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AUTONOMIC NERVOUS FUNCTION IN EXPERIMENTALLY DIABETIC RATS:
THE EFFECTS OF ALDOSE REDUCTASE INHIBITION, DIETARY MYO-INOSITOL AND THYROID HORMONE REPLACEMENT

By

ASIF MOHAMMED SARDAR

PhD

LOUGHBOROUGH

© A. M. SARDAR, 1992
TO
MY
FATHER
MOHAMMED SARDAR (Late)
&
MOTHER
NAZIRAN BEGUM
AKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr P D Lucas, for his continued support and encouragement throughout this project.

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I am also indebted to my wife Zarina Sindhu Sardar.

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'Ponalrestat' is a trademark, the property of Imperial Chemical Industries Pharmaceuticals.
SUMMARY

Neuropathy, a common complication of human diabetes is not prevented by current antidiabetic therapy. Several mechanisms, some reversible, have been proposed. Clinical assessment of drug efficacy in this condition is difficult because of its slow and unpredictable development & its possible irreversibility, once established. A reliable animal model of diabetic neuropathy would be very useful. Changes such as reduced nerve conduction velocity are used as models but their relationship to neuropathy is uncertain. The main purpose of this study was to examine autonomic changes in the experimentally diabetic rat with the aim of identifying more appropriate models. The effects of three treatments which correct specific biochemical abnormalities which may underlie diabetic complications, were also studied.

Autonomic function was assessed by measuring responses to nervous stimulation and exogenous transmitters. In some tissues $^3$H-noradrenaline uptake and release and Na$^+$K$^+$ ATPase activity was measured. Since diabetes induced changes are often time dependent, three periods of diabetes, six weeks, six months and one year were used.

Diabetes induced changes in sensitivity and maximum responses to noradrenaline were observed in aorta (increased sensitivity and reduced maximal responses in 6 month animals and decreased sensitivity in 1 year animals), anococcygeus (increased sensitivity in 6 month animals) & vas deferens (increased maximal response in 6 month animals). Reduced responses to both exogenous acetylcholine and nervous stimulation were observed in ileum, but not in oesophagus. Most of these changes appeared to be due to post-synaptic rather than nervous changes. These results, therefore, do not provide evidence of significant neuropathy in the tissues examined after up to 1 year of diabetes. This questions the link between changes which occur after only one or two weeks of diabetes such as reduced nerve conduction velocity and diabetic neuropathy and hence their validity as models of the latter. It may be that these changes, together with other unknown ones, contribute to neuropathy in humans.

Reduced Na$^+$K$^+$ ATPase activity may contribute to neuropathy and other diabetic complications. Activity was reduced in skeletal muscle from diabetic rats. The latter were hypothyroid & normalising plasma T3 levels by s.c. T3 also prevented Na$^+$K$^+$ ATPase activity depression. Hypothyroidism, which also occurs in human diabetics, might, therefore, underlie this change.
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INTRODUCTION
1.1 HISTORICAL BACKGROUND

Diabetes, the Greek for syphon, as a human condition was first identified in the First Century by Arteus of Cappadocia. It was only in the Seventeenth Century that Thomas Willis joined the term Mellitus, Latin for honey, to describe the excess glucose present in urine in diabetic patients (Lewis, 1980).

In the Eighteenth Century the condition was linked to a pancreatic disorder and this was confirmed by Minkowski & Meyer (1889). In a classic experiment they induced diabetes in a healthy dog by removing its pancreas.

Sharpey-Schäfer and de Meyer, both working independently in the 1910's, postulated that the previously identified Islets of Langerhans were responsible for secreting a hormone which controlled carbohydrate metabolism. They termed the hormone "INSULINE".

Banting & Best (1922) extracted the hormone from a foetal calf pancreas, and succeeded in reducing the blood glucose level of a diabetic dog. Previously insulin extraction had been hampered by digestive juices in the pancreas which destroyed the hormone during the process. A foetal pancreas however has no digestive juices and therefore the hormone was preserved. Soon after this, in 1927, the hormone was extracted, purified, and crystallised by John Abel (Murnaghan & Talalay, 1967).

