bolisable carbohydrates (Ashcroft, 1973a). Furthermore, inhibitors of metabolism such as dinitrophenol, oligomycin and antimycin A also inhibit insulin release (Georg, 1971; Malaisse, 1967; Grodsky, 1974).
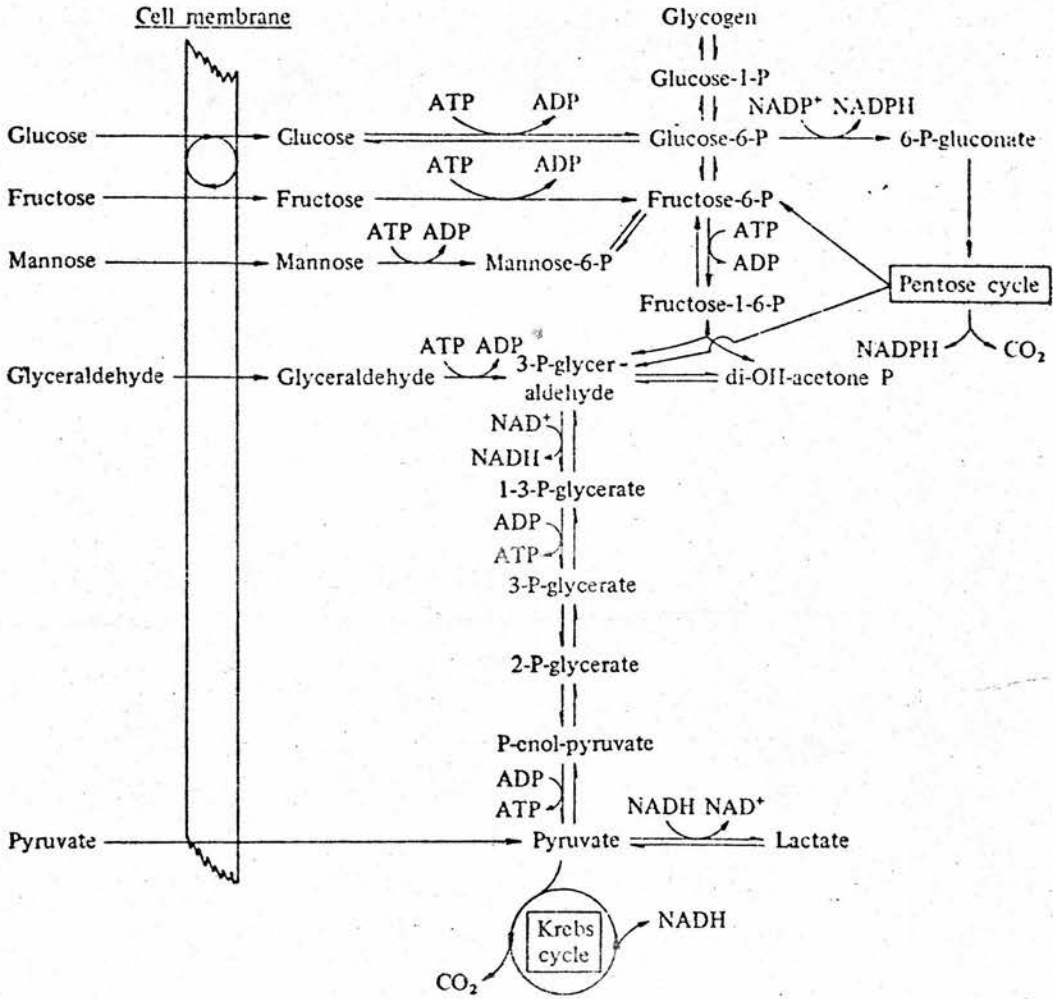
Figure 8 Schematic representation of metabolic pathways present in the pancreatic B cell. (From: A.E. Lambert, 1976).

(3)   Glucose penetrates rapidly into the B cell (Idahl, 1968; Matchinsky, 1968) and changes in glycolytic intermediates occur at least as early as the enhancement of insulin secretion by glucose (Idahl, 1973; Panten, 1973; Ashcroft, 1973b). Secretion correlates closely to glucose utilisation, glucose oxidation and islet oxygen consumption (Hellman, 1974). Reports that rat islet glucose metabolites do not change with extracellular glucose (Matchinsky, 1972) have not been confirmed by other workers, but in any event, concentrations of a presumptive insulin releasing metabolite may not need to show large variations if its rate of flux is the more important criterion for cellular mechanisms of insulin release.

The nature of the initiating metabolite is still speculative. The stimulation of insulin secretion by xylitol '*in vivo*' (Kuzuya, 1966) and '*in vitro*' (Montague, 1967 and 1968) led to the suggestion that the pentose cycle might provide the signal (Montague, 1969), particularly as the intra-islet concentration of 6-phosphogluconate correlated with insulin secretion. However, by far the largest fraction of glucose is metabolised via the Embden-Meyerhof pathway (Reese, 1973), and even the small amount of glucose which passes through the pentose cycle decreases with increasing concentrations of extracellular glucose (Snyder, 1970; Ashcroft, 1972a).

The action of glyceraldehyde in stimulating insulin release closely resembles that of glucose (Ashcroft, 1973a; Hellman, 1974d; Malaisse, 1974a), suggesting that the initiating metabolic signal is situated at or below the triose phosphate step of the glycolytic pathway (Hellman, 1974d), particularly as the stimulant action of glyceraldehyde is not inhibited by glucosamine or mannoheptulose (Ashcroft, 1973a; Hellman, 1974d). These two substances inhibit

islet glucose utilisation and glucose induced insulin secretion
(Ashcroft, 1972b), probably by blocking glucose phosphorylation, a
step circumvented by glyceraldehyde. Current evidence suggests that
phospho-enol-pyruvate is the most likely glycolytic intermediate
which triggers insulin release, if the "substrate-site" hypothesis
is the correct one. Intracellular concentrations of phospho-enol-
pyruvate (PEP) increase with raised extracellular glucose or
stimulation of the B cell with glyceraldehyde (Sugden, 1977). PEP
can elicit insulin release from B granules incubated with islet mem-
branes, while other glycolytic intermediates are ineffective (Davis
and Lazarus, 1976), and it is the only product of glycolysis able to
stimulate mouse adenylate cyclase (Capito, 1977). Finally, a rise
in B cell cytosolic calcium is thought to be closely linked to
insulin exocytosis, and observations of non-islet tissue show that
PEP stimulates calcium efflux from isolated liver, heart and islet
mitochondria (Chudapongse, 1973; Sul, 1976; Sugden and Ashcroft, 1978).

(d)   The "receptor-site" hypothesis

Although the "substrate-site" hypothesis has survived many
attempts at refutation, the glucoreceptor theory remains a subject
of conjecture. No decisive inferences can be established on the
indirect body of evidence available. The following observations
are pertinent to the possible existence of a "receptor-site" for
glucose.

(1)   Certain agents can inhibit insulin release without affecting
glucose metabolism (Cerasi, 1973), but these substances lack
specificity and their effects may be attributable to other com-
ponents of their action.

(2) Similarly, cytocholasin B stimulates insulin release while blocking glucose uptake and metabolism, but again, this effect may be due to its action on other intracellular events (Lacy, 1973; Levy, 1974).

(3) Insulin secretagogues can be non-metabolisable, like certain analogues of arginine and leucine (Fajans, 1974), or do not enter the B cell, as in the case of sulphonylureas (Hellman, 1973).

(4) The α-glucose anomer is a more effective stimulant of insulin secretion than the β anomer (Grodsky, 1974 and 1975), but previously the β anomer was reported to be more rapidly metabolised, since it caused a greater accumulation of glucose-6-phosphate in islets (Idahl, 1975). This implied a discrepancy between the more potent stimulant and cellular metabolism; the stereo-specificity providing good circumstantial evidence in favour of a glucoreceptor. However, although β-D-glucose is taken up more rapidly by the pentose pathway, phosphoglucose isomerase displays a preferential affinity for α-D-glucose, and in islets a relative metabolic block occurs in the handling of β-D-glucose-6-phosphatase (Malaisse, 1976). Thus the B cell possesses the same anomeric specificity for glucose metabolism and insulin release.

(5) The most convincing evidence for a glucose "receptor-site" comes from experiments using partially purified islet membranes. This membrane preparation was found to alter its UV absorbance in response to glucose, mannose and fructose, while non-stimulating sugars were ineffective (Price, 1973). However, no further characterisation of the membrane material has been achieved.

Amino Acids

Amino acids stimulate the release of both insulin and glucagon (Fajans, 1972; Gerich, 1974a), arginine being the most potent stimulator in humans, followed by lysine and leucine (Fajans, 1972). Amino acids evoke a greater glucagon response than an insulinogenic one, thus providing an effective mechanism for prevention of hypoglycaemia following a protein meal, when insulin secretion is also elicited (Unger, 1969). *In vitro*, amino-acid-stimulated insulin release is monophasic in the absence of glucose, but the addition of even small amounts of glucose (4-5 mmol/l) gives rise to a multiphasic response (Gerich, 1974a; Pagliara, 1974).

Free Fatty Acids and Ketones

Free fatty acids and ketone bodies do affect insulin (Balasse, 1968 and 1973; Crespin, 1969; Hawkins, 1971) and glucagon (Edwards, 1970; Gerich, 1974b) secretion, but the '*in vivo*' response cannot be typified from the inter-species and inter-experimental variations. '*In vitro*' studies show that short chain fatty acids, long chain fatty acids and ketones stimulate insulin release in the presence of non-stimulatory levels of glucose (Hawkins, 1971), and the possible rôle of these substrates is to ensure sufficient insulin release to prevent severe keto-acidosis in starvation (Seyffert, 1967).

Calcium

Calcium is known to link the process of stimulus recognition to hormone discharge in a wide variety of secretory tissues (Douglas, 1968). Certainly, the presence of extracellular calcium

is an essential factor in the initiation of B cell insulin release
by glucose (Hales, 1968a; Malaisse, 1973a), and intracellular calcium
plays a critical rôle in the regulation of insulin secretion
(Malaisse, 1973a). All insulinotropic agents affect the cellular
movements of calcium, with the exception of those which alter the
intracellular response to calcium through systems controlling the
migration and extrusion of secretory granules, such as cytochalasin B
(Malaisse, 1978). Furthermore, the divalent ionophore A23187
stimulates insulin release in the absence of glucose by facilitating
the entry of calcium into the B cell (Malaisse, 1973a). Detailed
studies on isolated islets using labelled ^{45}calcium have shown that
a glucose stimulus induces a rapid accumulation of radiolabelled
calcium from the extracellular medium (Malaisse-Lagae, 1971) and a
concomitant inhibition of calcium efflux from prelabelled cells
(Malaisse, 1973b). This sequence of events is followed by a sharp
rise in calcium efflux coincident with insulin release, although
cationic extrusion does not occur in the absence of extracellular
calcium (Malaisse, 1978). It is not clear whether these movements of
calcium are operated through voltage dependent ionic channels during
depolarisation of B cells, or by a modification of a native iono-
phoretic system in the cell membrane.

The concentration of cytosolic calcium appears to be the focal
trigger for the insulin secretory mechanism (Hellman, 1975). This is
determined partly by the influx and efflux of calcium across the cell
membrane and also by its uptake and release from intracellular com-
partments. X-Ray microanalysis of the calcium distribution in
frozen sections of islet tissue, have shown that B cell mitochondria,
storage granules and other organelles contain significant quantities

of calcium (Howell, 1975a and 1975b). The mitochondrial calcium
pool appears to be the most significant in terms of regulation of
hormonal release (Howell, 1975b). Thus, while potentiators of
insulin release do not alter the cellular uptake of calcium, they
may inhibit the accumulation or increase the efflux from intra-
cellular organelles to effect a rise in cytosolic calcium concentration
(Montague, 1977). This is unlikely to be a direct effect of secondary
stimuli, since some do not penetrate the B cell, and is probably
mediated by the cyclic AMP system. Glucose stimulation also affects
the movements of other ions in the islet cells; it provokes a sudden
release of inorganic phosphate (Freinkel, 1974), an increased uptake
of potassium (Howell, 1968) and an inhibition of potassium efflux
(Henquin, 1978). However, the significance of these ionic alterations,
or their relationship to the glucose-induced depolarisation of the
B cell membrane (Dean, 1970), is not clear.

Cyclic AMP

Cyclic AMP acts as a "second messenger" in secretory tissues
such as the thyroid and arterior pituitary, and is known to act as
an important intracellular mediator in glucose-induced insulin
release (Montague, 1977). The observation that cyclic AMP potentiated
insulin release in response to glucose originated from the finding that
glucagon was a potent insulinotropic agent, particularly during hyper-
glycaemia (Sharp, 1979). Glucagon acts by stimulating adenylate
cyclase, a membrane-bound enzyme catalysing the formation of cyclic
AMP from ATP (Howell, 1972b), and thus increasing intracellular cyclic
AMP levels. Subsequent studies have noted that substances which effect

a rise in intracellular cyclic AMP, such as β adrenergic agents, sulphonylureas, phosphodiesterase inhibitors, cholera toxin and exogenous cyclic AMP enhance glucose-induced insulin release, particularly at high glucose concentrations (Malaisse, 1967f; Montague, 1977; Sharp, 1979).

It is unlikely that a direct relationship exists between glucose, cyclic AMP and insulin release. The rise in intracellular cyclic AMP provoked by methylxanthines or cholera toxin is not associated with a significant enhancement of insulin secretion in the absence of a primary stimulus such as glucose (Montague, 1977).

After many contradictory observations, it is now generally agreed that glucose stimulation does increase B cell cyclic AMP levels (Sharp, 1979) providing extracellular calcium is present (Charles, 1975; Zawalich, 1975). However, theophylline has a far more pronounced effect on cyclic AMP than glucose, although it is not accompanied by a greater insulin release unless glucose is also present (Charles, 1973). This indicates that cyclic AMP positively modifies insulin secretion by having a potentiating effect on the B cell response to a primary stimulus such as glucose.

Adenylate cyclase activity provides an important focus of control of cyclic AMP levels. Glucose does not appear to directly affect adenylate cyclase in broken cell preparations (Kuo, 1973; Howell, 1973a),but its activity can be regulated through a variety of factors, including long-term substrate availability (Howell, 1973b), and various hormones (Kuo, 1973).

This significance of cyclic AMP in the sequential process which leads to insulin release appears to be a diverse interaction with cellular events. Cyclic AMP inhibits the uptake of calcium by

intracellular organelles (Howell, 1975a), so that it enhances the rise of cytosolic calcium if calcium efflux is simultaneously reduced by a glucose stimulus. Another important property of cyclic AMP is its ability to activate B cell protein kinases (Montague, 1972) which are functional in the effector system of hormonal secretion (Rasmussen, 1977; Gillespie, 1975). The means with which cyclic AMP levels are raised by glucose remain obscured by the rapid dynamics of the process, but the initial rise in cytosolic calcium may be instrumental, through activation of speculative calcium dependent regulator proteins (Sharp, 1979). These have been demonstrated in other tissues (Rasmussen, 1977), and may also be implicated in calcium induced extrusion of secretory organelles (Rasmussen, 1977; Sharp, 1979).

Discharge of Insulin

In common with all secretory products encased in granular membranes, insulin is released from the B cell by a process of exocytosis (Lacy, 1961). Calcium is a universal requisite for exocytosis (Rubin, 1970), which can be defined as the movement of membrane-bound storage granules to the cell border, fusion of the granule sac with the plasma membrane and emptying of the granule contents to the outside of the B cell (Lacy, 1970; Howell, 1971a; Orci, 1974). The dependency on calcium for insulin release and the presence of actin and myosin in secretory cells (Abramowitz, 1972; Blitz, 1974), may reflect the involvement of a contractile system for granule extrusion (Malaisse, 1973b), since an increase in cytosolic calcium is essential for exocytosis (Malaisse, 1970; Lacy, 1970). The so-called microtubular-microfilamentous network of B cells may constitute an active vectorial transport system for the translocation of B granules to the cell membrane, and their subsequent extrusion following glucose stimulation (Lacy, 1968; Malaisse, 1971; Orci, 1972; Lacy, 1975a).

Microtubules are linear, hollow rods composed of tubulin subunits (Olmsted, 1973; Montague, 1975a) with associated proteins (Sloboda, 1975), which appear as "fuzzy arms" on the completed microtubule surface (Dentler, 1975). It has been suggested that the observed saltatory granule movement towards the plasma membrane (Lacy, 1975b; Kanazawa, 1976; Malaisse, 1975) is achieved by binding of granules to the microtubular-associated proteins and directional orientation of granules along the microtubules to the cell surface (Ostlund, 1977). This idea is based on the inhibitory effects of

microtubule-disrupting agents, such as colchicine, vinblastine and vincristine, on insulin release (Lacy, 1968; Malaisse, 1971 and 1972). Unfortunately, these agents act on intracellular systems other than microtubular structure (Wilson, 1970; Mizel, 1972), which may also account for their effects. Calcium and cyclic nucleotides have been implicated in microtubular function (Gillespie, 1975), but their rôle is unknown.

Microfilaments are found in B cells as parallel arrays of lattice networks (Orci, 1972; Malaisse, 1972), and are thought to be made of actin, which has been identified immunologically in islet cells (Gabbiani, 1974). They constitute a web near the cell membrane, extending to surround and connect secretory granules, microtubules and the plasma membrane (Ostlund, 1977). Disruption of micro-filaments by cytochalasin B increases insulin secretion in rat islets (Lacy, 1973; Van Obberghan, 1973), so it has been proposed that the microfilamentous cell web normally acts as a barrier to granule release (Orci, 1972), but the effects of cytochalasin B are not essentially specific to actin-like structures (Sanger, 1972), and extrapolations from such experiments require more direct validation.

Present understanding of the microtubular-microfilamentous network has been incorporated into a theory of intracellular granule movement and exocytosis of insulin. It is suggested that a glucose stimulated rise in cytosolic calcium may trigger off the contraction of microfilaments (Lacy, 1970; Malaisse-Lagae, 1971b), resulting in extrusion of granules (Ostlund, 1977). Insulin secretion then continues by movement of granules along microtubules towards the cell surface. Models of the motive force for granule migration vary

from microfilamentous contraction (Ostlund, 1977), electrostatic force (Rebhun, 1972; Kinoshita, 1963) or rapid polymerisation of microtubules (Murphy, 1974), but so far the evidence for the involvement of the microtubular-microfilamentous network in insulin secretion is indirect.

The reverse process of endocytosis has been observed in B cells. Glucose stimulates an inward flow of vesicles that bud off from the plasma membrane, allowing membraneous material to be relocated into the cell concurrently with the addition of granule sacs to the plasma membrane during exocytosis (Orci, 1973).

THE STUDY OF THE ENDOCRINE PANCREAS "*IN VITRO*"

Early work on pancreatic endocrine function was based on estimations of plasma insulin and glucose in response to applied stimuli in animals and humans. The perfidious nature of this '*in vivo*' situation prompted attempts to construct '*in vitro*' models and amplify the details of pancreatic physiology and biochemistry.

Isolation of Endocrine Tissue

Anderson and Long (1947) were the first to isolate the rat pancreas, perfuse it with synthetic medium and demonstrate that the organ could be examined under controlled environmental conditions in the absence of complex neurohumoral influences. Insulin secretion has also been measured in dogs (Metz, 1960; Seltzer, 1962; Unger, 1967) and rats (Kilo, 1967) by perfusing the pancreas '*in situ*' with its nerve and blood supply intact, a method which was particularly useful for the investigation of the nervous control of insulin secretion (Kaneto, 1967; Frohman, 1967). The observation that small pancreatic pieces could provide viable experimental material (Bouman, 1960) enabled more systematic and detailed studies to be done on insulin release (Coore and Randle, 1964b) and synthesis (Taylor, 1964). The breakdown of insulin and destruction of islet cells by the proteolytic exocrine enzymes was minimised by addition of Trasylol (a trypsin inhibitor), so that minced pancreatic tissue could be used to elaborate on the dynamics of insulin release and the insulin response to a variety of agents (Burr, 1969a, 1969b, 1971a; Kikuchi, 1974).