It was nearly three decades later when the next pioneering steps were taken. Ryle and his colleagues, working at Cambridge, carried out the Nobel Prize-winning identification of the 51 amino acid residue sequences in bovine insulin (Ryle et al, 1955). Following this Blundell and co-workers successfully analysed the crystal structure of insulin (Blundell et al, 1972).
Progress has carried on a pace to the present day. Nowadays synthetic human insulin is commonly used in place of insulin from animal sources. The effects of diabetes are just beginning to be fully understood; but all this would not have been possible without the innovative work of the early researchers.

1.2 THE BIOSYNTHESIS, SECRETION & ACTION OF INSULIN.

1.2a The Pancreas.

The mammalian pancreas develops from two diverticula of the duodenum, one forming part of the head and the other forming the rest of the head, the body and the tail (Lacy & Greider, 1979). The islets of Langerhans are scattered throughout the mammalian pancreas, with the greatest number in the tail (Opie, 1900; Bellman, 1959 & 1970). The total mass of the islets comprises approximately 1-2% of the wet weight of the adult human and rat pancreas (Lacy & Greider, 1979).

The main part of islet population consists of medium-sized islets, approximately 200μm in diameter; and these number approximately 876,00 in the human adult (Clarke, 1913), and 13,500 per rat pancreas (Haist & Pugh, 1948).

The islets are arranged as cords along the capillary channels within the islets and individual cells are surrounded by distinct plasma membranes (Berlin et al, 1975).
1.2b Pancreatic Cell Types.

There are 6 types of cell occurring in the pancreas, of which only three are presently thought to be involved in blood glucose regulation.

The alpha (α) cells have granules which are round with a close membranous sac, and the center is extremely electronically dense with a less dense area surrounding it. The distribution of α cells varies in different species: in the rat & mouse they form a rim in the islet; whereas in man they are located in the center (Lacy & Greider, 1979). Alpha cells comprise only 15-20% of the islet population in mammals and are responsible for producing and storing glucagon.

Beta (β) cell granules vary in species (Lacy, 1957): in the rat and mouse they are round and relatively dense with a space between the granules and the membranous sacs; whereas in man they have a rectangular shape (Lacy & Greider, 1979). Beta cells comprise approximately 75-80% of the islet population in the adult human; and are responsible for producing, storing, and releasing insulin.

C cells were first described in 1911 (Bensley, 1911) as a clear cell in the islets of the guinea pig pancreas. The cell has no secretory granules, a few mitochondria, and a small amount of endoplasmic reticulum (Lacy, 1957; Caramia et al, 1965; Lacy & Greider, 1979).

Delta (δ) cells (Bloom, 1931) have large round secretory granules (250-450nm) with a low electron opacity. Although the gastrin-secreting cells in this group contain small granules (150-250nm) that are electron opaque (Lacy & Greider, 1979). The δ cells constitute roughly 5% of the population and are responsible for gastrin and somatostatin production (Brazeau et al, 1973; Koerker et al, 1974).

E cells have only been identified in the oppossum pancreas (Thomas,
1937) and are characterised by relatively large (350-400nm), irregular shaped granules of moderate electron density (Munger et al, 1965).

F cells, originally called X cells (Bencosme & Leipa, 1955), were located in the dog in the islets of the uncinate process of the pancreas and in the cat, where they are distributed throughout. The F cells have moderately electron dense secretory granules which have a wide range of shapes and sizes (Munger et al, 1965).

1.2c Proinsulin

The discovery of proinsulin dispelled the generally held idea that insulin was formed by the combination of separately synthesized A & B chains (Steiner et al, 1969; Steiner & Oyer, 1967). Proinsulin is formed in the rough endoplasmic reticulum and undergoes cleavage to yield insulin and C-peptide (See figure #1 & #2).