The difficult problem for those attempting to study islets, and one of unknown teleological significance, is that the islets of Langerhans are embedded in exocrine tissue, and comprise only 1-2% of the pancreas. Furthermore, the damage often incurred by endocrine cells and hormones from the powerful proteolytic enzymes released by disrupted acinar tissue precludes accurate estimations of islet function, and efforts were therefore made to isolate islets from the surrounding material. Initially, individual islets were microdissected from pancreata of non-mammalian species such as the teleost fish (Lazarow and Cooperstein, 1951; Hellman, 1964) or chickens (Mikami, 1962), but it was not feasible to extrapolate from these experiments to mammalian systems, since these islets were more sensitive to amino acids than glucose, as measured by insulin synthesis (Lazarow, 1964) and secretion (Schirner, 1964). Although freehand microdissection of mammalian islets was possible (Hellerström 1964), it proved a difficult and tedious task unless the larger islets of the genetically obese hyperglycaemic mouse were used (Hellman, 1964). Ingenious microanalytical techniques (Lowry, 1953, 1954, 1957) were applied to B cells microdissected from freeze-dried pancreatic sections (Lacy, 1962; Smith, 1962; Kissane, 1963) and have been useful in studying islet cell metabolism (Matschinsky, 1968a, 1971a, 1971b).

Nevertheless, the revolutionary step for this investigative field was the development of a simple method for the isolation of intact islets in large numbers, by digestion of the exocrine elements with collagenase enzymes, which freed the islets from the acinar cells and allowed them to be collected (Moskalewski, 1965). Originally tried in guinea pigs, the method was later modified for

rats and other small mammals (Kostianovsky, 1966; Howell, 1966).
Collagenase digestion has become the most widely-used system for
islet isolation, and the corollary of experiments which followed
its inception has formed the basic understanding of pancreatic
endocrine hormone synthesis and secretion.

However, although the essential separation of exocrine and
endocrine elements has been tackled, a further problem arises in
that the islets are themselves composed of a collection of hetero-
geneous cells. Efforts have been made to prepare free cell
suspensions by gentle squashing (Petersson, 1966), collagenase
hyaluronidase treatment (Hellman, 1971), tryptic digestion
(Petersson, 1973), application of mild shearing forces (Krause, 1973)
or shaking islets in EDTA (Lernmark, 1974), but islet cells
separated in these ways have not been demonstrably viable despite
being ultrastructurally intact. A tangential solution to these
difficulties has been the use of islets enriched with one or other
cell type, such as the islets from the genetically obese hyper-
glycaemic mouse which contain a raised proportion of B cells
(Hellman, 1964). Alternatively, B cells have been selectively
destroyed by streptozotocin, a B cell cytotoxic agent, leaving islets
composed predominantly of A cells, so that the structure, metabolism
and glucagon release of A cells could be studied (Howell, 1971b;
Edwards, 1971, 1972). Subcellular fractions such as secretory
granules (Howell, 1969a; Coore, 1969; Lambert, 1970) and plasma mem-
brane fractions (Price, 1973; Lazarus, 1975; Lernmark, 1976) have
been used to look at intracellular events, but these investigations
are hampered by the lack of sufficient volumes of starting material,

and difficulties in achieving a satisfactory fractionation of
islet homogenates.

Organ Culture of Endocrine Tissue

Once the techniques outlined above had facilitated a detailed
analysis of the acute events which occur during the biosynthesis,
storage and secretion of insulin and other islet hormones, it was
clearly important to develop methods whereby the endocrine pancreas
could be maintained in organ culture. Such a system is a pre-
requisite in the investigations of the long-term influences of
environmental situations on islet behaviour, the mechanisms
involved in islet cell regeneration and growth and the accumulation
of sufficient tissue for islet transplantation.

Foetal chick pancreas was cultured by Kappel in 1926, and
Chen (1954) obtained morphological data of the growth and
differentiation of islet cells in cultures of foetal rat pancreas.
Definitive evidence of the continued growth and development of
islets of such cultures was later established (Schweisthal, 1963;
Clark, 1972; Hegre, 1973), and insulin release from cultured cells
was demonstrated (Wells, 1967; Vecchio, 1967; Lambert, 1970). Orci
(1969, 1970) showed the normal integrity of B cell organelles in
ultrastructural studies of these organ cultures, and the trans-
plantation of dispersed foetal or neonatal rat pancreas reversed
diabetes in inbred rats both before or after organ culture (Leonard,
1973; Hegre, 1976). An interesting and elegant approach for studying
the induction of differentiation of exocrine and endocrine pancreatic
tissue was the 'in vitro' culture of gut tissue dissected from 10-11
day rat embryos (Spooner, 1970).

The monolayer culture of cell suspensions of foetal or neonatal pancreata (Hilwig, 1968; Macchi, 1969; Chick, 1973a) has been used in basic studies of islet ultrastructure (Orci, 1973a), hormonal secretion (Lambert, 1972) and B cell replication (Chick, 1973b), despite the difficulties in manipulating such cell suspensions, or tissue attached to culture dishes. Further problems arise because foetal and neonatal pancreata are still differentiating and have a different sensitivity to secretagogues than adult islets, being more responsive to amino acids than glucose (Milner, 1972; Grasso, 1973). However, monolayer cultures of a human B cell tumour have been maintained for 2-7 months with demonstrable insulin secretory capacity (Chick, 1973c; Yip, 1973; Adcock, 1975), and the establishment of a functional B cell tumour line would be of great importance in studies of insulin synthesis and secretion. More recently, pieces of a human islet cell adenoma were cultured for 7 days without loss of glucose sensitive insulin secretion (Bone, 1977a).

Moskalewski originally established that isolated guinea pig islets could be "organ cultured" for three weeks with satisfactory morphological preservation (Moskalewski, 1965, 1969, 1976). The small size of isolated islets (1-5 μm diameter) reduces the problems of central tissue necrosis inherent in the culture of larger organs, and insulin release can still be elicited from B cells in islets cultured for 6-12 weeks (Andersson, 1967; Westman, 1970; Kostianovsky, 1972; Andersson, 1972). The cellular composition of islets remains unchanged (Andersson, 1973) and the glucagon secretory response to arginine (Segerström 1976) is still present after culture. Therefore, despite some changes in B cells during tissue culture, which

will be elaborated later, this has proved to be a highly successful method for long-term investigation or storage of islets.

Monolayer cultures of adult islets from guinea pigs, mice and rats have been obtained (Andersson, 1967; Kostianovsky, 1974), and used for cinemicrographic studies on the movement of B granules within the cytoplasm of B cells (Lacy, 1975), but this approach is limited by fibroblast overgrowth of islet tissue. Artificial capillary units are proving to be a new and powerful adjunct to islet cell culture, and have been used to maintain neonatal pancreatic tissue (Chick, 1975) and adult rat islets (Tze, 1977). Not only does such a unit mimic the '*in vivo*' microvascular system of cell perfusion and environment, but it also obviates the possibility of any hormones in the culture medium affecting the B cells. Culture medium is pumped through the tiny capillaries, and the islets lie between them; the system shows a surprising lack of fibroblast proliferation. A reversal of the diabetic state can be achieved in rats by connecting their vasculature to such a capillary unit containing isolated islets (Sun, 1976; Tze, 1977).

Effect of Thyroid Hormones on Pancreatic B Cells

The question of direct damage to the endocrine pancreas by thyroid hormones was investigated by Houssay, in his classic experiments on dogs (Houssay, 1944 and 1948). He demonstrated that diabetes could be produced by the administration of thyroid extracts to animals after sub-total pancreatectomy. The dogs had 4/5 of their pancreata removed, but were normoglycaemic. When fed thyroid extracts, they became diabetic (thyroid diabetes) and in some cases permanently so,

even after cessation of thyroid treatment (metathyroid diabetes). In both events, the B cells of the pancreatic remnants showed histological damage, although the changes were reversible in thyroid diabetes.

These investigations were not amplified until techniques had been developed for the study of pancreatic endocrine function '*in vitro*', and the more recent findings will be discussed later. Attention is currently focussed on the long-term mechanisms for the control of insulin secretion. Thyroid hormones may directly influence such a regulatory system, but it is difficult to exclude the peripheral metabolic effects of hyperthyroidism, which may themselves evoke changes in insulin secretion. The following work employs an '*in vitro*' model whereby isolated islets can be maintained viable and functionally intact in tissue culture, so that it is possible to investigate the interaction between thyroid hormones and pancreatic islets in a controlled environment.

CHAPTER 2

MATERIALS AND GENERAL METHODS

Reagents and Apparatus

^{125}Iodine

$(\alpha^{32}P)ATP$

L-(4-5-3-H)leucine
}
Radiochemical Centre,

Amersham, Bucks.

Collagenase

L-Thyroxine

PPO (Diphenyloxazole)

'Analar' chemicals
}
Sigma (London) Chemical Co. Ltd.,

Poole, Dorset.

Kingston-upon-Thames, Surrey.

D-Glucose

Crystallised albumen

Ovalbumen

Theophylline hydrate

Triton X-100

Folin and Ciccalteu's reagent

Toluene
}
BDH Chemicals Ltd.,

Poole, Dorset.

Collagenase

Creatine phosphokinase

Phosphocreatinine
}
Boehringer Co. Ltd.,

Ealing, London

Insulin binding reagent

Human insulin standards
}
Wellcome Reagents Ltd.,

Beckenham, Kent.

Bovine serum albumen
}
Armour Pharaceutical Ltd.,

Eastbourne, Sussex.

Sterile disposable pipettes

Sterile petri dishes
}
Sterilin Ltd.,

Teddington, Middlesex.

Tissue culture medium 199   }   <u>Gibco</u>,

New-born calf serum   Paisley, Scotland.

3-Isobutyl 1-methyl xanthine   }   <u>Searle</u>,

                                   High Wycombe, Bucks.

Sephadex G-50 fine   }   <u>Pharmacia</u> (GB) Ltd.,

                                   Ealing, London.

Glass fibre filters   }   <u>Whatman</u> Biochemical Ltd.,

                                   Maidstone, Kent.

Streptomycin   }   <u>Glaxo</u> Labs. Ltd.,

                                   Greenford, Middlesex.

Animals

All animals used were female Sprague-Dawley albino rats of a weight range of 220-250 g. They were maintained on Spratts small animal diet (no. 1) comprised of 48% carbohydrate, 22% protein and 4.5% fat, and had free access to food and water up to the time of sacrifice.

Incubation media

The incubation medium used for the following studies was a bicarbonate-buffered simple salts solution (Gey and Gey, 1936), as described below:-

Salt	Concentration	
	g/L	mm
NaCl	6.5	111.0
KCl	0.37	4.97
$NaHCO_3$	2.27	27.0
$MgCl_2 . 6H_2O$	0.21	1.03
KH_2PO_4	0.03	0.22
$MgSO_4 . 7H_2O$	0.07	0.28
$Na_2HPO_4 . 2H_2O$	0.113	0.63
$CaCl_2 . 2H_2O$	0.294	2.0

In general, the medium contained 5.5 mmol/l D-glucose unless otherwise stated. Any additions made to the medium are described in the text. The medium was maintained at pH 7.4 by gassing with 95% O_2: 5% CO_2 immediately before use, and exposure to atmospheric air was kept to a minimum.

Tissue Culture Media

Tissue culture medium 199 with Hanks salts and 0.35 g/l sodium bicarbonate (Morgan, 1950) was used for all culture experiments. It was stored in sterile bottles at $+4^{\circ}C$ and supplemented with benzyl-penicillin (100 μ/ml) and streptomycin (0.1 mg/ml). The glucose concentration of the medium was 5.5 mmol/l. New-born calf serum was added to the medium immediately before use to give a concentration of 5%, under sterile conditions.

Thyroxine

L-Thyroxine was dissolved in 2.5×10^{-3} M-NaOH, and added to the incubation medium or the tissue culture medium to give a final concentration of 0.1 or 1.0 μg/ml. Media for experimental controls contained only an identical amount of the dilute NaOH.

Isolation of Islets of Langerhans

The technique used for the isolation of rat islets was originally described by Moskalewski (1965), and is essentially a modification of the collagenase digestion procedure (Howell and Taylor, 1966). The animal was sacrificed by cervical dislocation, and the pancreas with the spleen attached was rapidly removed into chilled incubation medium. Once the spleen and any excess fat had been removed from the pancreas, it was distended by subcapsular injection of medium through a 25 G needle, and finely chopped into small fragments. The pieces were centrifuged briefly for 2 minutes, and any remaining fat was drawn off with the supernatant. The tissue was

transferred with an approximately equal volume of medium into a 25 ml conical flask and collagenase was added. The collagenase consisted of a mixture of Boehringer and Sigma preparations in equal proportions; in general, 12 mg of enzyme was used per two pancreata, although this varied slightly with different batches of collagenase. The stoppered flask was incubated at 37° C in a rapidly shaking water bath (200 cycles/min) for approximately 10 minutes. The end-point of digestion was judged by examination of small aliquots of the tissue under a dissecting microscope at 10x magnification.

When sufficiently digested, the tissue was spun down gently in a bench centrifuge, the supernatant was discarded and the deposit was washed twice with incubation medium to remove any remaining collagenase.

Aliquots of this material were transferred into incubation medium on a plastic petri dish with a black painted base, and examined under a dissecting microscope. The islets appeared as discrete white spheres against a translucent background of acinar tissue. The islets were separated from the exocrine fragments using a finely drawn out pasteur pipette and collected in a test tube containing fresh medium.

They were then "re-picked" to allow complete separation from exocrine elements, and incubated in fresh medium for 30 minutes at 37° C prior to use in an experiment, to allow stabilisation of insulin release after the isolation procedure. In general, two pancreata were processed together, and approximately 150-200 islets could be isolated from each pancreas.

Insulin Release from Islets During One Hour Batch Incubation

The secretory capacity of isolated islets was assessed using
short-term periods of incubation with various concentrations of
glucose, and measuring the insulin released into the incubation
media after 1 hour. Theophylline, a phosphodiesterase inhibitor
(Butcher, 1962), was also used to stimulate release. Following 30
minutes pre-incubation as described in the islet isolation procedure,
groups of three islets were transferred to flat-bottom tubes con-
taining 0.6 ml of bicarbonate-buffered medium, pH 7.4 (Gey and Gey,
1936) together with 1 mg/ml albumen, 2, 6 or 20 mmol/l glucose with or without
5 mmol/l theophylline. The tubes were gassed with 95% O_2, 5% CO_2,
stoppered, and incubated for one hour at 37° C. At the end of this
period, 100 µl samples of the incubation media were gently withdrawn,
taking care not to remove the islets, and diluted with 900 µl of
phosphate-buffered medium (as described for insulin assay). The
samples were then assayed for insulin. The islets were considered to
be viable if the controls showed a significantly increased insulin
secretion to the high glucose concentration, when compared to basal
levels at 2 mmol/l glucose, and data was only used from experiments
where this was the case. The mean insulin secretion from islets was
relatively constant for a series of experiments, so that results from
experiments carried out on different days could be accumulated
together.

ASSAY OF INSULIN

(a)   Principle

Measurement of insulin throughout this study was by radio-
immunoassay (Yalow and Berson, 1960; Hales and Randle, 1963). This

entails the formation of insulin anti-insulin antibody complexes
which can be separated by filtration through glass fibre discs. The
anti-insulin antibody is mixed with the sample to be assayed and a
known amount of radio-iodinated pure insulin. Both labelled and
unlabelled hormones compete equally for antibody binding sites during
a period of incubation, and once equilibrium is reached, the insulin
antibody complex can be separated by filtration and counted for radio-
activity. Radio-labelled insulin will be present in the filtrate in
inverse proportions to the insulin concentration of the unknown
sample. Thus, the amount of insulin in the sample can be estimated
from a standard curve of known concentrations of insulin plotted
against counts of radioactivity in the filtrate.

(b) Reagents

1. Phosphate buffer (50 mM). This was used for dilution of
iodinated insulin, insulin standards and unknown samples, and for
washing the filtrate. It contains, per litre:-

Na_2HPO_4	5.7 g
NaH_2PO_4	1.56 g
Bovine Serum Albumen Fraction V	1.0 g
Ethylmercurithiosalicylate	0.25 g

2. Anti-insulin antibody, or insulin binding reagent, is a
freeze-dried stable preparation available commercially. It is raised
against bovine insulin and precipitated with anti-guinea pig globulin.
It has previously been shown not to discriminate significantly between
rat and human insulin over the concentration range 0-4 ng/ml used in
these assays (Montague, 1969a). Each bottle of binding reagent was
reconstituted with 8 ml distilled water.

3. Insulin standards were prepared from crystallised human insulin dissolved in phosphate buffer to give standard solutions of 0.5 to 6.0 ng/ml of insulin. These could be stored at $-20°$ C for several months without any detectable loss of immunoreactivity. Rat insulin is comprised of two structurally different molecules (Smith, 1966; Humbel, 1972) which are synthesised in approximately equal amounts (Steiner, 1967), with a very similar biological activity (Taylor, 1964). The structural variation is that of different amino acids at positions 9 and 29 on the B chain where methionine (form II) is substituted for lysine (form I) (Steiner, 1972) but it is believed to have little effect on immunological specificity. For the purpose of this study, it was assumed that the two insulins are not significantly different in their release characteritics or their immunoreactivity.

4. ^{125}Iodinated ox-insulin was prepared by a modification (Ewart and Tylor, 1971) of the chloramine T procedure for iodination of human growth hormone (Hunter and Greenwood, 1962) as described below.

(c) Preparation of ^{125}I-labelled ox-insulin

Insulin can be iodinated at both tyrosyl and histidinyl residues, providing that iodine incorporation is kept low to avoid molecular damage and consequent loss of immunological activity. The procedure was carried out in a fume cupboard with careful pre-cautions against radioactive contamination. 1 mCi of Na ^{125}I in 10 μl of solution was added to 20 μl of 50 mM phosphate buffer, albumen free, containing 2.5 μg insulin, in a small assay tube. The reaction was started by the addition of 20 μl of a freshly prepared solution of chloramine T (3.5 mg/ml in 50 mM phosphate buffer).

This was shaken for 10 seconds and the reaction was immediately stopped by addition of 100 µl of sodium metabisulphite solution (1.75 mg/ml in 50 mM phosphate buffer), again with shaking. 100 µl of KI solution (10 mg/ml) and 100 µl of 5% albumen, both in 50 mM phosphate buffer, were added, and the contents of the reaction tube were mixed.

[^{125}I]-Insulin was separated from ^{125}I-iodine and [^{125}I]-proinsulin by gel filtration. The contents of the reaction tube were fractionated on a Sephadex G-50 column (1 x 50 cm) using 50 mM phosphate buffer, with albumen, as eluant. 10 µl of each 1 ml fraction collected were assayed for radioactivity (Fig. 9). Fractions containing ^{125}I-insulin (peak 2) were pooled, diluted with eluant buffer to give an activity of approximately 2 x 10^6 cpm/ml, and stored at -20°C in small aliquots.

(d)   Procedure

100 µl of unknown sample of insulin standard solution was transferred to bacteriological tubes in triplicate, 100 µl of reconstituted insulin binding reagent was added to each tube, the tubes were shaken on a whirlimixer and allowed to equilibrate for 4 hours at 4°C. ^{125}I-Labelled insulin was diluted with 50 mM phosphate buffer to give approximately 1.5 x 10^5 cpm/ml, and 100 µl was added to each tube. After thorough mixing, the tubes were stored at 4°C for a minimum of 18 hours. Following this second incubation, the insulin-antibody complex was separated by filtration under vacuum through glass fibre filters. The tubes and the precipitate were each washed once with ice-cold phosphate buffer, containing 0.5% ovalbumen. The filter discs were transferred to plastic tubes and their radioactivity was counted directly in a gamma counter. A
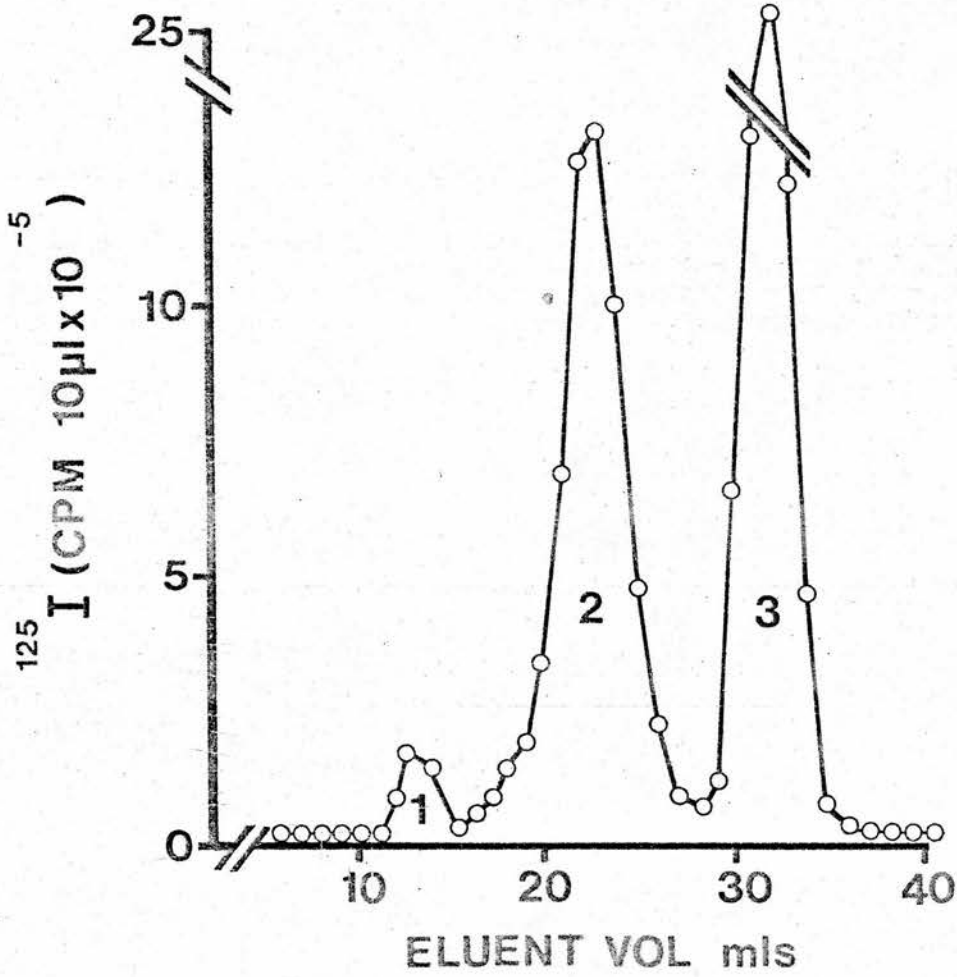
Figure 9 Gel filtration profile of iodinated insulin. Insulin
(peak 2) was iodinated as described in the text, and separated from
[125]iodide (peak 3) on a Sephadex G-50 column, using 50 mM phosphate
buffer as eluant. (Courtesy of Dr. K. Pedley).

standard curve was drawn of ng/ml insulin plotted against radioactivity in cpm (Fig. 10), and the concentration of insulin in the samples was then estimated from the graph, as a mean of triplicate observations.

Tissue Culture of Islets

The method of culture of isolated islets of Langerhans was described by Andersson and Hellerström (1972) and the optimal conditions for islet survival have been further investigated in our laboratory (Whittaker, 1977).

Islets were isolated and pre-incubated for 30 minutes in bicarbonate-buffered medium. Forty to fifty islets were then transferred to 5 ml of tissue culture medium 199 (with supplements as described above) in 5 cm plastic petri dishes. L-Thyroxine was added to test dishes to achieve a final concentration of 0.1 or 1.0 μg/ml. Each experiment included a control group of islets in culture medium to which only dilute NaOH had been added. The islets were then incubated at 37°C, in a humidified atmosphere of 5% CO_2:95% air, for 24 hours. Subsequently the islets were again pre-incubated for 30 minutes in bicarbonate-buffered medium prior to their use in experiments, to wash them free of insulin and serum accumulated in the culture medium, and also to accustom them to a new medium.

The concentration of thyroxine during tissue culture of islets was monitored over the 24 hour period. The total thyroxine content of the samples was determined by immunoassay at the Department of Biochemistry, Royal Sussex County Hospital, Brighton, Sussex; the free thyroxine and free triiodothyronine levels were measured by equilibrium dialysis by Dr. A. B. Kurtz, Department of Nuclear Medicine, Middlesex Hospital, London.
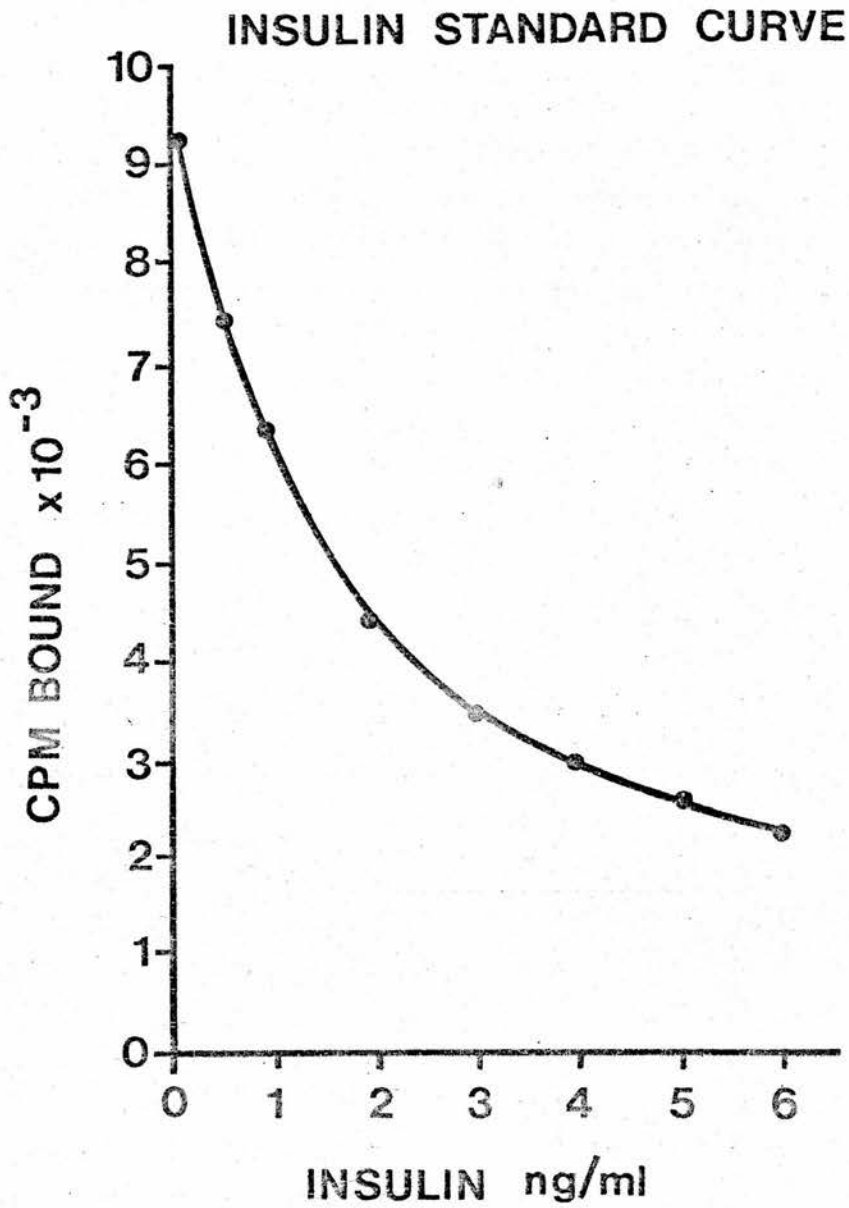
Figure 10 Standard solutions of insulin dissolved in 50 mM phosphate buffer were assayed according to the procedure described in the text. Each point is the mean of triplicate observations.

Measurement of Radioactivity

^{125}I was counted directly in a Nuclear Chicago Automatic Gamma System (Model 1195); with a counting efficiency of approximately 55%. Tritium was counted in vials in a scintillation solution of toluene 700 ml: Triton X-100 300 ml: PPO 5 g using a Beckman LS 233 liquid scintillation spectrometer, with a counting efficiency of approximately 25%.

Expression of Results

Results are expressed throughout as the mean ± the standard error of the mean (SEM) of the number of observations shown in parentheses. Differences between means were assessed by the use of Students 't' test or analysis of variance, and were considered statistically significant when the calculated P value was less than 0.05 (Winer, 1964).

CHAPTER 3

THE DIRECT EFFECT OF THYROID HORMONES ON FRESHLY ISOLATED ISLETS

Introduction

Some hormones directly influence insulin secretion, while others modify the insulin response to stimuli '*in vivo*', presumably by an indirect effect through long-term changes in the islet environment. Glucagon, corticotropin (ACTH), thyrotropin (TSH) and the prostaglandins have been demonstrated to augment the '*in vitro*' insulin response to glucose from pancreatic pieces or isolated islets in short-term incubations (Malaisse, 1967b; Johnson, 1973), possibly by activating the adenylate cyclase system (Howell, 1973a; Kuo, 1973). Conversely, somatostatin inhibits insulin secretion from isolated islets (Efendic, 1975; Claro, 1977), an effect that is more apparent if islets are exposed to the hormone for longer periods, in tissue culture (Wollheim, 1977).

The short-term effect of thyroid hormones on the glucose stimulated insulin release from isolated islets has not previously been documented. Malaisse and colleagues (1967c) concluded that the addition of thyroxine to the incubation media did not alter the insulin secretion from rat pancreatic pieces over 90-minute periods. Nevertheless, the authors have noted that the enzymes released by the exocrine elements of pancreatic pieces (Malaisse, 1967d) may lyse both test hormones and the insulin secreted into the medium (Malaisse, 1967b), and the acinar tissue will itself metabolise thyroxine. It was therefore important to establish whether any immediate changes in islet insulin secretion were precipitated by thyroid hormones.

The two prevailing means of investigating insulin secretion by

isolated islets are 'continuous perfusion' and 'static batch incubation'. The former method, whereby fresh incubation medium is continuously pumped over the islets, is technically more exacting, but invaluable in gauging the post-stimulatory metabolic changes and ionic fluxes during very short exposures to stimuli, where the immediate dynamic effects are more pronounced than long-term ones. Static batch incubation of islets is the simplest and most widely used technique for measuring insulin release. It has two major drawbacks. Firstly, any insulin leaking out from dead or damaged cells accumulates in the static incubation medium together with the secreted insulin, and may distort eventual conclusions drawn from the experiment; this is of doubtful significance if small numbers of test islets are used together with controls. Secondly, the system is insensitive to the multiphasic nature of insulin release and the inhibitory effects of exogenous insulin. The negative feedback of insulin on further hormonal secretion is detectable after approximately one hour of incubation (Malaisse, 1972). However, where very transient changes are not of primary importance and incubation periods are limited to 60 minutes, this particular method provides a simple and readily available approach, and has been adopted in the following work. The experiments described in this chapter were set up to investigate the possibility of a direct short-term effect of thyroid hormones on glucose-stimulated insulin secretion from isolated islets of euthyroid rats.

Methods

Details of the methods are described in Chapter 2. The islets were isolated from adult rats by the collagenase procedure and pre-

incubated for 30 minutes at 37°C. Islets were then batch incubated
in groups of three in bicarbonate-buffered medium containing 2, 6 and
20 mmol/l glucose with or without theophylline (5 mmol/l), for 60
minutes at 37°C. L-Thyroxine was added to the incubation media of
the test groups prior to incubation, to give a final concentration of
1 µg/ml. All experiments comprised a test group and a control group
at each concentration of glucose. At the end of the one-hour period,
samples of incubation media were withdrawn and assayed for insulin.
Samples could be stored at -20°C for several weeks with no apparent
loss of insulin immunoreactivity.

Results

The results are shown in Figure 11. Control islets incubated
without thyroxine show a characteristic and significant enhancement
of insulin release with increasing concentrations of glucose. Maxi-
mal stimulation of insulin release with 20 mmol/l glucose and
theophylline resulted in a 2.4-fold rise of insulin secretion above
basal levels (at 2 mmol/l glucose). A similar pattern was also
observed from the islets incubated with thyroxine, with significant
enhancement of secretion at 20 mmol/l glucose, and a 2.2-fold rise in
basal insulin secretion on maximal stimulation. No significant differ-
ences were demonstrated between the glucose-stimulated insulin
secretion of control islets and islets incubated with thyroxine.

Discussion

One of the earlier criticisms of the collagenase digestion method
for islet isolation was that the proteolytic enzymes contained in
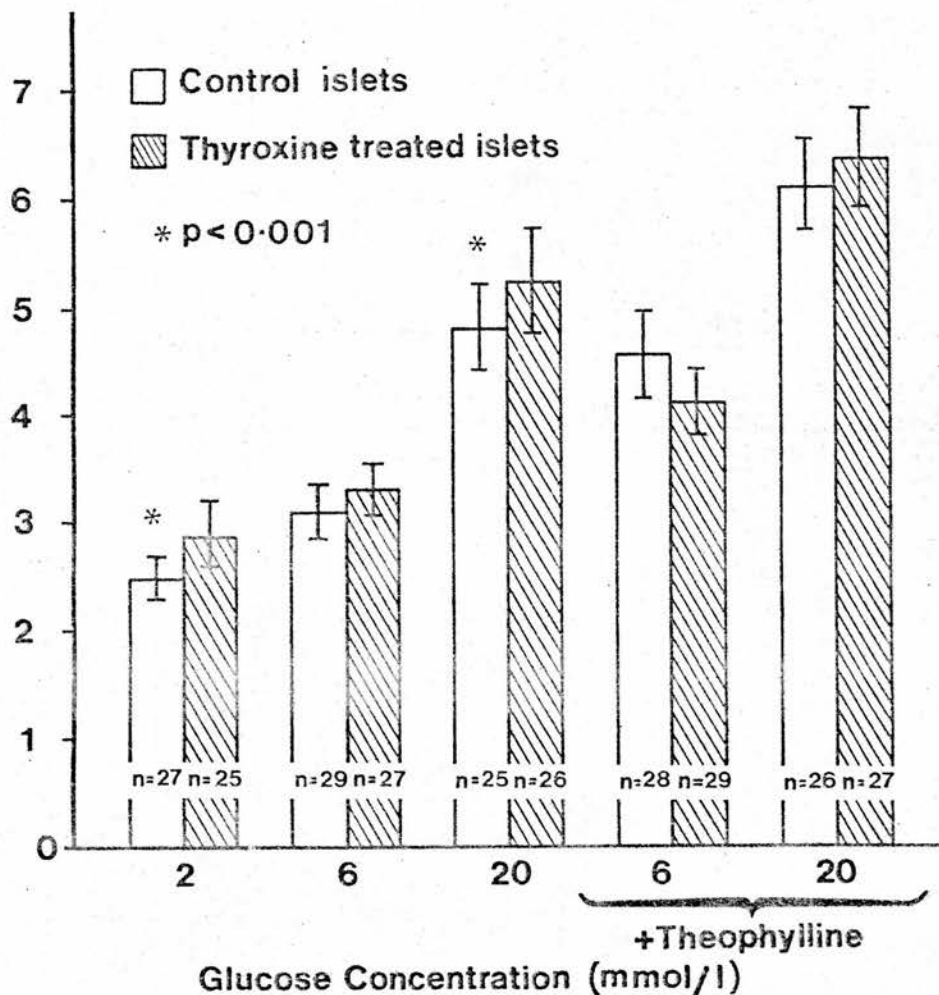
Figure 11 Direct effect of thyroxine (1 μg/ml) on insulin secretion
from isolated rat islets. Islets were incubated for 60 min at 37°C
in bicarbonate-buffered media containing thyroxine (1 μg/ml), theo-
phylline (5 mmol/l) and glucose in the concentrations shown. Each
result is the mean insulin secretion in ng insulin/islet/hour ± SEM
of the number of observations shown (n).

collagenase preparations may damage islets and alter their functional
integrity. The ability of B cells to secrete insulin in response to
changes of secretagogue concentration is probably the most indicative
feature of islet viability. Islets which can modulate their insulin
release must have an intact cell membrane and operative intracellular
organelles. Experiments showing that collagenase isolated islets
exhibit a similar response to agents such as glucose, as islets in rat
or rabbit pancreatic slices (Malaisse, 1967a; Howell, 1967) demonstrate
that collagenase does not adversely affect this parameter of B cell
function. Studies of the ultrastructure of collagenase isolated islets
show normal cells with no evidence of swollen mitochondria or cell
membrane disruption (Lacy, 1967) and this has been confirmed in our
laboratory (Fig. 12). Comparisons between microdissected and collagen-
ase prepared islets show close similarities in oxygen consumption
(Hellerström, 1968) and glucose metabolism (Montague, 1968; Matchinsky,
1968). Evidently, the collagenase isolation procedure is not del-
eterious to islet function providing the cells are not overexposed to
the enzymes and the end-point of digestion is carefully assessed.

The results show that the islets were responsive to glucose and
theophylline, and the maximal levels of insulin secreted compare
favourably with those of other studies (Whittaker, 1977). The islets
treated with thyroxine behaved very similarly. In conclusion, these
experiments confirm the former studies of Malaisse (1967c) on
pancreatic pieces, that there was no immediate effect of thyroid hor-
mones at the concentrations used on insulin secretion during short-
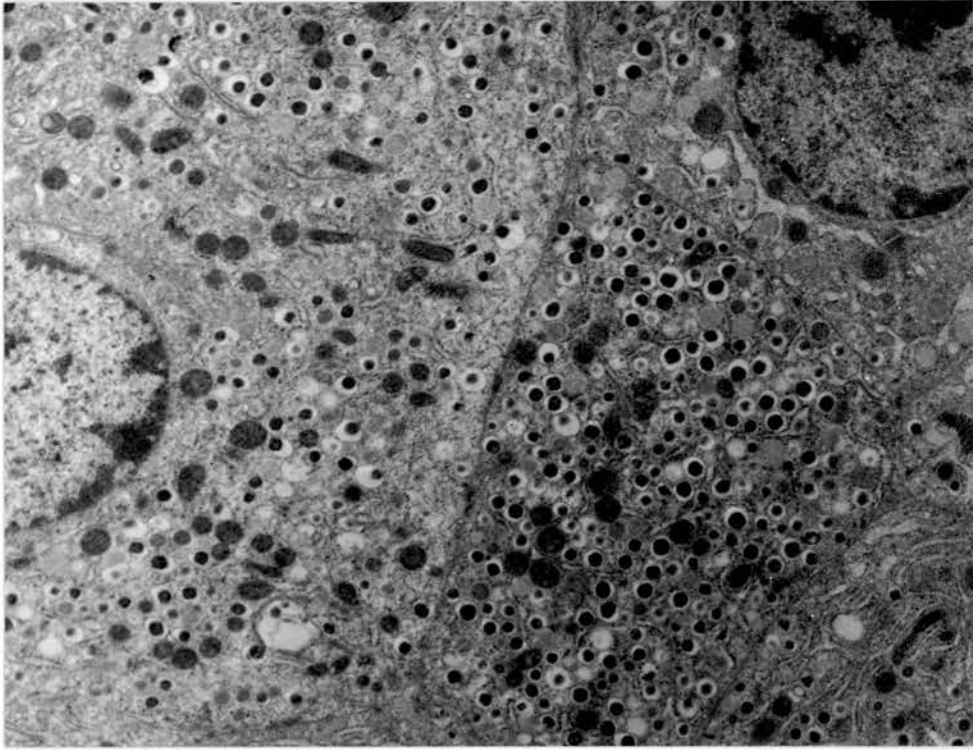term periods of exposure to the hormones.

Figure 12 (x9000 magnification). Electron micrograph of B cells from an islet immediately after isolation by the collagenase method, as described in the text.

CHAPTER 4

THE LONG-TERM DIRECT EFFECT OF THYROID HORMONES ON PANCREATIC INSULIN SECRETION

Introduction

The effects of experimentally produced hyperthyroidism on subsequent insulin secretion were originally described by Malaisse (1967c). He noted that *in vitro* insulin secretion and pancreatic islet content were reduced by pretreatment of rats with thyroxine, and postulated a long-term deleterious effect of thyroid hormones on insulin secretion, possibly through changes in insulin biosynthesis. Lenzen and colleagues subsequently persued a variety of these aspects in more detail, using isolated perfused pancreata from rats with experimental hyperthyroidism following thyroid hormone administration. In these preparations, thyroid hormones were found to inhibit the second phase of insulin release in response to glucose concentrations over 8.3 mmol/l (Lenzen, 1975; Lenzen, 1978a), although first phase release (which in fact represents only 1-2% of the insulin secreted over a one-hour period) was unaffected (Lenzen, 1976a). The reverse situation, using pancreata from hypothyroid rats following thyroidectomy or propylthiouracil treatment, resulted in an augmentation of glucose induced insulin secretion above that of normal (Lenzen, 1976b). However, these experiments fail to discriminate between the possible direct effects of thyroid hormones on pancreatic B cells and the secondary physiological and metabolic disturbances of hyperthyroidism, which may damage B cell function.