Wheat germ ribosomal systems have been used to show the existence of a precursor to proinsulin, preproinsulin, which is a peptide with a molecular mass of 11,500 (Lernmark et al, 1976; Chan et al, 1964 & 1976; Permutt et al, 1976; Lomedico & Saunders, 1976). Preproinsulin consists of proinsulin with N-terminal peptide extensions that are 23 residues long (Chan et al, 1976). This peptide has a very rapid turnover (Patzelt et al, 1980) and is probably cleaved even before the peptide chain is completed (Steiner & Tager, 1979).

Proinsulin is very similar to insulin in many properties, including solubility, isoelectric point (Steiner et al, 1972), self-associative properties (Steiner et al, 1971), and reactivity to insulin antisera (Steiner et al, 1969; Rubenstein et al, 1969 & 1970). It has been suggested that this similarity in properties is due to the conformation of the insulin moiety in proinsulin being very nearly identical to
**FIGURE #1**

**STRUCTURE OF HUMAN PROINSULIN**

![Diagram of human proinsulin structure](image)

Human insulin consists of chains A & B, the connecting peptide being between X & Y; whilst the C-peptide is between P & Q.

<table>
<thead>
<tr>
<th>AA Res. - Amino Acid Residues</th>
<th>Points of variation in insulin structure in species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue Numbers</td>
<td></td>
</tr>
<tr>
<td>A Chain</td>
<td></td>
</tr>
<tr>
<td>1  8  9  10  1</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Thr  Ser  Ileu</td>
</tr>
<tr>
<td>Porcine</td>
<td>Thr  Ser  Ileu</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Thr  Ser  Ileu</td>
</tr>
<tr>
<td>Beef</td>
<td>Ala  Ser  Val</td>
</tr>
<tr>
<td>B Chain</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td></td>
</tr>
<tr>
<td>Asp - Aspartic acid</td>
<td>Cys - Cysteine</td>
</tr>
<tr>
<td>Glu - Glutamic acid</td>
<td>Gln - Glutamine</td>
</tr>
<tr>
<td>Ile - Isoleucine</td>
<td>Leu - Leucine</td>
</tr>
<tr>
<td>Met - Methionine</td>
<td>Phe - Phenylalanine</td>
</tr>
<tr>
<td>Ser - Serine</td>
<td>Thr - Threonine</td>
</tr>
<tr>
<td>Tyr - Tyrosine</td>
<td>Val - Valine</td>
</tr>
</tbody>
</table>

**CODE FOR AMINO ACIDS**

- Ala - Alanine
- Asp - Aspartic acid
- Glu - Glutamic acid
- Ile - Isoleucine
- Met - Methionine
- Ser - Serine
- Tyr - Tyrosine

- Arg - Arginine
- Cys - Cysteine
- Gln - Glutamine
- Leu - Leucine
- Phe - Phenylalanine
- Thr - Threonine
- Val - Valine

- Asn - Asparagine
- Gly - Glycine
- His - Histidine
- Lys - Lysine
- Pro - Proline
- Trp - Tryptophan

(Figure from Lewis, 1989)
The structure of glucagon is shown and consists of 29 amino acid residues whilst that of the proglucagon extension is shown in brackets. (Both figures from Steiner & Tager, 1979)

See figure #1 for amino acid codes.
insulin itself (Steiner et al, 1972).

1.2d Biosynthesis of Insulin

Following biosynthesis in the endoplasmic reticulum and cleavage of the preproinsulin chain, peptide chain folding, and sulphydryl oxidation occur rapidly in the cisternal spaces. The folded proinsulin is then transported to the Golgi apparatus (a process requiring about 20 minutes) (Steiner et al, 1972; Howell et al, 1969; Orci et al, 1971; Jamieson & Palade, 1967a & 1967b; see figure #3). Newly synthesized peptide material approaches the Golgi body, as "progranules" or condensing vacuoles, and is taken in and formed into secretion granules which then pass out and migrate towards the membrane (Howell et al, 1969; Orci et al, 1971).

The conversion of proinsulin to insulin, a first-order reaction (Steiner, 1967), requires the joint action of trypsin like protease and carboxypeptidase B. The latter enzyme is necessary to remove the C-terminal basic residues left after tryptic cleavage, giving rise to C-peptide and insulin (Steiner & Tager, 1979) (see figure #2). As the secretory products move to the Golgi body and proteolysis begins, the cation arginine and lysine residues liberated during conversion may diffuse out of the granules and be replaced by hydrogen ions, resulting in a decrease in the intracellular pH (Steiner & Tager, 1979).