Modern developments in organ culture techniques for endocrine pancreatic tissue have created the potential to simulate an *in vivo* environment which excludes the effects of the numerous variables arising

in a living animal. Thus, it is possible to examine the long-term direct effects of any one factor on pancreatic endocrine secretion whilst maintaining the stability of cellular surroundings. Careful observations of B cells in tissue culture have established that they retain the qualitative features which characterise their viability, so that such *in vitro* models can be compared to *in vivo* conditions.

Andersson has elaborated on Moskalewski's findings of good morphological preservation of guinea pig islets maintained in tissue culture for three weeks (Moskalewski, 1965, 1969 and 1970), with microscopical details of mouse islets. The islets were histologically normal after twelve days in tissue culture. There was no evidence of necrotic areas, and the islets merely showed a diminution of capillary spaces and a lack of contaminating fibroblasts (Andersson, 1972). Electron micrographs confirmed the absence of structural derangements in A_2 or B cells (Andersson, 1974), and the cellular composition of the islets was unchanged (Andersson, 1973a). The continuing production of insulin has been shown in cultured rabbit islets (Boder, 1969) and monolayer cultures of B cells, several weeks after explantation (Andersson, 1970), with retention of the regulatory mechanisms of insulin release (Lambert, 1971). Measurements of basal rates of islet oxygen consumption and glucose oxidation were higher in cultured islets, but showed a similar increase in response to a raised extracellular glucose as did freshly isolated islets (Andersson, 1972). Mitoses were observed in islets in tissue culture (Andersson, 1973b) and DNA synthesis can be demonstrated with the addition of H_3-thymidine to the culture medium (Andersson, 1975). Other aspects of islet function and metabolism which retained similar characteritics in tissue culture were adenylate cyclase activity (Howell, 1973b), the levels of ATP and

glycogen, and the activity of the glucose phosphokinases
(Andersson, 1974b).

The major changes which occur during tissue culture are a
reduction of islet insulin content (Andersson, 1972; Ziegler, 1974),
and a quantitative decrease in insulin secretion and biosynthesis,
particularly in response to raised concentrations of glucose
(Andersson, 1974). This co-incides with a relative degranulation of
B cells during culture (Moskalewski, 1965, 1969; Westman, 1970;
Andersson, 1972), although A_2 cells are little, if at all, affected
(Andersson, 1974 ; Moskalewski, 1976). Nevertheless, mouse islets
cultured for 8 months have remained functional (Andersson, 1978), as
have human islets, which still retain a biphasic insulin release to
an acute glucose load after several months of tissue culture
(Andersson, 1977).

Isolated islets from euthyroid animals were used in the
following experiments in a tissue culture system, to explore any long-
term direct effects of thyroid hormones on glucose stimulated insulin
secretion.

Methods

The details of the methods are described in Chapter 2. The
islets were isolated from normal adult rats. Following a 30 minute
pre-incubation period, groups of 40-50 islets were transferred to
petri dishes containing tissue culture medium (t.c.m.) 199. L-Thyroxine
0.1 or 1.0 µg/ml was added to the test dishes, each experiment
including a control group of islets in hormone-free medium, and the
islets were cultured for 24 hours. Subsequently the islets were again

pre-incubated for 30 minutes in bicarbonate-buffered medium prior to
their use in the experiments. Glucose stimulated insulin secretion
was then determined by batch incubation of islets as previously
described. After one hour, samples of incubation media were with-
drawn and immunoassayed for insulin. It should be noted that
thyroxine was not added to the incubation media during these secretion
tests performed after a period of culture.

The concentration of thyroxine during tissue culture of islets
was monitored over the 24-hour period. 0.5 ml of culture media were
taken at 0, 20 and 24 hours and assayed for total thyroxine content
and free thyroxine levels, by immunoassay and equilibrium dialysis
respectively.

Results

Islets were cultured for 24 hours with thyroxine and their
subsequent insulin secretory response to 2, 6 or 20 mmol/l glucose
with or without theophylline is shown in Fig. 13 and 14. Both
groups of control islets were generally less responsive to glucose
than freshly isolated islets. In addition there was some variability
between the control values of insulin secretion shown in Fig. 13
and those in Fig. 14, although the islets had undergone the same
experimental procedure. The exact reasons for this are not known,
but it may reflect the influence of different batches of collagenase
(Toledo-Pereyra, 1979) and calf serum on the secretory response of
islets after tissue culture (Whittaker, 1977). For this reason,
each experiment was always accompanied by a control group of islets
isolated at the same time as thyroxine treated islets and cultured in
identical conditions.

Figure 13 Insulin secretion from isolated islets cultured for 24 hours
with 0.1 µg/ml thyroxine. Islets were transferred from the culture
media and incubated for 60 minutes at 37°C in bicarbonate-buffered
media containing theophylline (5 mmol/l) and glucose in the concentrations
shown, but no thyroxine. Each result is the mean insulin secretion in
ng insulin/islet/hour ± SEM of the number of observations shown (n).

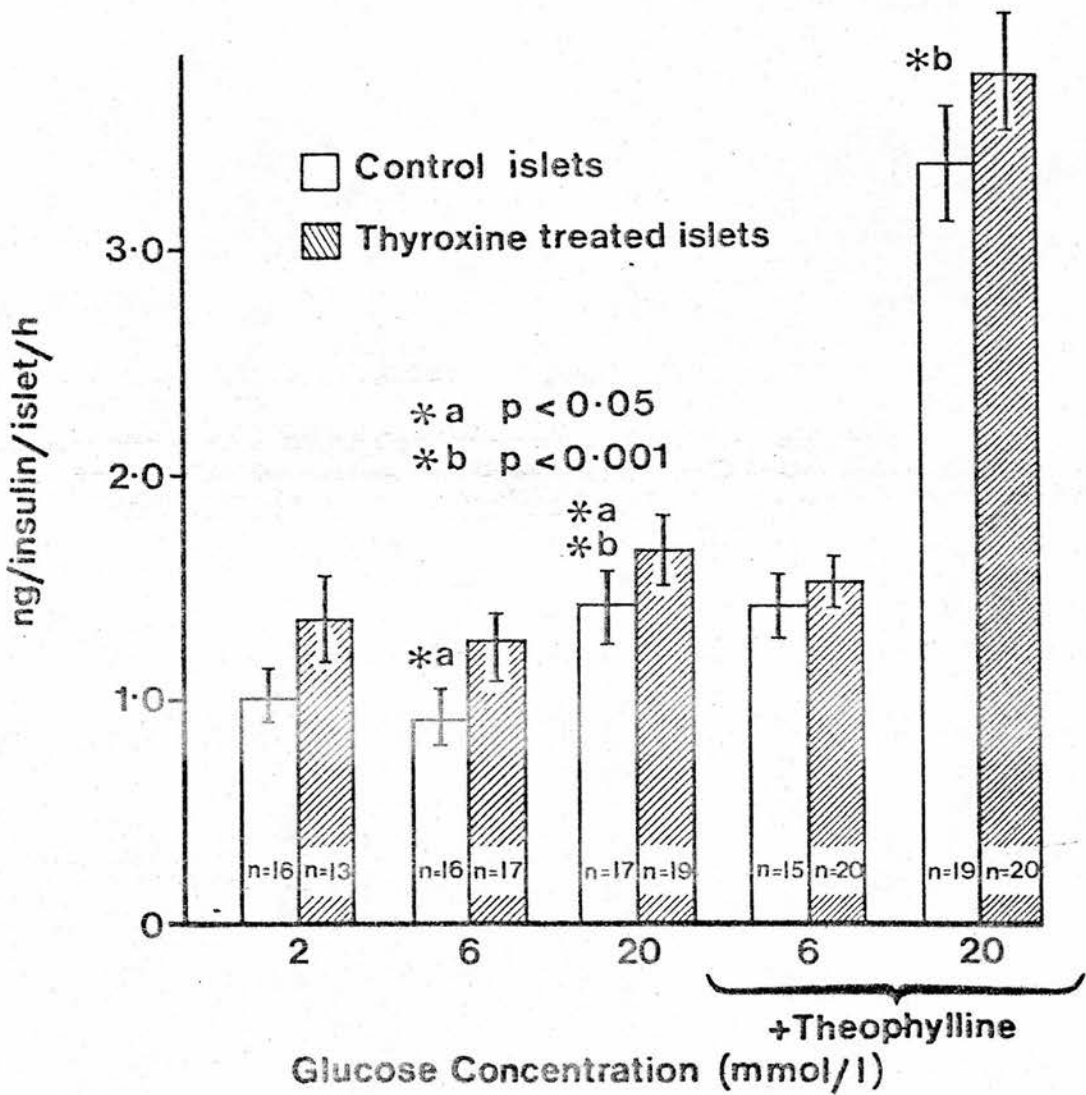Figure 14 Insulin secretion from isolated islets cultured for 24 hours
with 1.0 μg/ml thyroxine. Islets were transferred from the culture
media and incubated for 60 minutes at 37°C in bicarbonate-buffered
media containing theophylline (5 mmol/l) and glucose in the concentrations
shown, but no thyroxine. Each result is the mean insulin secretion in
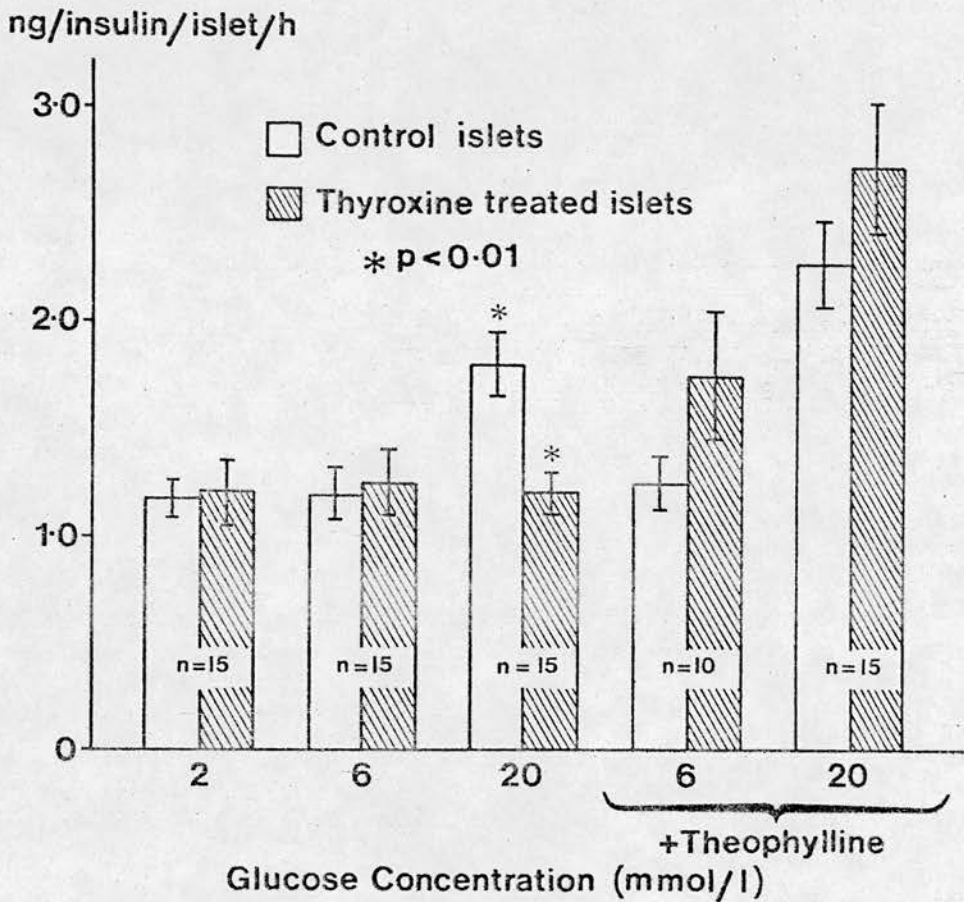ng insulin/islet/hour ± SEM of the number of observations shown (n).

Fig. 14 shows that the islets cultured with 1.0 µg/ml thyroxine did not respond to an increase in glucose concentration from 2-20 mmol/l, and this inhibition of insulin release was highly significant compared to cultured control islets ($p < 0.01$). The addition of theophylline to the incubation media during tests of secretory capacity restored the insulin response of thyroxine treated islets to normal levels. Thus, at high glucose concentrations with theophylline there was no significant difference between the insulin release from control islets, or those treated with thyroxine (1 µg/ml).

The increase in insulin secretion that was elicited by the addition of theophylline to 20 mmol/l glucose media was 0.45±0.28 ng insulin/islet/hour for control islets, and 1.51±0.34 ng insulin/islet/hour for the thyroxine treated islets. The enhancement of insulin release by theophylline was, therefore, significantly higher in the thyroxine (1 µg/ml) treated group ($p < 0.001$).

Thyroxine concentration in the culture media

The total thyroxine in the culture media for experiments using an estimated concentration of 1.0 µg/ml was as follows:

At the start of the culture period 0.95±0.15 µg/ml (n = 3), at 20 h 0.81±0.15 µg/ml (n = 3), at 24 h 0.79±0.12 µg/ml (n = 3). Approximately 99% of the thyroxine in the culture media was bound to the plasma protein fraction of the added new-born calf serum. The free thyroxine concentration was 14.4 ng/ml at the start of the culture period, and 9.6 ng/ml at the end of culture. Analyses of the free hormone levels in the media revealed that tri-iodothyronine was present in small quantities in the thyroxine preparation: The value

being 0.15 ng/ml of tri-iodothyronine at the start of incubation, and 0.13 ng/ml after 24 hours.

Discussion

It has been established that the 'free floating' culture system used here, where islets do not become adherent to the bottom of the dish, is the most suitable for islet incubations (Andersson, 1978). Extrapolations from data of rates of glucose oxidation by islets in culture show that the islet tissue has a negligible influence on substrate and metabolite levels of the culture medium during incubations of up to four days (Andersson, 1972). Therefore, it can be assumed that the thyroxine treated islets were maintained at a physiological glucose concentration throughout the 24-hour period. There was no gross degradation of thyroxine in this time, as two thirds of the free T_4 was still present after 24 hours. Approximately 1% of the free hormone in the media was tri-iodothyronine, and the results therefore relect the effects of a combination of T_4 and T_3.

One consistent aspect of tissue culture of islets which is still poorly understood is the diminution of glucose sensitivity and a decreased capacity to secrete insulin after a period of culture (Andersson, 1972; Rabinovitch, 1978). The addition of a phosphodiesterase inhibitor such as theophylline (Butcher, 1962) or isobutylmethylxanthine (IBMX) to the incubation media during subsequent secretion tests can restore the glucose induced insulin secretion to more normal levels (Andersson, 1972; Rabinovitch, 1978), which implies that tissue culture may involve changes in the islet cyclic AMP system. The activity of islet adenylate cyclase is said to be

unaltered during culture at 5.5 mmol/l glucose (Howell, 1973b; Whittaker, 1977), as is the basalcAMP content (Rabinovitch, 1978). Interestingly, the rise in cyclic AMP that accompanies glucose stimulated insulin release in freshly isolated islets (Gerich, 1976) was not detected in islets after tissue culture (Rabinovitch, 1978). The normalisation of the glucose induced insulin response by phospho-diesterase inhibitors suggests that the effector mechanisms for insulin release are unaltered by tissue culture and continue to be responsive to the postulated signal derived from cyclic AMP. It appears that an increase in cyclic AMP is not a prerequisite for insulin secretion to take place, but it may be essential for the establishment of a normal response to glucose, and the initiatory events including the cyclic AMP system on exposure to glucose may be altered by tissue culture. Whether the changes take place at the B cell membrane or reflect a metabolic deficiency is still conjectural. The presence of serum during culture at 5.5 mmol/l glucose is an acknowledged necessity for islet survival (Andersson, 1978); possibly it contains essential chemical factors which are not included in TCM 199. Unfortunately, culture with different batches of sera results in variations of islet function (Whittaker, 1977) as was seen in the control groups of islets indicating that the sera are not standardised for these unidentifed requirements.

In the experiments in this chapter, the glucose induced insulin secretion by control islets after tissue culture was less than one half that of freshly isolated islets (Chapter 3). Nevertheless, despite a diminished sensitivity, the control groups were evidently viable after 24 hours, since high concentrations of glucose and the inclusion of theophylline could elicit a significant increase in insulin secretion. In the case of thyroxine treated islets, insulin secretion in response

to 2 and 6 mmol/l glucose was not affected by culture with thyroxine, and was comparable to the control values. However, thyroid hormones inhibited insulin secretion at 20 mmol/l glucose, and this pattern closely parallels observations of a reduced secretory response to glucose by the perfused pancreas isolated from thyroxine treated rats (Lenzen, 1976b). In these dose-response experiments, an inverse correlation was found between the thyroid status of the animals and the subsequent pancreatic insulin secretion. When theophylline was added to the incubation media, the insulin release of thyroxine (1 µg/ml) treated islets was restored to normal control levels, indicating that the secretory capacity of the B cells was still intact. This reversal of the inhibitory action of thyroid hormones suggests that they have a specific effect on the regulation of insulin secretion in response to glucose which can be reversed by a phosphodiesterase inhibitor, rather than acting as a general toxic factor.

There are some striking similarities between the inhibition of insulin secretion seen in starvation and that reported here as a result of thyroxine treatment at normal and constant glucose concentrations. An impairment of glucose stimulated insulin secretion as a result of fasting is evident *in vivo* (Grey, 1970), in pancreatic pieces from starved animals (Malaisse, 1967e) and in tissue culture experiments in which islets are maintained in low glucose concentrations (Andersson, 1976a). The latter experiment indicates that these alterations in islet function are a direct result of prolonged hypoglycaemia, rather than the other complex metabolic changes that occur during fasting. Studies on B cell function from pancreata of hyperthyroid or starved animals show that the defective insulin secretion can be normalised by intermediates of glycolysis in both cases (Lenzen, 1975, 1976a;

Levy, 1976). Furthermore, the pattern of enhancement of insulin secretion by theophylline is similar in islets from starved animals (Levy, 1976) and the thyroxine treated islets described here.

The alterations of insulin secretion during starvation imply the existence of long-term mechanisms of control and regulation, possibly through changes in glucose metabolism (Levy, 1976) or the cyclic AMP system (Howell, 1973b). These will be discussed in more detail later, but it appears that thyroid hormones influence these mechanisms directly, giving rise to an impaired insulin release.

CHAPTER 5

THE EFFECT OF THYROID HORMONES ON THE INSULIN AND PROTEIN CONTENT, AND ULTRASTRUCTURAL APPEARANCE OF PANCREATIC ISLETS

Introduction

It seemed possible that any depletion of B cell insulin stores might play a rôle in the alteration of glucose induced insulin secretion by thyroid hormones. Therefore the insulin and protein content of isolated islets cultured for 24 hours in the presence of thyroxine (1 µg/ml) was investigated. Studies were also made of the ultrastructural appearance of thyroxine treated islets to detect any intracellular derangement.

Methods

Isolated islets from euthyroid animals were cultured for 24 hours with 1.0 µg/ml thyroxine as previously described. At the end of the culture period, they were washed three times in bicarbonate-buffered medium, and a few islets were collected after the washing procedure for electron microscopic studies. The protein and insulin content of the remaining islets was then estimated.

Groups of ten islets were homogenised in a glass tissue homogeniser with 0.2 ml ethanol:HCl:water (23.5:0.45:7.0) and 0.2 ml water. Samples of the homogenate were taken for protein estimation by the method of Lowry (1951) using crystalline albumen standards, and insulin content by immunoassay after dilution with 50 mM phosphate buffer.

Measurement of Islet Protein Content

Principle

The method is based on Lowry's (1951) calorimetric reaction between the tryptophan and tyrosine residues of the protein in alkaline copper solution and phosphomolybdic-phosphotungstic reagents commercially prepared as Folin Ciocalteu's reagent.

Reagents

1. Na_2CO_3 (2%) in 0.1 M-NaOH.

2. $CuSO_4.5H_2O$ (1%).

3. NaK tartrate (2%).

4. Fresh alkaline copper reagent prepared by mixing 1, 2 and 3 in a ration of 100:1:1 by volume.

5. Folin-Ciocalteu's reagent.

Procedure

Samples of 0.01 ml islet homogenate were diluted with 0.1 M-NaOH to ensure that they were alkaline. Standard protein solutions of 0-300 µg albumen/ml were prepared from crystalline bovine albumen in 0.1 M-NaOH.

10 µl of unknown sample or protein standard was added to flat bottomed tubes in duplicate. 1 ml of reagent 4 was added to each tube, mixed and allowed to stand for 10 minutes for hydrolysis to take place. 100 µl of Folin-Ciocalteu's reagent was then added to each tube, mixed and left to develop colour at room temperature for 1 hour. The optical density of the blue solutions was read in a Unicam SP 1800 Ultraviolet Spectrophotometer at a wavelength of 750 nm. The absorption of the standard protein solutions was plotted against their protein concentration (Fig. 15) and the protein contents of the unknown samples were read directly from the graph.
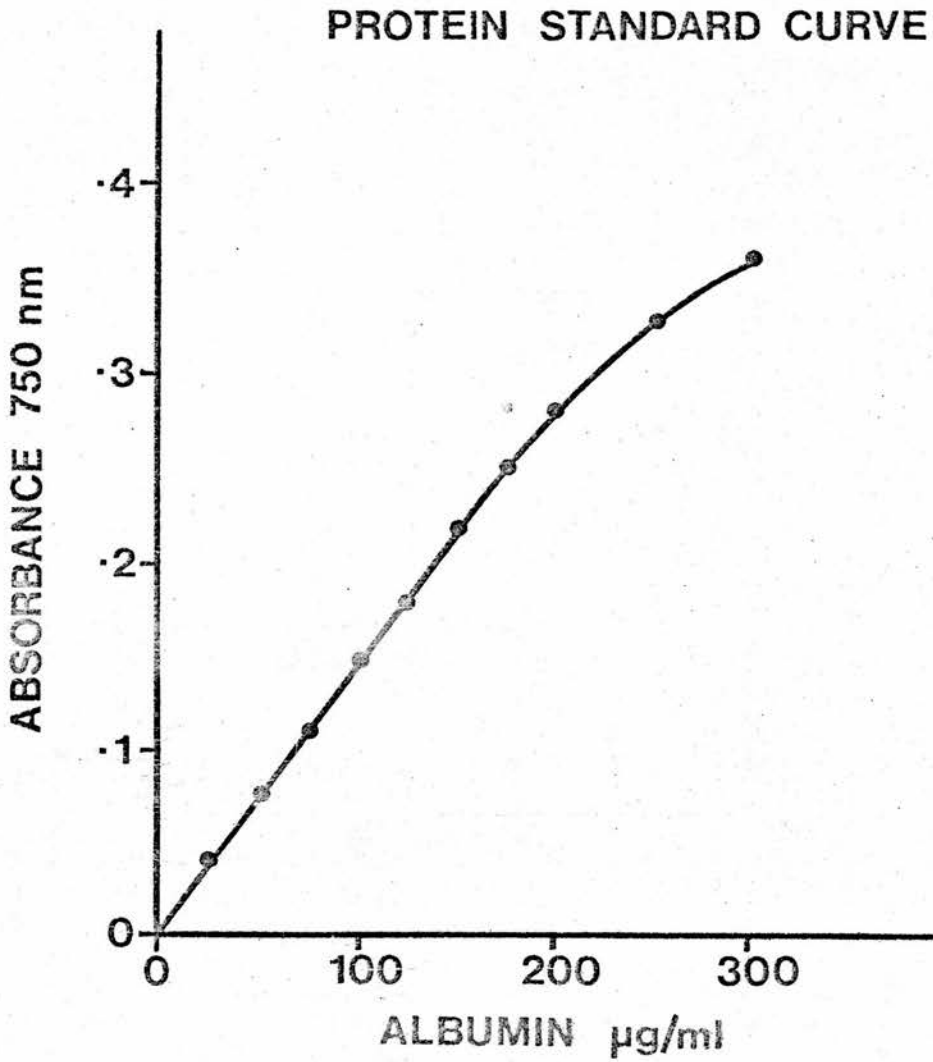
Figure 15 Standard solutions of crystalline albumin were assayed as described in the text. The absorption of protein containing solutions was read in an SP 1800 spectrophotometer at a wavelength of 750 nm. Each point is the mean of duplicate observations.

Electron Microscopy

Islets cultured with or without thyroxine were fixed with 3% glutaraldehyde in 0.1 M-phosphate buffer, pH 7.4, post-fixed in 2% osmium tetroxide and embedded in epoxy resin. This sections were prepared and stained with a saturated solution of uranyl acetate in 50% ethanol before examination in a Jeol 100 S electron microscope.

Results

Insulin and Protein Content of Islets After Culture

The total insulin content of islets following 24 hour culture with thyroxine (1 μg/ml) was no different from normal islets cultured in the same conditions without thyroxine. The corresponding results for insulin content were as follows: thyroxine treated = 16.0 ± 2.03 ng insulin/islet (55 observations); controls = 16.61 ± 2.14 ng insulin/ islet (63 observations). $p > 0.05$. However, thyroxine treated islets showed a significant diminution of total protein content, observed to be 80% of normal. Protein content of controls was 1.71 ± 0.19 μg protein/islet (63 observations); protein content of thyroxine treated islets was 1.39 ± 0.12 μg protein/islet (55 observations) ($p < 0.05$).

Ultrastructure of Islets After Culture

Electron micrographs of randomly selected areas of islets which had been cultured for 24 hours in the absence (Fig. 16) or in the presence (Fig. 17) of 1.0 μg/ml thyroxine were examined. It is clear that there were no major structural differences in either A or B cells, between islets cultured in the presence or absence of thyroid hormones. In addition, the isolation and culture procedure had no adverse effects

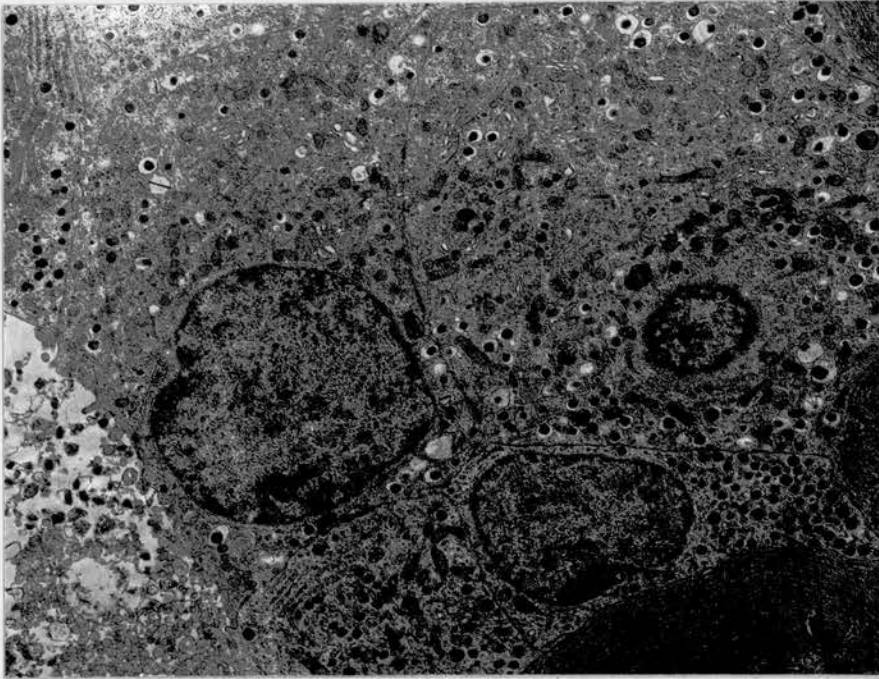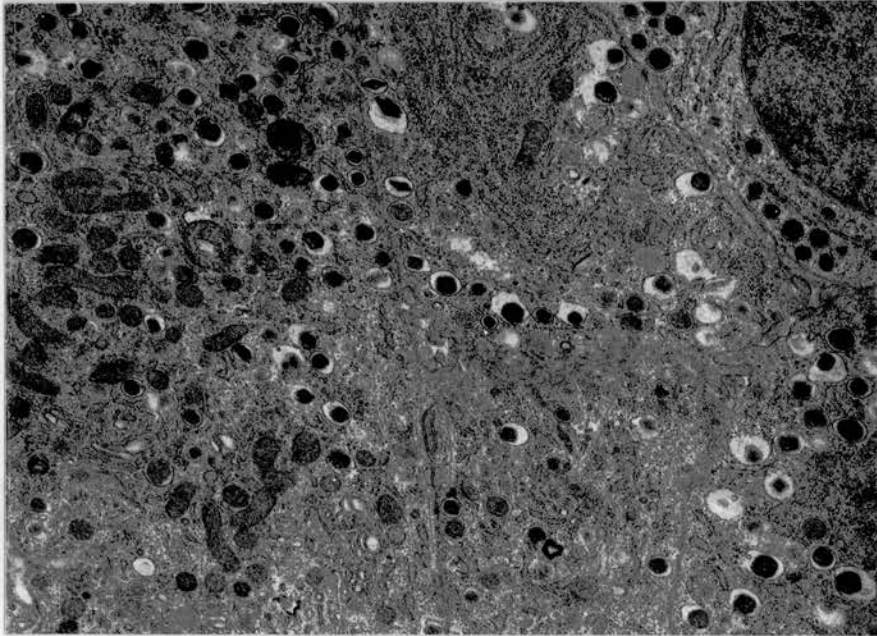Figure 16a (x4000 magnification)

Figure 16b (x8000 magnification)

Electron micrographs of B cells from islets cultured for 24 hours in the conditions described in the text, without thyroxine.
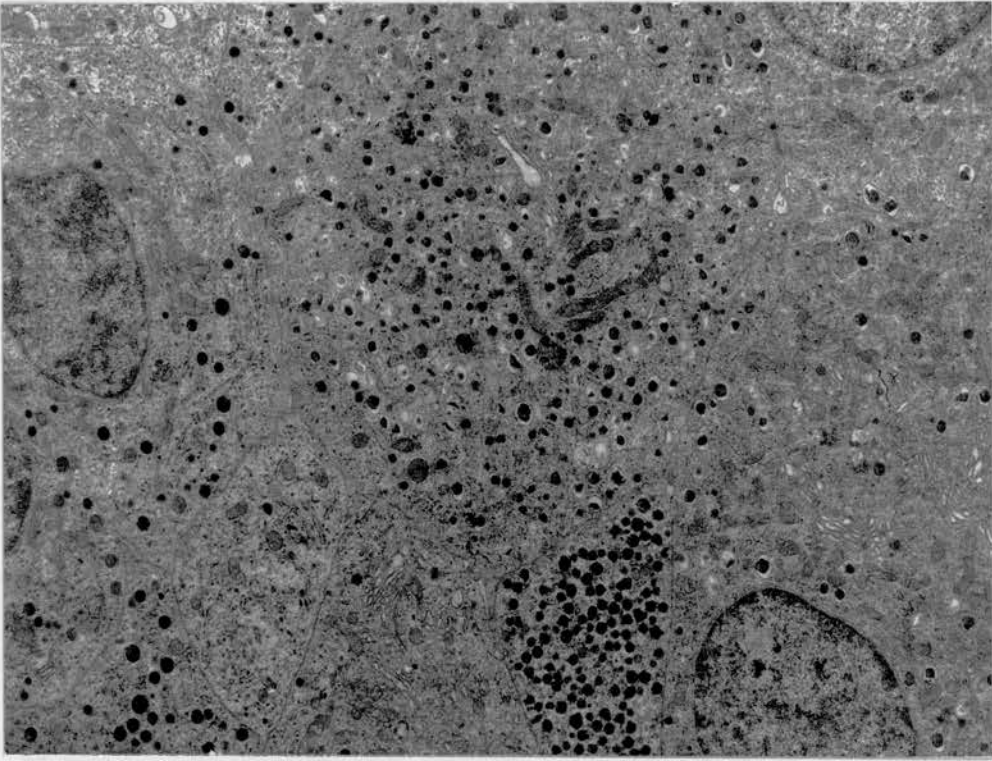
Figure 17a (x5400 magnification)
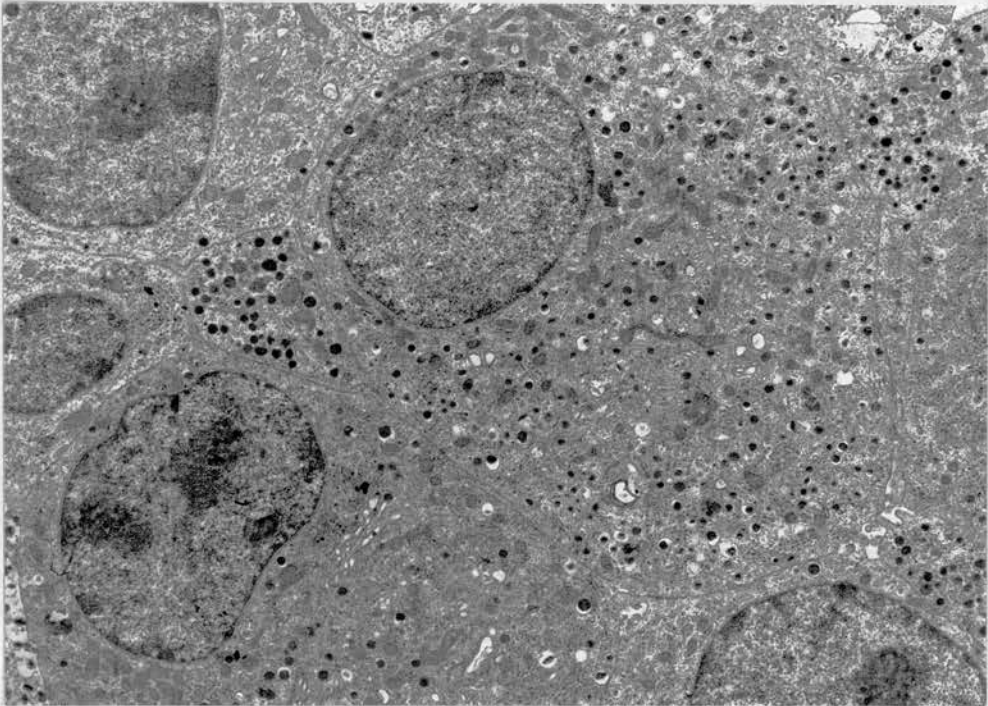
Figure 17b (x4000 magnification)

Electron micrographs of B cells from islets cultured for 24 hours with 1.0 µg/ml thyroxine in the conditions described in the text.

on the ultrastructure of A or B cells, whose appearance remained
closely similar to that seen in islets in whole pancreas (Fig. 12).

Discussion

There was no alteration in the insulin content of thyroxine
treated islets as compared to cultured controls, in accordance with
previous reports of a normal islet insulin content in experimentally
hyperthyroid ob/ob mice (Lenzen, 1978b). Similarities between the
effects of thyroid hormone treatment and starvation on B cells were
again apparent, in that starved animals also have a normal islet
insulin content (Malaisse, 1967e). In addition, the protein content
of islets cultured with thyroid hormones was reduced by 20% while
Levy reported a 12% reduction of protein content in islets from
starved rats (Levy, 1976). The decrease in islet protein content is
consistent with the predominance of protein catabolism over protein
synthesis, which is generally found in hyperthyroidism, particularly
in muscle tissue (Hoch, 1974).

The electron micrographic studies demonstrated that tissue
culture of islets with thyroid hormones did not give rise to any
deleterious structural changes in B cells. This further excludes
the possibility that these hormones have a purely toxic effect, in
agreement with the results of histological examinations of the
pancreatic tissue from hyperthyroid animals (Lenzen, 1978b).

CHAPTER 6

THE LONG-TERM DIRECT EFFECT OF THYROID HORMONES ON (PRO)-INSULIN BIOSYNTHESIS

Introduction

The long-term influences on insulin secretion by a variety of agents are paralleled by comparable changes in insulin biosynthesis. For instance, enhanced insulin secretion of late pregnancy (Green, 1972) is accompanied by an increased rate of insulin synthesis (Bone, 1977) and starvation similarly impairs both glucose-induced insulin secretion (Malaisse, 1967e) and biosynthesis (Andersson, 1974). At present, there is scant information regarding the biosynthetic capacity of B cells during hyperthyroidism. In view of the depression of glucose-induced insulin secretion by thyroid hormones, and the close interaction between mechanisms of hormonal secretion and production, the following experiments were designed to investigate possible long-term effects of thyroid hormones on insulin biosynthesis.

Methods

Isolated islets were cultured for 24 hours with or without 1 µg/ml thyroxine. They were then pre-incubated for 30 minutes in bicarbonate-buffered medium, prior to their use in the experiments. The (pro)-insulin biosynthetic capacity of the islets after culture was measured by the method of Schatz et al. (1973).

(Pro)-Insulin Biosynthesis

Groups of 40 islets were incubated in 1 ml bicarbonate-buffered media with 2 mg/ml albumen, 17 naturally-occurring amino acids (20 µg/ml of each amino acid excluding leucine), glucose to give concentrations

of 6 or 20 mmol/l and 4-5-H3-L-leucine, 50 µCi/ml (58 Ci/mmol specific activity). Incubations were carried out in 95% O_2: 5% CO_2, at 37°C for 3 hours with gentle shaking.

After incubation, the islets and the incubation media were extracted together to measure the total synthetic capacity of the islets. Ice-cold trichloroacetic acid (TCA) was added to the samples to give a final concentration of 10%. The samples were sonicated for 10 seconds (MSE Ultrasonic Disintegrator) at high power, amplitude 3, and the resultant precipitate was washed three times with 5% TCA containing 1 mg/ml unlabelled leucine. The final precipitate was redissolved in 0.5 ml 1 M acetic acid.

Separation of the islet proteins in solution was achieved on a Sephadex G-50 fine column, 1.2 x 100 cm, eluting with 1 M acetic acid at a rate of approximately 10 ml/h. Fractions of 1 ml were collected, 1 ml of scintillation fluid (700 ml toluene:300 ml Triton X-100:5 g PPO) was added to 0.1 ml of each fraction, and the radioactivity was counted. The two intermediate peaks obtained after separation of TCA-precipitable islet proteins were identified as pro-insulin and insulin by polyacrylamide-gel electrophoresis (Davis, 1964). Six fractions of each peak from individual samples were pooled to estimate the total counts per minute of radioactivity in the void volume, pro-insulin and insulin peaks.

Polyacrylamide Gel Electrophoresis

Preparation of gel columns

»   Solution A: 48 ml 1 M HCl

                                36.6 g Tris

                                0.23 ml Temed (N,N,N',N'-Tetramethylethyl-enediamine) diluted to 100 ml, pH 8.9.

Solution B: 32 g Acrylamide

                         0.8 g Bis-acrylamide, diluted to 100 ml

                         for 8% solution.

Buffer Solution C: 6.0 g Tris

                         28.8 g Glycine diluted to 1000 ml

                         used in 1:10 dilution.

Gel solution was made up by mixing 1 ml solution A, 2 ml solution B, 3 ml distilled water and 2 ml ammonium persulphate (0.14 g in 100 ml). 2 ml of gel solution was placed into each gel column and a small amount of distilled water was layered on top of each gel to obtain a flat interface. The columns were allowed to set.

Preparation of samples

The eluant fractions of peak 2 and peak 3 (Fig. 18) were pooled into two separate samples, lyophilized and reconstituted with 150 µl 0.01 M HCl together with porcine pro-insulin and bovine insulin markers.