As the insulin is liberated from proinsulin it crystallizes with zinc atoms (Greider et al, 1969; Lange et al, 1972). The insulin is stored as a crystal complex of 6 molecules of insulin to 2 atoms of zinc. Together with insulin and C-peptide there are also present small amounts of proinsulin and a variety of other proteins in the granule (Steiner et al, 1972; Geller et al, 1972; Judah et al, 1973; Jacobson & Baltimore, 1972).
SCHEMATIC SUMMARY OF INSULIN BIOSYNTHESIS IN THE BETA CELL

AMINO ACIDS
TRANSFER RNA
ATP, GTP, Mg²⁺
ENZYMES

ANTI-MYCEIN BLOCKS

TRANSFER STEP 1
(energy dependent)

TRANSFER STEP 2

EARLY GRANULES

PROGRESSIVE
CONVERSION
(tₘ = -1 hour)

MEMBRANE
BOUND
PROTEASES

Zinc²⁺

Arg²⁺

Lys²⁺

MAINLY INSULIN
(Crystalloid) +
C-PEPTIDE

NATURAL
GRANULES

TRANSFER STEP 3
(energy & Ca²⁺
dependent)

SECRETED PRODUCTS
Insulin & C-peptide = 94%
Proinsulin + others = 6%

ENDOCYTOSIS
(EXOCYTOSIS)

PLASMA MEMBRANE

MEMBRANE
RECYCLING?

Figure from Steiner & Tager, 1979
(10)
Due to the localization of the proinsulin conversion process within secretion granules, the C-peptide accumulates along with insulin in equimolar amounts (Steiner et al., 1971) and is secreted along with the hormone by exocytosis of the granule contents (Klostermeyer & Hambel, 1966).

1.2c Release of Insulin

Beta cells secrete insulin in response to physiological levels of D-glucose and in the presence of substimulatory levels of glucose L-amino acids, fatty acids, and ketone bodies (Matschinsky et al., 1975; Pagliari et al., 1974; see figure #4). Acetylcholine, once again in the presence of substimulatory levels of glucose, also stimulates insulin release in amounts and duration very similar to glucose (Loubatieres-Mariani et al., 1973).

The cells release insulin in a biphasic manner in response to stimulus (see figure #5). Initially there is a rapid burst of insulin release following stimulation which reaches a peak after 1 minute; this is followed by a resting phase lasting several minutes and then by a slowly rising second phase (Matschinsky et al., 1979).

The dose-response curve for insulin release compared to glucose concentration is sigmoidal in shape, reaching a plateau at approximately 25mM glucose concentration, and corresponds to a decrease in glucagon secretion with the same levels of glucose (see figure #5). Some mechanisms have been proposed to account for the insulin release occurring in β cells (Ashcroft & Randle, 1975; Matschinsky et al., 1971; Matschinsky et al., 1972; Lambert, 1976).

1) The Receptor or Regulator Site Model proposes that the islets...
HYPOTHETICAL MECHANISM FOR INSULIN RELEASE

(Figure from Matschinsky, Pagliari, Zawalich, & Trus, 1979) (12)
FIGURE #5

INSULIN RELEASE and GLUCOSE EFFECT ON INSULIN & GLUCAGON RELEASE

STIMULATED INSULIN RELEASE FROM PANCREATIC BETA CELLS

EFFECT OF GLUCOSE ON INSULIN & GLUCAGON RELEASE

Relative Rate of Hormone Release

0 10 20 30 40 50 60 70

INSULIN

GLUCAGON
have receptors on their membrane surfaces capable of recognising the different agonists.

2) The Substrate Site Model suggests that recognition of a "trigger" molecule may be mediated through events connected with the metabolites of this molecule.

3) A combination of the above two working together simultaneously.

It is most likely that the third option offers a working model.