Gel electrophoresis

When the gels had set, the water was removed from the surface, and the samples, mixed with bromophenol blue, were layered on top of the gels, which were then topped up with Tris/glycine buffer (solution C). Electrophoresis was carried out at room temperature at 5 mA per tube. On removal from the gel columns the gels were stained with 10% TCA and sliced at 3 mm intervals. Each slice was dissolved in 0.2 ml of 100-volume H_2O_2 at 60°C for 8 hours. 2 ml of Triton-based scintillant (700 ml toluene:300 ml Triton X-100: 5 g PPO) were added to each sample, and they were assayed for radio-activity. The pro-insulin and insulin markers were observed as white bands on the stained gels, and the slice numbers of these bands were noted.
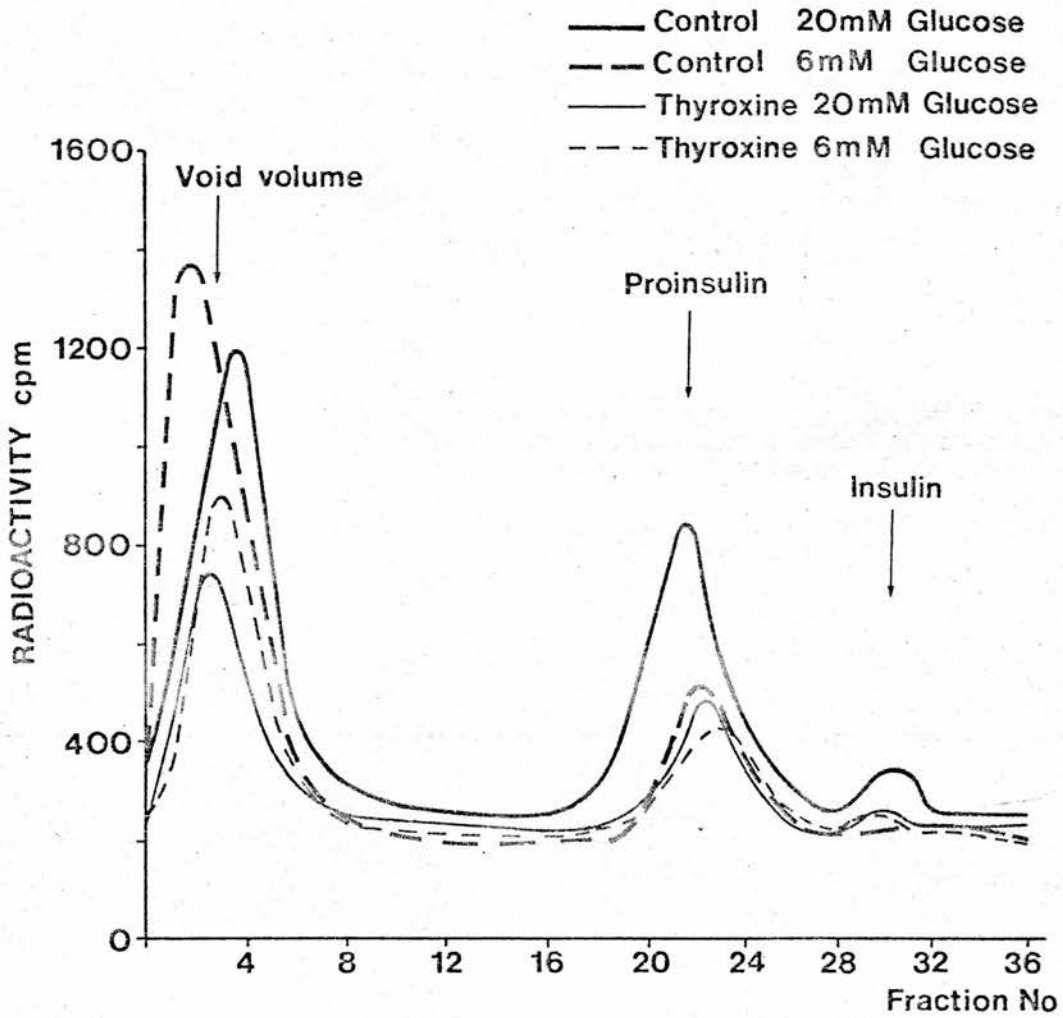
Figure 18 The effect of glucose (6 or 20 mmol/l) on the incorporation of H-3-leucine into the proteins of isolated islets. The islets were cultured for 24 hours with 1 μg/ml thyroxine prior to incubation with glucose. Following precipitation with trichloroacetic acid, the islet proteins were fractionated on a Sephadex G-50 fine column. Mean curves are shown, derived from three experiments each.

Results

The incorporation of H-3-leucine into islet proteins at 6 or
20 mmol/l glucose is shown in Fig. 18. The elution pattern from the
Sephadex column forms three major peaks. The first peak represents
the void volume eluted with dextran blue. Polyacrylamide gel electro-
phoresis of pooled fractions of the second and third peaks is shown
in Fig.19 and Fig.20. The intermediate peak gave the greatest radio-
activity in the area of the pro-insulin marker (Fig. 19) while the
bulk of radioactivity of the third peak coincided with the insulin
marker (Fig. 20). Thus, the intermediate peak and the last peak com-
prise the incorporation of labelled leucine into pro-insulin and
insulin respectively. It has previously been observed that the radio-
activity recovered in the insulin region includes the radioactivity of
C-peptide, which is eluted together with insulin from Sephadex G-50
columns (Sando, 1972).

In the control islets the incorporation of radioactivity into
TCA-precipitable non-specific proteins eluted with the void volume was
not significantly affected by increasing the glucose concentration from
6 to 20 mmol/l (Table IVa and IVb). However, the uptake of H-3-leucine
into the pro-insulin and insulin fractions rose by 66% with an
increased concentration of glucose. 24 hour culture with thyroxine
did not affect the pro-insulin and insulin biosynthetic capacity of
islets at 6 mmol/l glucose (Table IVa), but, incubation in 20 mmol/l
glucose resulted in a significant ($p < 0.005$) 41% inhibition of non-
specific protein biosynthesis by thyroid hormones (Table IVb). In
the presence of this concentration of glucose the thyroxine treated
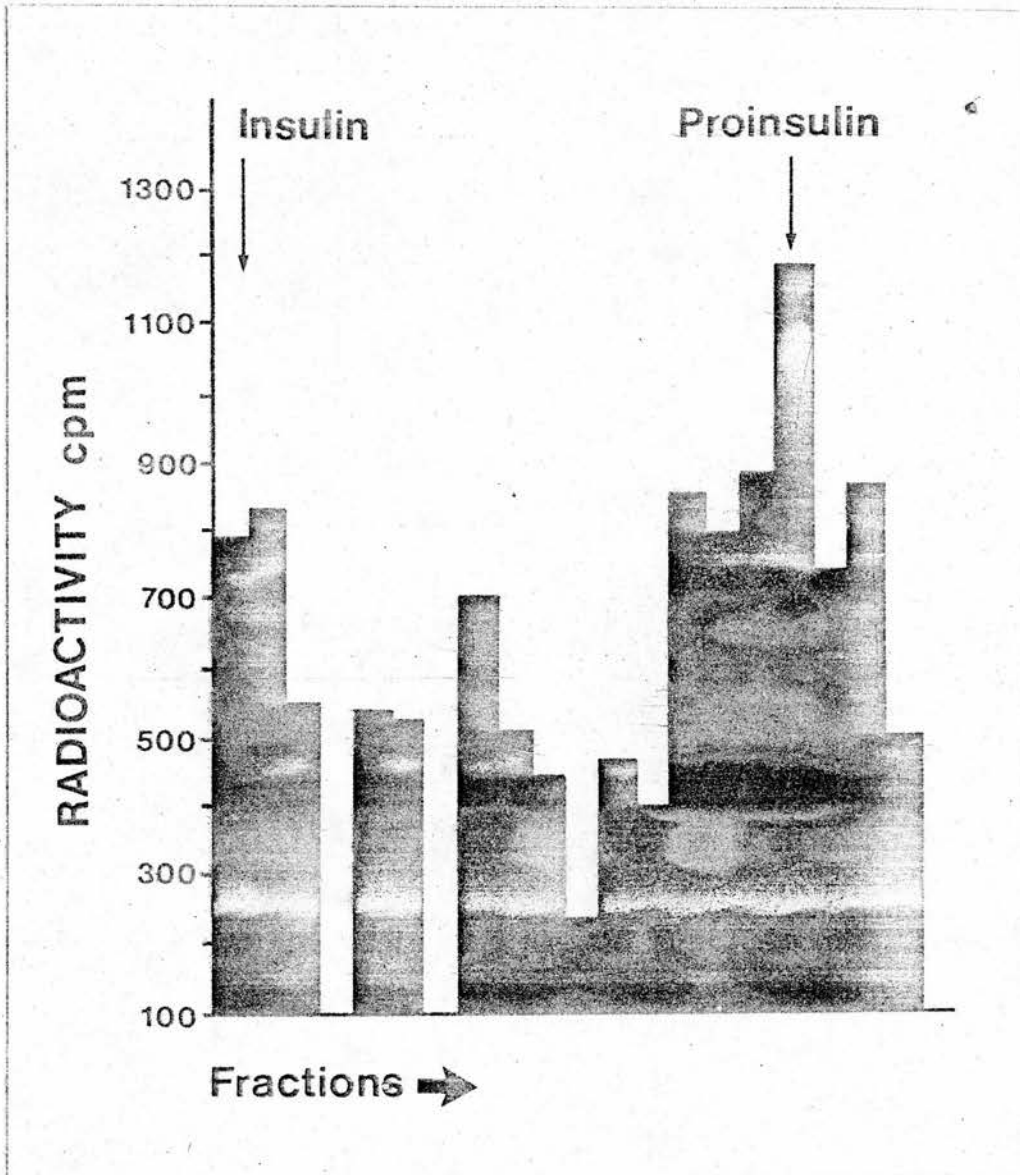islets also showed a significant ($p < 0.005$) 38% reduction of pro-

Figure 19 Polyacrylamide gel electrophoresis of protein fractions
constituting the second peak collected after elution of TCA-
precipitable islet proteins labelled with H-3-leucine, from a control
sample of cultured islets, on a Sephadex G-50 column. Porcine pro-
insulin and bovine insuline were added to the samples as markers. The
maximum radioactivity of the intermediate peak was associated with the
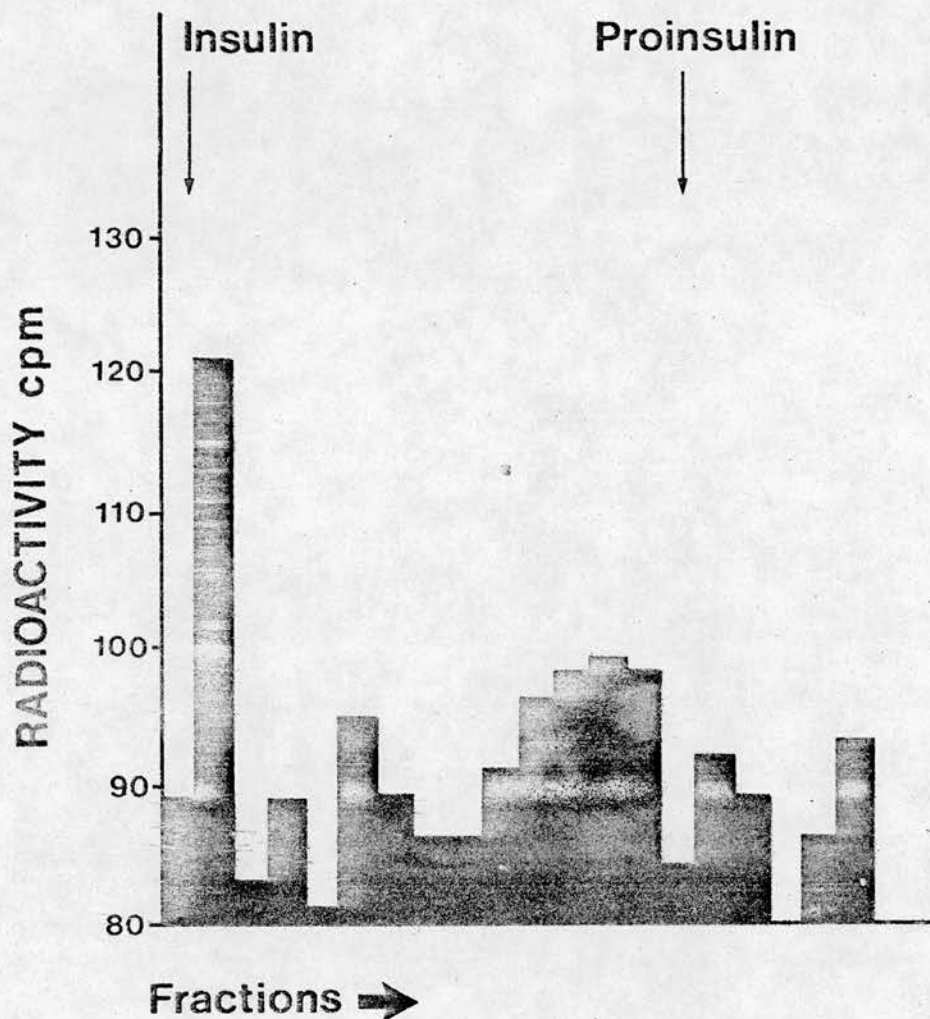pro-insulin marker.

Figure 20 Polyacrylamide gel electrophoresis of protein fractions
constituting the third peak collected after elution of TCA-precipitable
islet proteins labelled with H-3-leucine, from a control sample of
cultured islets, on a Sephadex G-50 column. Porcine pro-insulin and
bovine insulin were added to the samples as markers. The maximum
radioactivity of the third peak was associated with the insulin
marker.

TABLE IVa and IVb

Effect of 24 hour culture with thyroxine (1 µg/ml) on the subsequent incorporation of radioactivity into 3 fractions of islet proteins in response to 6 or 20 mmol/l glucose. After culture islets were incubated for 3 hours in bicarbonate-buffered media containing 6 or 20 mmol/l glucose, albumen and 50 µCi/ml H-3-leucine. TCA-precipitable proteins were separated on a Sephadex G-50 fine column. The radio-activity of the protein, pro-insulin and insulin peaks was estimated by pooling 6 eluted fractions constituting each peak. The mean values of the total counts per minute in the pooled peaks are shown ± SEM of the number of observations in parentheses. The observed difference between paired control and thyroxine treated samples (c.p.m.) is calculated as a percentage of the control value, and is expressed as the percentage inhibition of protein, pro-insulin or insulin bio-synthesis by thyroxine.

TABLE IV (Cont.)

(a) 6 mmol/l glucose

Peaks	Control	Thyroxine 1 µg/ml	% Inhibition of biosynthesis by T_4
Protein	14,510±6,600 (3)	12,030±4,560 (3)	24.0%±19.9 (3)
Pro-insulin	6,357± 971 (4)	5,740±1,422 (4)	12.87%±9.34 (4)
Insulin	3,925± 551 (4)	4,007± 599 (4)	-1.82%±3.10 (4)

(b) 20 mmol/l glucose

Peaks	Control	Thyroxine 1 µg/ml	% Inhibition of biosynthesis by T_4
Protein	15,170±2,320 (3)	9,050±1,800 (3)	*41.03%±3.05 (3)
Pro-insulin	9,835±2,300 (4)	5,815± 856 (4)	*38.19%±4.94 (4)
Insulin	4,657± 798 (4)	3,975± 811 (4)	11.44%±6.31 (4)

*$p < 0.005$

insulin synthesis (Table IVb). The pro-insulin uptake of H-3-leucine
reaches a steady state within a few hours of incubation, while
labelling of the insulin pool continues at a linear rate for at
least 12 hours (Steiner, 1969). Therefore, any increase in the ratio
between the total radioactivity in pro-insulin and insulin and that
in insulin alone reflects a change in the rate of conversion of pro-
insulin. The results show that, despite an inhibition of pro-insulin
synthesis at high concentrations of glucose by thyroid hormones, there
was no appreciable effect on the degree of conversion of pro-insulin
to insulin, which was maintained at approximately 40%, and the
incorporation of radioactivity into insulin at the end of the incu-
bation period with 20 mmol/l glucose was unaffected (Table IVb).

Discussion

Glucose has been shown to promote amino acid incorporation into
rabbit pancreas slices (Parry, 1966), and isolated islets (Howell, 1966;
Morris, 1970), by preferential stimulation of pro-insulin and insulin
biosynthesis (Morris, 1970; Bone, 1977). The effect is rapid and
appears to depend on a selective enhancement of tRNA rather than
synthesis of new mRNA (Bone, 1977). Although the uptake of labelled
amino acids into other islet proteins is also dependent on the pres-
ence of glucose, the influence of increasing glucose levels is
relatively insignificant, as was the case in these experiments,
whereas there is marked concurrent stimulation of pro-insulin and
insulin synthesis (Schatz, 1973).

In addition to the changes of B cell insulin secretion incurred
during a period of tissue culture, the alterations of glucose sensi-
tivity are reflected in the pattern of hormonal synthesis by isolated

islets. Unless the islets are cultured in media containing a high concentration of glucose, there is an overall reduction in (pro)-insulin biosynthesis, although the characteristic ratios of amino acid incorporation into pro-insulin and insulin are not grossly affected (Andersson, 1974). Furthermore, after tissue culture the islets do not show the same marked increase in insulin biosynthesis, which generally accompanies a rise in glucose concentration in freshly isolated islets (Andersson, 1974).

In the experiments here, the biosynthesis of (pro)-insulin by control islets was increased in 20 mmol/l glucose, but the rise was not statistically significant. This was possibly due to the large standard errors inherent in this particular method of separating islet proteins. However, thyroid hormones evidently exert a highly significant ($p < 0.005$) inhibitory effect on the synthesis of TCA-precipitable proteins and pro-insulin, when compared to non-treated islets at a raised concentration of glucose. It is possible that thyroid hormones stimulate the breakdown of islet proteins with a consequent increase in the intracellular pool of free amino acids. This may lead to a dilution of the free H-3-leucine pool and a decrease in the observed incorporation of radioactivity into (pro)-insulin. It has been suggested that this phenomenon occurs in starvation (Gylfe, 1974), but isotope dilution seems unlikely in the above experiments, since incubation of islets with thyroid hormones did not alter the incorporation of labelled leucine into TCA-precipitable proteins at a physiological concentration of glucose.

Thus the changes in (pro)-insulin biosynthesis produced by thyroid hormones are again analogous to the decreased biosynthesis of (pro)-insulin observed in islets from starved animals (Tijoe, 1976;

Bone, 1977), or isolated islets cultured in low glucose concentrations (Andersson, 1974 and 1976a), although the effect of starvation on total islet protein synthesis remains controversial (Levy, 1976; Bone, 1977). The conversion of pro-insulin to insulin is a process which does not require the presence of glucose (Steiner, 1972; Jain, 1977), but may be activated by high concentrations of glucose (Jain, 1977; Andersson, 1974). Neither thyroid hormones nor starvation appear to influence this phase in insulin synthesis (Levy, 1976).

CHAPTER 7

THE LONG-TERM EFFECT OF THYROID HORMONES ON ISLET ADENYLATE CYCLASE
ACTIVITY

Introduction

It has been proposed that an increased sympathetic activity may
account for some of the features observed in hyperthyroidism and that
the action of thyroid hormones is mediated through catecholamines
(Brewster, 1956). The possibility that thyroid hormones enhance the
tissue response to catecholamines through the adenylate cyclase-cyclic
AMP system was investigated in epididymal fat pads from hyperthyroid
rats (Krishna, 1968). These authors concluded that thyroid hormones
induce the formation of adenylate cyclase in adipose tissue, thus
potentiating catecholamine effects. Many analogies can be drawn between
the changes in islet function due to the prolonged lack of an adequate
glucose stimulus, and the long-term effects of thyroid hormones already
demonstrated. A diminution of adenylate cyclase activity is strongly
implicated as at least one of the causal events leading to the reduced
insulin secretion and biosynthesis in starvation (Howell, 1973b).
Furthermore, the reversal of thyroxine-induced inhibition of insulin
secretion by theophylline (Chapter 4) also suggests that the adenylate
cyclase-cyclic AMP system may be involved. Thyroid hormones do not
appear to significantly alter islet ATP levels (Lenzen, 1978b), nor the
activity of phosphodiesterase in adipose tissue (Krishna, 1968).
Therefore, it seemed important to establish whether islet adenylate
cyclase activity was affected.

Methods

Isolated islets from non-fasted euthyroid animals were cultured for 24 hours with or without 1.0 µg/ml thyroxine as previously described. At the end of the culture period, they were washed three times in bicarbonate-buffered medium prior to their use in the experiments.

The activity of islet adenylate cyclase was measured by the method of Howell and Montague (1973a) using a broken cell preparation which is incubated in the presence of labelled ATP, and separating the newly formed labelled cyclic AMP on a neutral alumina column. The adenylate cyclase of the broken cell preparation shows a marked stimulation with 10 mM sodium fluoride, although the mechanism of this effect is uncertain, and the activity of adenylate cyclase in the presence of NaF indicates the total amount of enzyme present (Montague, 1975b).

Two buffers were made up as follows:-

Buffer A: 25 mM Tris HCl; 5 mM $MgCl_2$; 1 mM EDTA.

Buffer B: 25 mM Tris HCl; 1 mM EDTA; 0.1% (w/v) albumen,

5 mM $MgCl_2$; 1 mM isomethylbutylxanthine;

1 mg/ml creatine phosphokinase, 7 mg/ml

phosphocreatine; 10 µM ATP and 20 µl/ml

$(\alpha-^{32}p)$ ATP.

After tissue culture, batches of 50 islets were homogenised using a glass homogeniser in 0.5 ml buffer A.

50 µl of buffer B was added to assay tubes, with or without 10 µl NaF (10 mM). 50 µl of islet homogenate was then placed in each tube and the tubes were incubated at room temperature for 30 minutes.