In insulin release the primary event is an alteration in ion conductivity caused by a molecule attaching itself to the receptor (Matschinsky et al., 1979; Mathews, 1975). This leads to opening of the calcium gates, which together with cAMP (Malaisse et al., 1971), acts as the signal for insulin release.

1.2f The Insulin Receptor

The insulin receptor is present in most human cells (Reddy & Kahn, 1988). The receptor is a large transmembranous glycoprotein of approximately 300kD and is made up of 2 extracellular α-subunits (135kD) and 2 transmembrane β-subunits (95kD each) united by disulphide bonds (see figure #6a). Both subunits contain carbohydrate chains capped by terminal sialic acid residues. The α-subunits contain the binding domain while the β-subunits contain the tyrosine kinase domain (Reddy & Kahn, 1988).

It is currently proposed that insulin binding to the α-subunit stimulates autophosphorylation of tyrosine residues on the intracellular domain of the β-subunit which, in turn, triggers the activation of phosphotransferases and catalyses tyrosine phosphorylation of specific substrates (Carpentier, 1989; Rosen et al., 1983; Yu & Czech, 1984; Herrera & Rosen, 1986).
Schematic view of the insulin receptor with its two external binding domains (α-subunits) linked by two disulfide bonds to two transmembrane domains (β-subunits) extending into the cytoplasm. On the outer portion of the β-subunits tyrosine residues become phosphorylated (P-Tyr) upon insulin binding to the α-subunits.

(Figure from Carpentier, 1989)
Following binding of the receptor and insulin, the aggregate moves in the plane of the plasma membrane to reach membrane invaginations or pits. These pits are lined with a protein coat, clathrin, which is associated with the cytoplasmic leaflet of the membrane (Carpentier, 1989; Pearse & Bretscher, 1981; Carpentier et al, 1986). The bound insulin is then internalized, through endocytosis, which results in the formation of a coated vesicle free in the subplasmalemmal cytoplasm; the life-time of the vesicle is only 1-2 minutes or less and the process by which this coat is shed is unknown (Carpentier, 1989; see figure #5b).

The endosomes, possessing proton pumps, quickly acidify the organelle; this acts to dissociate the insulin ligand from the receptor (Tycko & Mansfield, 1982). The liberated insulin then travels to the late endosomes which contain lysosomal enzymes and mannose-6-phosphate receptors. The insulin finally reaches a digestive vacuole where it is degraded, but the receptor itself is recycled to the cell surface (Brown & Farquhar, 1984; Gruenberg et al, 1989; Carpentier et al, 1979a & 1979b; Carpentier, 1989).

1.2g Action of Insulin

The principal action of insulin is at the cell surface where it regulates the redox-potential gradient across the membrane. It thus controls the energy available to the cell for its active glucose transport into the cell (Krahl, 1961). Insulin also inhibits the breakdown of glycogen, in the liver, to glucose.

In the absence of insulin this process is responsible for maintaining blood glucose levels. The transamination of amino acids to pyruvic acid and thence to glucose via the Embden-Meyerhof pathway is also inhibited.
Schematic representation of the intracellular pathway of insulin following binding to its plasma membrane receptor on microvillus. Also represented is the recycling route of the receptor from the endosomal-lysosomal compartment and the possible insertion of the newly-synthesized receptors. MVB= multivesicular body.

(Figures from Carpentier, 1989)
by insulin (Young et al, 1960; see figure #7).

It can be seen that insulin actively acts to reduce blood glucose levels and that its absence would, and does, have severe consequences for blood glucose regulation.

1.3 DIABETES AND ITS DIAGNOSIS

1.3a Classification.

There are two main forms of diabetes, Diabetes Mellitus and Diabetes Insipidus. Diabetes Insipidus is a rare condition which is caused either by an insufficient secretion of Antidiuretic Hormone (ADH or vasopressin) or a renal insensitivity to it. ADH is an essential hormone required for the reabsorption of water from the kidney tubules. An absence of this hormone leads to excessive water loss; and the disease is characterised by the production of large volumes of pale-colourless urine (De Wardener, 1973).

Diabetes Mellitus afflicts between 20-30 million people in Europe and the U.S.A., nearly 1 in 50, although there are considerable regional variations (Marks et al; 1971). A recent report from the Office of Health Economics (1989), the research arm of the pharmaceutical industry, estimated that, in the U.K. alone, there were more than 500,000 undiagnosed diabetics.

In diabetes mellitus there are two forms, Juvenile Diabetes and Maturity Onset Diabetes.

Juvenile diabetes occurs either in childhood or adolescence, may be due to viral infection (Craighead, 1975; Rubenstein, 1979), and is characterised by a total failure to secrete insulin (Parker et al,
FIGURE #7

SCHEMATIC REPRESENTATION OF HORMONAL CONTROL OF METABOLISM

DIET

GLUCOSE

GL

I

I

G

I, T3

AMINO ACIDS

GL

GH

Protein

FAT

GLYCOGEN

OXIDATION

EXPLANATION OF SYMBOLS

I: Insulin, T3: Triiodothyronine, GH: Growth Hormone, G1: Glucocorticoids, & G: Glucagon.
The onset may be very quick or conversely very slow, and so it's occurrence is very unpredictable (Baker & Stanley, 1979). Treatment for this disease consists of intravenous injections of soluble insulin to reduce blood glucose levels (Oakley et al; 1975). The incidences of this form of diabetes has increased in recent times.

Maturity onset diabetes varies in its severity and may be characterised by a total failure of the pancreas or by an inappropriate amount or pattern of insulin secretion; in either case treatment is required (Oakley et al, 1975; Kipnis, 1968).

Diabetes mellitus is caused by certain dysfunctions (Marks et al, 1971; Fajams, 1979) (see figure #8).

In diabetes mellitus glucose, fat, and protein metabolism are all affected. Glucose blood levels are increased above the renal threshold so that glucose starts to appear in the urine (glucosuria). Deficiencies in fat metabolism results in a large production of ketone bodies which leads to ketosis and ketonuria (see below). In mild form ketosis causes nausea, vomiting, and drowsiness; but in a severe form it can cause coma and possibly death (Oakley et al, 1975; Liljenquist et al, 1979).

1.3b Fatty Acid Metabolism

Normally in fatty tissue triglycerides are broken down to fatty acids, but are reincorporated into triglycerides by the action of glycerophosphate; which is synthesized from glucose (McGarry & Foster, 1979).

In diabetes the fatty tissue glucose content decreases so that glycerophosphate production also falls. This leads to a build-up of fatty acids which then enter the blood stream. The fatty acids in blood
FIGURE #8

PROPOSED AETIOLOGICAL AND PATHOGENIC FACTORS IN DIABETES MELLITUS

A. Genetically determined abnormality of beta cell function or number.
1. Delayed and decreased insulin secretory response to nutrients.
   (i) Decreased number of beta cell receptor sites to glucose or amino acids
   (ii) Decreased affinity of receptor sites.
   (iii) Altered microtubular response.
   (iv) Altered microfilamentous activity.
   (v) Altered cation shifts.
   (vi) Abnormality in cAMP.
2. Decreased insulin biosynthesis.
3. Abnormality in conversion of proinsulin to insulin.
4. Decrease in beta (ß) cell replication—early senescence.
5. Synthesis of an abnormal insulin with impaired biological activity.
6. Increased susceptibility of ß cells to environmental factors linked to HLA system.

B. Environmental factors altering beta cell integrity & function.
1. Infective agents—viruses.
2. Autoimmunity—antipancreatic cell-mediated immunity; increased susceptibility to ß cell damage by viral agents due to defective immune response influenced by genes in the HLA chromosomal region and leading to an autoimmune process (in proportion of juvenile-onset-type diabetes).
3. Diet (calories) leading to obesity; pregnancy (maturity-onset type).
4. Autonomic nervous system—increased adrenergic activity.

C. Abnormality of insulin action
1. Insensitivity to endogenous insulin.
   (i) Release of insulin with impaired biological activity.
   (ii) Decreased number of insulin receptor sites.
   (iii) Interference with binding of insulin to its receptor sites—antibodies to insulin receptors.
   (iv) Decreased activity of key enzyme(s).