The reaction was terminated at the end of this period by placing the tubes in a boiling water bath for 3 minutes. 50 µl of 1 mg/ml unlabelled cyclic AMP was then added to each tube as a carrier, and the contents of the tubes were run through alumina columns, eluted with 6 ml of 10 mM Tris HCl (pH 7.6). This separated the newly synthesised cyclic $[^{32}P]AMP$, which was collected with the eluant in scintillation vials, and counted for radioactivity. All observations were in triplicate. The protein concentration of 50 µl of each islet homogenate was measured by the method of Lowry (1951).

Results

Estimations of adenylate cyclase activity of islet homogenates after 24 hour incubations with thyroxine (1 µg/ml) are shown in Table V. Basal values of adenylate cyclase activity after tissue culture were low, but adequate stimulation of activity was achieved with sodium fluoride. There was no significant effect of thyroid hormones on either the basal or stimulated adenylate cyclase activity.

Discussion

The low basal values of adenylate cyclase activity in both control and thyroxine treated islets were probably a consequence of tissue culture; the glucose stimulated rise in cyclic AMP observed in freshly isolated islets is not apparent in islets after tissue culture, despite their maintenance at a physiological glucose concentration (Rabinovitch, 1978). Thyroid hormones did not significantly affect the basal enzyme activity, nor did they influence the maximal adenylate cyclase activity demonstrated by stimulation with sodium fluoride,

TABLE V

Adenylate cyclase activity of rat islet homogenates after 24 hour
culture of islets with or without thyroxine (1 μg/ml). Enzyme
activity at basal control value and after stimulation with sodium
fluoride is expressed as pmoles cyclic AMP formed/mg protein/20 min
± SEM of the number of observations in parentheses.

	Control	Thyroxine 1 μg/ml
Basal control	0.88±0.74 (4)	1.43±0.82 (5)
NaF stimulation 10 mmol/1	17.95±1.64 (5)	18.65±3.15 (5)

when islets were cultured in the presence of 5.5 mmol/l glucose.

Many of the hormonal adjustments of insulin secretion are made through modifications of adenylate cyclase activity. This enzyme can be regulated by the interaction of hormones with specific receptor molecules at the B cell plasma membrane (Goldfinch, 1972; Sharp, 1979). Polypeptide hormones such as ACTH, glucagon, secretin and pancreozymin stimulate adenylate cyclase activity and raise intracellular cyclic AMP (Sharp, 1979). Consequently, insulin release in response to glucose is potentiated through the augmentation of the calcium-dependent secretory events (Montague, 1977; Sharp, 1979). Insulin itself exerts a negative feedback inhibition on the B cell, and so far is the only hormone known to depress adenylate cyclase activity in islets (Kuo, 1973). Somatostatin and α-adrenergic agents are also purported to lower cyclic AMP levels by inhibition of adenylate cyclase (Kuo, 1973; Howell, 1973a; Efendic, 1978), but this has been disputed since raising cyclic AMP or activating adenylate cyclase does not reverse the blocked insulin release produced by these agents (Malaisse, 1970a; Gerich, 1977).

Isolated islets from pregnant rats also have an increased adenylate cyclase activity (Green, 1973), which presents a possible explanation for the enhancement of insulin release in response to gestational hormones (Howell, 1977). However, pregnant rats in pair-feeding experiments with control animals did not show a comparable magnitude of insulin secretion as did pregnant rats on an unrestricted diet (Green, 1974). Therefore, at least part of the effect of pregnancy on insulin release can be accounted for by the 20% rise in food intake during late gestation (Green, 1972).

It is now evident that the long-term influence of carbohydrate ingestion on insulin secretion, as manifest during starvation or glucose-loading, is accompanied by alterations in islet adenylate cyclase activity (Howell, 1973b) and glucose metabolism (Levy, 1976). It was originally suggested that the poor insulin response to glucose in rats fasted for 48 hours was due to changes in a glucose-inducible enzyme system, since the restoration of normal insulin secretion was blocked by actinomycin D (Grey, 1970). Certainly, basal and glucagon-stimulated adenylate cyclase activity is reduced by starvation (Howell, 1973b). In addition, the lack of a glucose induced rise in cyclic AMP in islets from starved rats, which precedes any alterations in glucose metabolism (Wolters, 1977), and the restoration of normal insulin release by phosphodiesterase inhibitors (Grey, 1970), implicates the adenylate cyclase-cyclic AMP system in the starvation induced changes of insulin secretion, rather than a defect in glycolysis (Bouman, 1979). This may also account for the reduced insulin biosynthesis in starvation (Bone, 1977), since cyclic AMP appears to have a regulatory rôle on the glucose sensitive insulin-synthesising mechanism (Maldonato, 1977). Conversely, the long-term trophic effect of glucose on insulin secretion is reflected in the rise in adenylate cyclase activity after exposure of B cells to hyperglycaemia (Howell, 1973b) or high glucose concentrations *in vitro* (Howell, 1973b; Andersson, 1972).

In view of the changes in the pattern of insulin secretion and biosynthesis produced by thyroid hormones, and its similarity to that observed after starvation, it seemed possible that thyroid hormones could evoke alterations in cyclic AMP though a direct effect on adenylate cyclase activity. The results have failed to show any

significant change in the activity of this enzyme, in keeping with other reports of a lack of effect of thyroid hormones on adenylate cyclase activity in the hepatic and cardiac tissue from hyperthyroid animals (Jones, 1972; Sobel, 1969). Cyclic AMP concentrations may be affected by substrate ATP availability. However, despite a 20% fall in the hepatic ATP content of euthyroid rats treated with tri-iodothyronine (Ismail-Beigi, 1973), a similar decrease has not been found in the endocrine pancreas (Lenzen, 1978b), liver (Chilson, 1959) and heart muscle (Piatnek-Leunissen, 1967) from hyperthyroid animals. In these experiments, the finding that insulin secretion, itself an ATP dependent process, can be restored by theophylline makes an overall fall in cellular ATP concentration seem unlikely. However, thyroid hormones may lead to a speculative fall in local ATP levels close to the plasma membrane. The existence of a local pool of ATP has pre-viously been suggested in rat muscle cells (Reporter, 1972) and rat superior cervical ganglia (Lindl, 1975). Davis and Lazarus (1976) have reinforced this theory using *in vitro* preparations of mouse islet B-granules and cod islet plasma membranes. Their studies on the con-ditions leading to granule-membrane interactions and subsequent insulin release suggest that compartmentation of ATP at the plasma membrane is crucial both for initiating and maintaining insulin secretion. Thus, if such a membrane-associated ATP pool was reduced by thyroid hormones, the additional stimulus produced by theophylline, in raising cyclic AMP levels, may be necessary for normal insulin secretion to take place.

CHAPTER 8

GENERAL DISCUSSION

Thyroid hormones are known to impair glucose tolerance. Hyperthyroidism may precipitate overt diabetes in susceptible animals (Houssay, 1944 and 1948) and humans (Regan, 1940), and frequently exacerbates the metabolic derangement in pre-existing diabetes (Lakin, 1961). Various theories put forward to explain these phenomena have failed to differentiate between the possible primary effects of an excess of thyroid hormones on insulin secretion, and the multitude of diverse metabolic changes that occur in hyperthyroidism, with secondary sequelae. Consequently, '*in vivo*' studies of hyperthyroidism and carbohydrate tolerance have yielded complex and contradictory results. The work here has, within the methodological limitations outlined below, contributed both to the biochemical information of the basis of thyroid hormone and B cell interactions, and to clinical understanding of the relationship between hyperthyroidism and diabetes.

Methodological Considerations

The development of organ culture techniques for endocrine pancreatic tissue has encouraged an '*in vitro*' appraisal of insulin secretion, and has made it possible to examine the direct effects of any one factor on B cell function, whilst maintaining the stability of cellular surroundings. How far one is justified in extrapolating the conclusions from this particular approach to the intact animal is still questionable. Living tissues exist in dynamic interactions, and this has to be borne in mind when interpreting such '*in vitro*' models. Nevertheless, these methods have provided a valuable way

to disentangle the numerous elements involved in the control of hormone release.

The following considerations emerged in a retrospective analysis of experimental methods:-

1.   The tissue culture technique used in this study demonstrated the general drawbacks which are known to occur in the '*in vitro*' maintenance of tissues other than islets; although cellular viability is retained, there is a loss of the more specific features of cellular activity.   In this case, the sensitivity and capacity of islets to respond to glucose was diminished, even after 24 hours.   It seems that indeterminate constituents of serum can abrogate these disadvantages to some degree (Whittaker, 1977; Andersson, 1978), and in retrospect, perhaps a higher concentration of serum should have been included in the tissue culture media.   The maintenance of islets in artificial capillary units (Amikon Ltd.) with continuous perfusion in fresh medium (Tze, 1977) may provide a means of producing a closer approximation to stable physiological conditions.

2.   The measurement of (pro)-insulin biosynthesis (Chapter 6) by gel filtration of TCA-precipitable islet proteins did not give highly accurate results.   It has now been superseded in most laboratories by the use of anti-insulin serum covalently coupled to a solid phase, usually cyanogen bromide-activated Sepharose 4B (Pharmacia Ltd.) which is used to separate immunoreactive pro-insulin and insulin from other islet proteins (Berne, 1975).   However, attempts to utilise the latter technique proved unsuccessful.   Sepharose-bound normal guinea pig serum designated as a control in the separation of (pro)-insulin had a high binding affinity for radio-labelled islet proteins, so that

represent a similar long-term influence of thyroid hormones on pancreatic B cells. By using a tissue culture system for isolated islets from euthyroid rats, it was possible to investigate the direct effects of thyroid hormones on islet function, without the secondary metabolic complications of hyperthyroidism.

The present studies on the short-term influence of thyroid hormones on isolated islets showed that thyroid hormones, in the concentrations used, had no immediate effect on insulin secretion in response to glucose. However, after 24 hour exposure of islets to the presence of thyroid hormones, both the subsequent glucose stimulated insulin biosynthesis, and secretion were significantly reduced. This effect was apparent at higher concentrations of glucose (20 mmol/l) and was consistent with similar reports of selective inhibition of insulin secretion from pancreatic pieces or the perfused pancreas from hyperthyroid rats (Malaisse, 1967c; Lenzen, 1975). These observations may have been reflected in a fall in islet insulin content after thyroxine treatment. While this work was in progress, Lenzen reported that hyperthyroid ob/ob mice did not develop changes in the insulin content of isolated islets, although he detected a fall in total pancreatic insulin content, and postulated a reduction of individual islet cell volume in relation to the total pancreatic parenchyma, probably due to shrinkage of B cells rather than a quantitative change in cell number (Lenzen, 1978b). In accordance with this proposal, rat islets treated with thyroid hormones for 24 hours *in vitro* also had a normal insulin content, but a 20% fall in protein content. A lack of degenerative changes in the islets from hyperthyroid ob/ob mice on histological examination (Lenzen, 1978b), together with the normal ultrastructural appearance of thyroxine treated rat islets,

precludes the possibility of a simple toxic effect of thyroid hormones on B cells. In addition, the reversibility of the suppression of glucose induced insulin secretion in thyroxine treated islets by theophylline, or by intermediates of glycolysis in islets from hyperthyroid rats (Lenzen, 1976a), further implies a specific effect of thyroid hormones on B cell metabolism.

The characteristics of islets resulting from thyroid hormone treatment are strikingly similar to those observed after starvation (Malaisse, 1967e; Levy, 1976). Malaisse and collaborators have proposed that reduction of glucose phosphorylating activity and of phosphofructokinase in B cells may be critically important in mediating the reduced secretory response of islets from starved rats (Levy, 1976). Reports of thyroid hormone action on carbohydrate metabolism almost uniformly suggest that an increase in glucose oxidation and glycolysis occurs (Hoch, 1974; Lenzen, 1978b), so that a reduction of glycolytic activity of the type demonstrated in starvation (Malaisse, 1976) would be unlikely to account for the inhibition of insulin secretion by thyroid hormones. Adenylate cyclase activity is reduced in islets from starved animals, probably as a result of hypoglycaemia (Howell, 1973b). Glucose normally exerts a long-term "trophic" influence on this enzyme in islets (Howell, 1973b), and in the presence of a normal glucose concentration, thyroid hormones did not elicit a similar change in adenylate cyclase activity.

Possible Mechanisms of Thyroid Hormone Action on B Cell Metabolism

Magnus-Levy introduced the fundamental observation in 1895 that oxygen consumption was higher in hyperthyroid patients. Originally,

thyroid hormones were postulated to uncouple mitochondrial oxidative phosphorylation (Loomis and Lipman, 1948), but this hypothesis has not been upheld. Coupled oxidative phosphorylation was demonstrated in mitochondria from hyperthyroid patients (Stocker, 1968), and thyroxine administration *in vivo* increased the respiratory and phosphorylating capacity of rat mitochondria (Tata, 1963; Gustafsson, 1965). In fact, the actions of thyroid hormones at the intracellular level are so diverse that no single coherent explanation exists at present to account for all the observed effects. A wide variety of binding proteins for thyroxine and tri-iodo-thyronine have been identified in the cytosol and nuclear material of several tissues known to be sensitive to thyroid hormones (Rall, 1978). Generally, it is held that thyroid hormones enhance the synthesis of messenger RNA by binding to nuclear proteins, with possibly similar, but at present still obscure, effects on mitochondrial protein synthesis (Tata, 1966; Rall, 1978). The net result is the induction and increased activity of various enzyme systems, including the glycolytic enzymes, α-glycerophosphate dehydrogenase being the most strikingly affected (Rall, 1978).

Recent evidence suggests that thyroid hormones regulate oxygen consumption and thermogenesis by stimulating the mechanisms involved in maintaining the intracellular ionic composition of hormone-sensitive tissues (Ismail-Beigi, 1977). Cells establish and maintain a constant potential difference through the selective transport of Na^+ and K^+ ions. The membrane-bound Na^+ K^+-ATPase enzyme system constitutes an integral part of this process, and 20-45% of the total cellular energy expenditure is used by this 'sodium pump' (Skou, 1965; Glynn, 1975). Thyroid hormones increase sodium pump activity.

Muscle tissue from experimentally hyperthyroid rats has a raised membrane potential difference for sodium and potassium, and a fall in intracellular sodium (Ismail-Beigi, 1971). Studies on $^{22}Na^+$ pre-labelled rat liver slices show that the rate of sodium efflux is stimulated in hyperthyroidism (Ismail-Beigi, 1973). Finally, direct measurements of $Na^+ K^+$-ATPase in rat skeletal, hepatic and renal tissue after administration of tri-iodo-thyronine to euthyroid animals show a significant increase in $Na^+ K^+$-ATPase activity (Israel, 1973; Ismail-Beigi, 1977). Thus a major mode of action of thyroid hormones is the stimulation of energy utilisation by activating transmembrane sodium transport, leading in turn to increased ADP formation and a consequent rise in mitochondrial oxygen consumption (Rall, 1978).

The implications of such a theory of thyroid hormone action are two-fold in respect to B cell function. Firstly, $Na^+ K^+$-ATPase has been identified in the plasma membrane fractions of rat pancreatic islets (Lernmark, 1976; Kemmler, 1977), with an activity comparable to that in other mammalian tissues (Kemmler, 1977). A thyroid hormone stimulated rise in enzyme activity could result in the reduction of a hypothetical membrane-associated ATP pool (Davis, 1976) through enhanced adenine nucleotide dephosphorylation, and reduced cyclic AMP production, as discussed in Chapter 7.

Secondly, the control of sodium entry into the B cell may be a fundamental event in the stimulation of insulin secretion (Milner, 1967). Insulin release is influenced both by intra- and extracellular sodium concentrations. Low extracellular sodium inhibits insulin secretion (Hales, 1968a), and agents such as oubain, or high extra-cellular potassium, which raise intracellular sodium, potentiate insulin release (Hales, 1968a and 1968b). Current evididence suggests

that sodium influx into the B cell modulates the cytosolic calcium

concentrations (Lowe, 1976; Donatsch, 1977), which may itself be an

essential event in the initiation of insulin secretion (as outlined

in Chapter 1). Measurements of islet insulin release in the presence

of classical potentiators or inhibitors of the sodium permeability of

excitable membranes, such as veratridine (Ohta, 1973) or tetrodotoxin

(Donatsch, 1977), indicate that a similar mechanism operates for

calcium translocation in the B cell as in the giant squid axon

(Donatsch, 1977).

The B cell membrane is depolarised by a glucose stimulus (Dean,

1970), and calcium enters the cell as part of this electrochemical

event (Donatsch, 1977). Early in the generated action potential, the

patent sodium channels allow calcium entry. Membrane depolarisation

enhances further calcium influx late in the action potential, through

voltage-dependent calcium channels. These can be blocked by cobalt

chloride or D600 (Baker, 1975), and the subsequent reduction of insulin

release (Henquin, 1975; Malaisse, 1976a) implicates the voltage-

dependent channels as being the most significant means of calcium entry

(Donatsch, 1977). Consequently, the activity of the sodium pump may

represent an important factor in the control of membrane permeability

to calcium. The sodium-calcium counter transport system (Blaustein,

1974) may also be involved in modulating cytosolic calcium levels,

through the inhibition of calcium efflux, as a result of an increased

competition for the transmembrane carrier by high intracellular sodium

(Donatsch, 1977). Intracellular potassium levels are also important

for insulin secretion (Hales, 1968a); it has been postulated that

early membrane depolarisation is due to a reduction in the potassium

permeability of the B cell membrane (Henquin, 1978), since glucose

markedly diminished the efflux rate of $^{42}K^+$ from prelabelled islets (Henquin, 1978).

In summary, the Na^+ K^+-ATPase system may constitute an integral part of the regulation of B cell cytosolic calcium concentration. Despite the lack of effect of important insulin secretagogues, including glucose and sulphonylureas, on Na^+ K^+-ATPase activity (Kemmler, 1977), the function of the enzyme is altered by other agents influential in B cell secretion. Oubain inhibits Na^+ K^+-ATPase activity in rat pancreatic islets (Kemmler, 1977). Interestingly, although the observation has not been verified in pancreatic islets, cyclic nucleotides are highly effective inhibitors of Na^+ K^+-ATPase in rat liver plasma membranes (Tria, 1974) and human gastric mucosal cells (Mozsik, 1970). Of the various cyclic nucleotides tested, cyclic AMP was the most effective and its inhibitory action was further reinforced by theophylline (Tria, 1974). In the absence of cyclic nucleotides, phosphodiesterase inhibitors *per se* depressed Na^+ K^+-ATPase activity (Tria, 1974).

Thus, thyroid hormone induction of Na^+ K^+-ATPase activity or reduction of cyclic AMP levels, may have profound effects on movements and concentrations of cations, in particular through decreasing cytosolic calcium accumulation. Consistent with this is the finding of a significant decrease in glucose stimulated $^{45}Ca^{++}$ uptake in islets from hyperthyroid ob/ob mice, after fasting for three days (Lenzen, 1978b), but further evidence of changes in B cell calcium regulation in hyperthyroidism is required to substantiate this hypothesis. It is not known whether the reduction in islet cyclic AMP levels in starvation (Bouman, 1979), through alterations in adenylate cyclase activity, also lead to an inhibition of insulin secretion by analogous mechanisms.

Clinical Implications

The long-term influence of thyroid hormones on pancreatic islets described in this work has important clinical implications. The possible development of diabetes mellitus during uncontrolled hyperthyroidism should be borne in mind, particularly in view of the similarity of some of the clinical features of both diseases. Although the inhibition of insulin secretion brought about by thyroid hormones may not be evident during fasting, it may be that B cells cannot respond adequately to the fluctuations in blood glucose levels, which result from the effect of an excess of thyroid hormones on other tissues. This is particularly evident shortly after meals when hyperthyroid patients show raised blood sugar levels (Loeb, 1978). Both the results presented here and the observations on pancreata from hyperthyroid animals (Lenzen, 1978a) indicate that the adverse effect of thyroid hormones on B cell function occurs at higher glucose concentrations. Thus, a consistently poor insulin response to carbohydrate intake may ultimately lead to the irreversible "exhaustion" of B cells which was proposed by Houssay. In his experiments with partially depancreatectomised dogs, he demonstrated that "thyroid diabetes" could progress to permanent B cell destruction if administration of thyroid extracts to the animals was continued (Houssay, 1944 and 1948). This suggests that the duration of excess circulating thyroid hormones may have repercussions on the reversibility of alterations in B cell function. Perhaps the conflicting data on subsequent glucose tolerance when hyperthyroid patients become euthyroid is a reflection of this, since authors have rarely reported the estimated duration of abnormal thyroid function when

assessing their conclusions. Also, as mentioned earlier, the
higher incidence of diabetes in patients with toxic nodular goire
rather than Graves' disease (Regan, 1940) may be due to the earlier
treatment of the latter group, who tend to exhibit more pronounced
signs of hyperthyroidism. Therefore, hyperthyroidism should be
promptly controlled, and a surveilance kept for carbohydrate intoler-
ance. Similarly, in diabetic patients, metabolic instability can be
a sign of the onset of concurrent hyperthyroidism, and may indicate
further B cell damage.

Further research into thyroid hormone action on pancreatic islets
may add to the current theories of the long-term control and mechanisms
of insulin secretion. Insulin secretion from the perfused pancreas of
hyperthyroid rats is in many ways reminiscent of the pattern of
secretion in non-insulin dependent diabetes (Lenzen, 1978a) and this
has led to the suggestion that hyperthyroid animals could constitute a
valid experimental model for the investigation of maturity onset
diabetes (Lenzen, 1978a).

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PUBLICATIONS

Vrbova, H., Theodorou, N.A., Tyhurst, M. and Howell, S.L. (1979). Transplantation of Islets of Langerhans from Pilocarpine Pre-Treated Rats - A Method of Enhancing Islet Yield. Transplantation (in press).

APPENDIX

This work was done in collaboration with N.A. Theodorou, M. Tyhurst and S.L. Howell.

THE USE OF PILOCARPINE TO ENHANCE ISLET YIELD FROM RAT PANCREAS

Introduction

The low yield of islets that was obtainable from each rat pancreas presented a problem throughout the experiments described in this thesis, and is one of the major drawbacks in studies using isolated islets. In general, less than 20% of the endocrine pancreas can be separated from exocrine tissue using current techniques.

Kuo and colleagues (1973) achieved significant improvements in the yield of isolated islets by pretreatment of animals with pilocarpine to reduce the exocrine enzyme content, but their method has not been widely adopted for studies of islet metabolism '*in vitro*' because of alterations in insulin secretion from islets isolated in this way (Atkins, 1975). We have investigated the feasibility of using pilocarpine to enhance islet yield in more detail, and attempted to reverse the possible deleterious effects of pilocarpine on islet function by maintaining islets in tissue culture following isolation.

Methods

Pretreatment of rats and islet isolation

Fed rats (inbred WAG strain, 200-250 g) were injected intraperitoneally with a solution of 4% pilocarpine (Sigma, London) in normal saline (40 mg/kg body weight), 1-1½ hours prior to sacrifice by cervical dislocation, and their pancreata were removed for islet isolation by the collagenase digestion procedure described in Chapter 2. A standard bicarbonate-buffered medium (Gey and Gey, 1936) containing 5.5 mmol/l glucose and gassed with 95% O_2: 5% CO_2 was used throughout,

and the number of islets was noted at each isolation.

Insulin content of islets during 24-hour tissue culture

The insulin content of isolated islets from control and pilocarpine pretreated rats was determined at isolation and after tissue culture. According to the previously described method, the islets were incubated in TCM 199 supplemented with 5% new-born calf serum, antibiotics and glucose to achieve a concentration of 20 mmol/l, at 37°C. Groups of islets were removed and homogenised in acid-ethanol (as described in Chapter 5) at 0, 2, 4, 6 and 24 hours of culture and the extracted insulin was measured by immunoassay (Chapter 2).

Pancreatic morphology

Freshly isolated islets from pilocarpine pretreated animals were prepared as described in Chapter 5 for examination under the electron microscope.

Transplantation of islets

Streptozotocin 6 5 mg/kg in 0.05 M citrate buffer (pH 4.5) was injected into the tail vein of inbred WAG rats (200 g), at least 2 weeks prior to their use as recipients of transplanted islets. Only animals with at least 2 successive random blood glucose levels over 20 mmol/l and a urine volume greater than 50 ml/24 hours were used.

1000 or more islets, isolated from 4 pilocarpine pretreated donors, were suspended in 2 ml bicarbonate-buffered medium and injected into the superior mesenteric vein of a diabetic recipient (Fig. 21). The recipients were covered with penicillin (150,000 i.u.
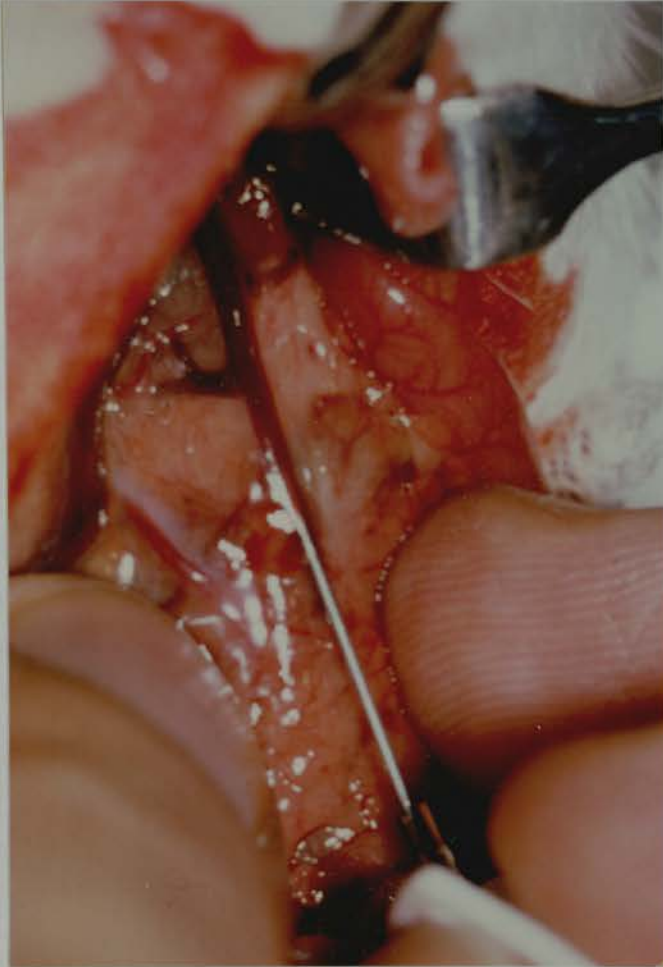
Figure 21   Intraportal transplantation of rat islets.   Isolated islets
were injected into the superior mesenteric vein of a diabetic rat
recipient, through a 25 G butterfly needle.

procainepenicillin, 150,000 i.u. benzathine penicillin) for 48 hours post-operatively, and their blood glucose concentrations and 24-hour urine volumes were subsequently measured at regular intervals. Blood glucose estimations were performed on non-fasting morning samples of blood using a glucose oxidase method (God-Perid, Boehringer, London).

Results

Islet yield and insulin content

The collagenase procedure for isolation of islets gave a mean of 686±45 islets in 10 consecutive isolations from groups of 4 non-pilocarpine pretreated control donors. Pretreatment of rats with pilocarpine significantly increased the mean islet yield to 1189±34 from 4 pancreata in 34 successive isolations ($p < 0.001$).

Fig. 22 shows the insulin content of islets at isolation and after 24 hours. The insulin content of freshly isolated islets from pilocarpine pretreated donors was considerably lower than that of controls ($p < 0.05$). The insulin content of control islets fell progressively during the 24 hour culture period (Fig. 22), and the final content was 60% of the original ($p < 0.001$). However, the disparity between test and control islets was not reversed after tissue culture since both lost insulin at similar rates, and the significant decrease in the insulin levels of test islets persisted throughout the 24-hour period ($p < 0.02$), as shown in Fig. 22.

Ultrastructure of islets

The ultrastructural appearance of islets isolated from pilocarpine pretreated rat pancreas did not show any abnormalities, as illustrated in Fig. 23.
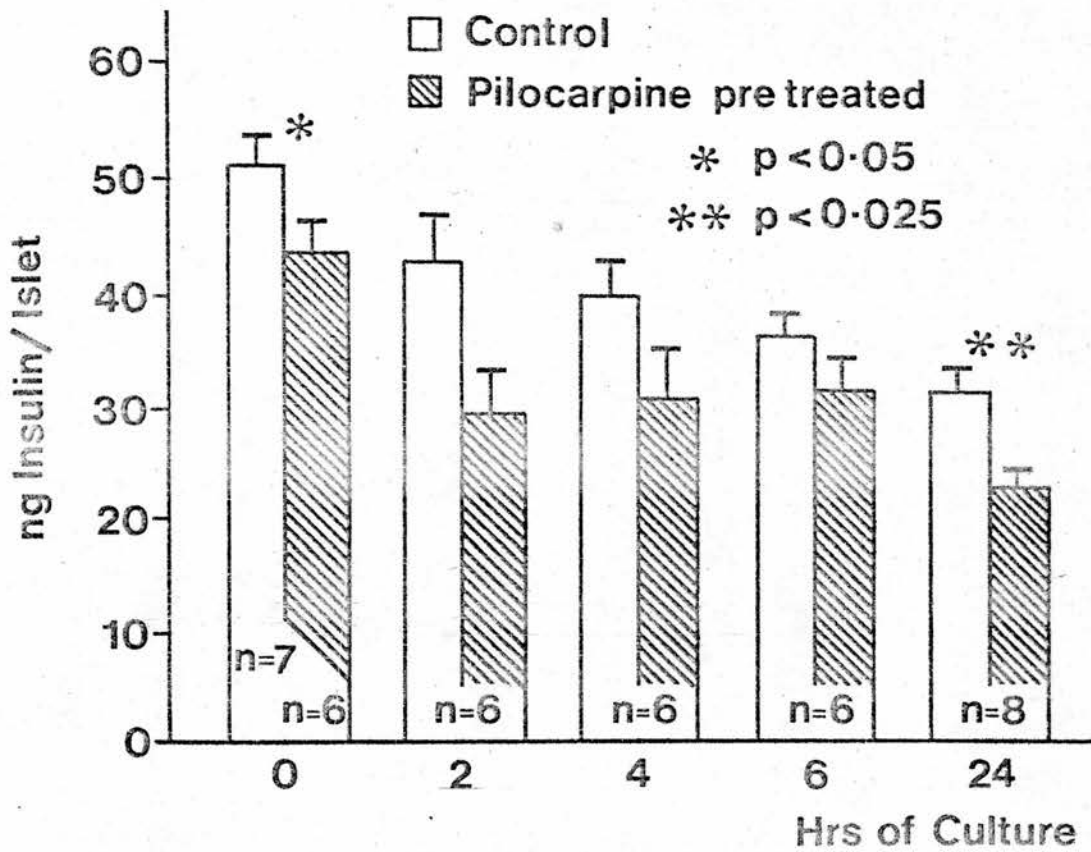
Figure 22 Insulin content of isolated islets from normal and pilocarpine-pretreated rats, at isolation and after culture for up to 24 hours. Results are expressed as Mean ± SEM of the number of observations shown. * and ** indicate significant difference (p < 0.05 and <0.025) between insulin content of islets from control and pilocarpine pretreated animals at the same time.
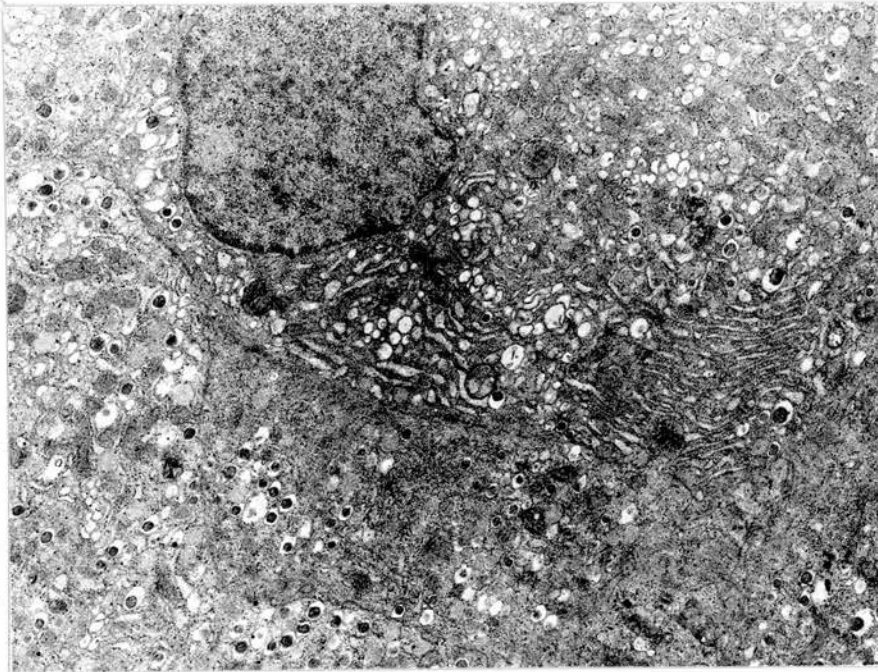
Figure 23 (x7000 magnification)

Electron micrograph of B cells from an islet isolated from the pancreas of a rat which had been pretreated with pilocarpine (40 mg/kg) 1-1½ hours before sacrifice, as described in the text.

Transplantation of islets

The blood glucose concentrations of recipient rats before and after transplantation of islets from pilocarpine pretreated donors are shown in Fig. 24. In six successive experiments the streptzotocin-induced diabetes was effectively reversed within two weeks of transplantation, as judged by random blood glucose estimations of less than 10 mmol/l, the absence of glycosuria and urine volumes of less than 10 ml/24 hours. These six animals have remained normoglycaemic for at least 6 months. No amelioration of diabetes has been observed in three control diabetic animals which did not undergo transplantation. One of these animals has developed bilateral corneal cataracts, a characteristic complication of long-standing diabetes, shown in Fig. 25b, and compared with the appearance of a non-diabetic rat in Fig. 25a.

Discussion

The number of islets obtained from control donors was comparable to that reported by Pipeleers *et al.* (1976), and the results here confirmed the findings of Kuo *et al.* (1973) that pilocarpine was clearly useful for enhancing islet yield from rat pancreas. Nevertheless, the reduced insulin content of islets from pretreated rats indicated that they were not comparable to normal islets. In an attempt to restore the depleted insulin content of islets isolated from pilocarpine pretreated rats to normal levels, the islets were maintained in tissue culture for 24 hours. The insulin content of control islets fell during this period, as described by other workers (Andersson, 1974),but the comparative reduction of insulin stores produced by pilocarpine pretreatment was not reversed by a period of tissue culture. Therefore,
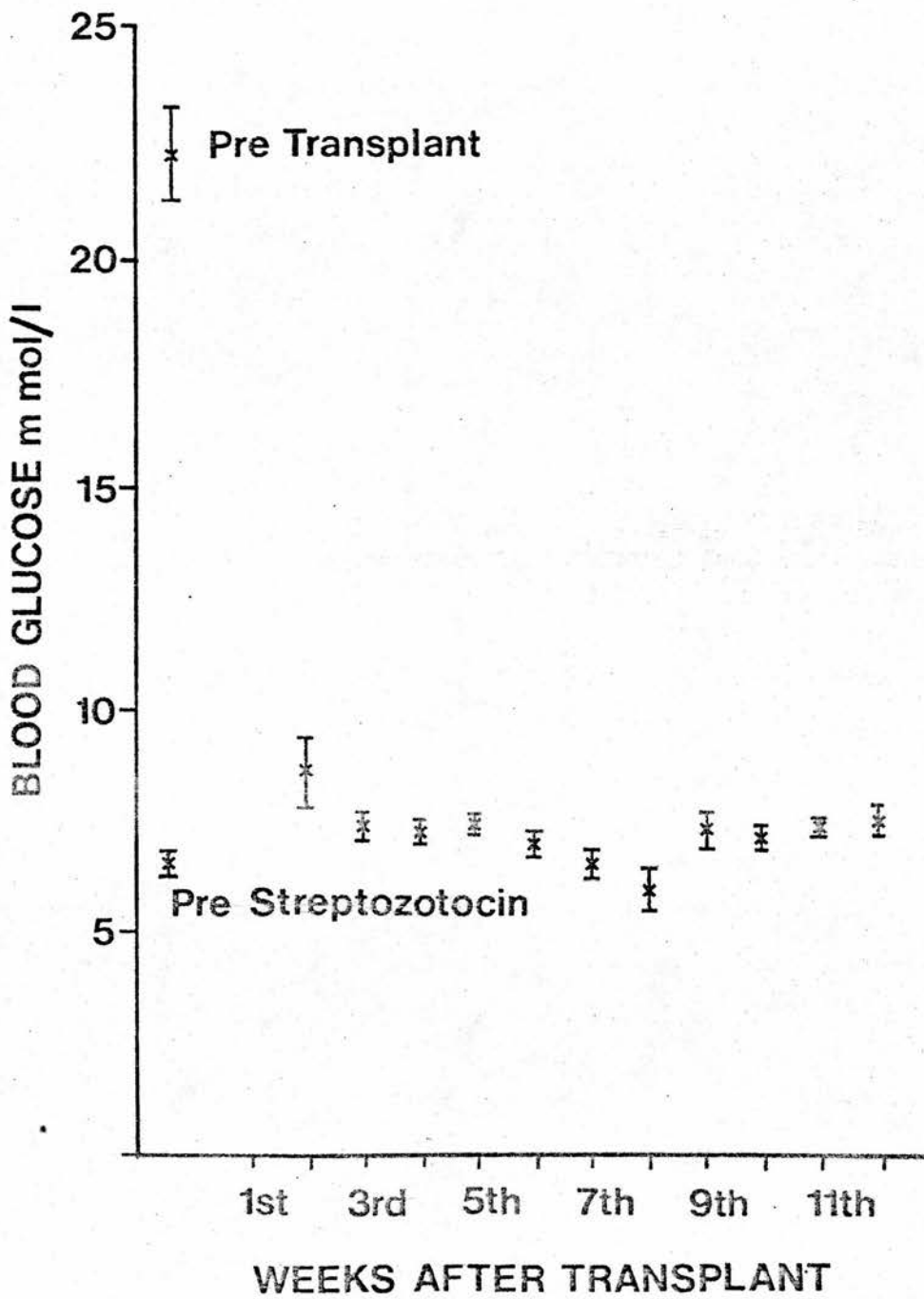
Figure 24 Random blood glucose concentrations of rats before and after transplantation of 1000+ islets from pilocarpine-pre-treated rats via the portal vein. Results shown are Mean ± SEM for 6 consecutive transplants.

Figure 25a Normal, non-diabetic rat.

Figure 25b Diabetic rat which has developed bilateral corneal
cataracts.

islets from pilocarpine pretreated animals were not considered
suitable for the investigation of islet function and metabolism in
short-term '*in vitro*' studies, even after a 'recovery' period of
24 hours in tissue culture.

However, pilocarpine pretreatment of rats undoubtedly enhanced
islet yield from the pancreas. In view of the normal ultrastructure
of B cells from islets of pilocarpine pretreated rat pancreas, it
seemed possible that the method could have an application in experi-
ments where a large quantity of islets and their long-term survival
were the important criteria - islet transplantation provides one
such application.

Transplantation of isolated rat islets from adult animals to
diabetic recipients has become a valuable and widely-used model for
the investigation of transplantation of the endocrine pancreas. The
reversal of streptozotocin-induced diabetes in rats by syngeneic
intraperitoneal transplantation of collagenase isolated islets was
first reported by Ballinger and Lacy (1972), but it was clear that the
amount of donor islet tissue used was an important factor in the
degree of amelioration of diabetes (Ziegler, 1974). If the number of
viable transplanted islets was below a minimal level, the experimental
animals reverted to a diabetic state, probably through exhaustion of
the B cells by overstimulation (Ziegler, 1974). Successful trans-
plantation relies on the long-term viability of islets, and an initial
disturbance of B cell response to hyperglycaemia may be unimportant.
Therefore we investigated the possibility of using islets from pilo-
carpine pretreated rats for transplantation.

Preliminary studies, and the available literature (Schulak,
1978) indicated that intraportal transplantation of 1000 or more islets

from untreated rats was sufficient to consistently reverse streptozotocin-induced diabetes in syngeneic recipients. An identical procedure was adopted for transplantation of islets from pilocarpine pretreated donors. It was clear that such islets could effectively reverse the diabetic state in the recipients, and pilocarpine pretreatment was evidently useful in experimental work on transplantation.

Conclusion

Pretreatment of rats with pilocarpine effectively enhanced the subsequent yield of isolated islets, but these islets were judged to be unsuitable for use in metabolic studies in view of the observed depletion of insulin stores. Nevertheless, they evidently retain their capacity to secrete insulin, and pilocarpine pretreatment provides a valuable means of increasing the yield of islets from rat pancreas for transplantation.

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