D. Abnormality in glucagon secretion
1. Secondary to insulin insufficiency.
2. Primary abnormality.

E. Abnormal formation or degradation of basement membrane
1. Primary abnormality on genetic basis.
2. Secondary to insulin insufficiency.

(Figure from Fajans, 1979)
are dealt with by the liver in several ways. They can either be formed into neutral fats or long chain fatty acids or ketone bodies such as acetoacetic acid, β-hydroxybutyric acid and acetone (see figure #9). Indeed in severe uncontrolled diabetics one can discern the smell of acetone on the breath of patients (Williamson & Hems, 1970; Lewis, 1980).

Ketone bodies are usually absorbed by muscle tissue, but this process is slow and unable to cope with the excess production occurring in diabetes (McGarry & Foster, 1979). This failure to absorb ketone bodies not only leads to ketosis, but their presence in the blood inhibits the production of long chain fatty acids; thus increasing the production of more ketone bodies (Liljenquist et al, 1979). The increased circulating ketone bodies can be utilised by most extrahepatic tissues for energy. This displacement of glucose for energy increases plasma glucose levels and thus leads to an increase in hyperglycaemia (Liljenquist et al, 1979; see figure #9).

1.3c Maturity Onset

In maturity onset diabetes insulin deficiency may be coupled with tissue insensitivity to insulin. Weight loss, which is common in untreated Juvenile onset, is less frequent in this form.

The underlying causes may vary but the results are the same. In some diabetics obesity may contribute to tissue insensitivity to insulin. Since adipose tissue stores are increased in obesity, and adipose tissue is insensitive to insulin, the normal pattern of insulin secretion is insufficient to regulate blood glucose levels (Rubenstein, 1979). Thus there is an increase in insulin secretion to compensate for this; which over time may lead to exhaustion of the β cells (Best et al;1959).
FIGURE #9

SCHEMATIC REPRESENTATION OF FAT

METABOLISM IN DIABETES MELLITUS

Glycerophosphate

TRIGLYCERIDES  ←  FATTY ACIDS

↓

Large increase in blood fatty acid levels.

↓

LIVER

Increase in KETONE BODIES  -Ve  →  NEUTRAL FATS

↓

Poor uptake into muscles

LONG CHAIN FATTY ACIDS

KETONE BODIES: Acetoacetic Acid, β-hydroxybutyric Acid, & Acetone.

(Figure summarised from Lewis, 1980)

(23)
It has been shown that tissue sensitivity to insulin is inversely related to degree of obesity.

Some diabetics are however not obese and there may be several reasons to explain hyperglycaemia (see above), an absence of the first rapid phase of insulin secretion which occurs when glucose is presented to the β cells (Matschinsky et al, 1979); it has also been suggested that one of the basic abnormalities that characterize the diabetic state is accelerated aging or senescence (Vracko & Benditt, 1974) and that this is responsible.

1.3d Glucose Tolerance Test

Certain people show glucose intolerance only in exceptional circumstances such as pregnancy or very severe stress. These people can be described as latent Diabetics and a glucose tolerance test (GTT) is a very effective means of assessing the degree and susceptibility to diabetes. However the age of the subject should be borne in mind, especially those over 50, as tolerance to glucose decreases with advancing age (Fajans, 1965; Andres, 1971).

The test involves a person fasting, 8-10 hours, to reduce the base level of glucose and then being given 50g of glucose, ideally in the morning. The blood glucose level is then monitored over a certain period and a graph versus time is drawn (Fajans, 1979).

Normal people have a low base level which rises sharply following glucose ingestion and then falls away rapidly. In latent diabetics there is a similar initial phase but the decline phase is more gradual. In mild diabetics the basal level is elevated, approaching the renal threshold of 180mg/100ml blood, the rise is much slower but is more elevated; whilst the decline gradient is equivalent to the incline. In
severe diabetics glucose intolerance is profound. The base level is far in excess of the renal threshold, subsequently the final plateau following ingestion is grossly elevated. The incline and decline phases are more gradual (see figure #10).

Bearing in mind such factors as time of day, age, physical activity, coffee, smoking, acute or chronic illness all affect the GTT, it is not suprising that the GTT is one of the most misused tests in performance and interpretation (Fajans, 1979). But these problems can be overcome if the correct procedure is adhered to and one of the many published criteria is used to interpret the results. Briefly the criteria for establishing diabetes is a test with a 1-hour value of 195 mg/dl, and a 2-hour value of 140 mg/dl or more (Fajans, 1979).

1.3g Hypoglycaemia

Whilst diabetes mellitus is characterised by insufficient insulin, the converse condition is equally serious.

If insulin secretion out paces carbohydrate intake there is a resultant low blood glucose level. Though several mechanisms come into play, such as the conversion of glycogen and amino acids to glucose, to correct this aberration; a continued fall has serious implications (Best et al, 1959). The condition may also be caused by other factors, such as a failure of either glycogenolysis or glucogenesis, and also possibly, by excess secretion of the thyroid hormones (Arky, 1979).

If the blood glucose level falls below 70 mg/100ml blood there appear characteristic signs of hunger, cold sweats, tremors and weakness. Should the blood glucose level fall far below the minimum limit then the brain suffers glucose deprivation leading to convulsions, which may be fatal (Arky, 1979).
FIGURE 10
TYPICAL GLUCOSE TOLERANCE CURVES FOR A RANGE OF SUBJECTS

Blood glucose (mg/dl)

Time after taking glucose (hours)

A: Normal Subject. Blood glucose returns to fasting level after 1.5hrs.
B: Latent Diabetic. Slower return to fasting levels.
C: Mild Diabetic.
D: Severe Diabetic. There is glycosuria at all times and blood glucose concentrations of 300-400mg per cent or more may be reached after glucose ingestion.

(Figure from Lewis, 1980)
1.4 HORMONES AFFECTING BLOOD GLUCOSE LEVELS

1.4a Glucagon

Its existence was first postulated in 1923 by Kimball & Mullin as a contaminant present in extracted insulin, since this "impure" insulin initially caused hyperglycaemia in subjects.

It was another thirty years before its composition was established as consisting of a single chain polypeptide of 29 amino acid residues (Staub et al., 1955; see figure #2).

It is secreted, as a precursor proglucagon (Steiner & Tager, 1979), by the α cells of the pancreas and by α-like cells present in the stomach and the duodenum. Secretion is stimulated by a fall in the blood glucose level, decrease in free fatty acid blood content, an increase in sympathetic nervous activity, and also by physical exercise (Best, 1959; Lefebvre & Unger, 1972).

Glucagon acts at a variety of sites, the main outcome of which is an elevation in blood glucose levels (Devrim & Recant, 1966). Besides its main actions (see figure #11), it also acts to decrease gastrointestinal movement and greatly increase the rate & force of cardiac contractions (Lewis, 1980).

The secretion of glucagon and insulin are interlinked and function as a homeostatic mechanism: Glucagon invokes insulin secretion whilst insulin inhibits glucagon secretion (Devrim & Recant, 1966; see figure).

It has been postulated (Unger & Orci, 1975) that glucagon is involved in diabetes mellitus. Injections of somatostatin, a potent inhibitor of both glucagon & insulin, doesn't produce hyperglycaemia. It could be that low insulin secretion raises blood glucose levels by preventing the utilization of glucose, and that concurrent with this glucagon acts on
FIGURE #11

EFFECTS OF GLUCAGON

1. Greatly increases blood glucose levels.

2. Increases circulating free fatty acid levels.

3. Increases GLYCOGENOLYSIS in the liver, the conversion of glycogen to glucose.

4. Increases GLUCONEOGENESIS, the transformation of amino acids (from protein breakdown) to glucose.

5. Stimulates the secretion of adrenaline.


7. Increases feeling of saition.

8. Stimulates the secretion of growth hormone.

9. Greatly increases the rate of cardiac contraction.

10. Increases the force of cardiac contraction.

11. Causes hypocalcaemia.

12. Induces insulin releases.

13. Increases the release of cAMP from the liver.

(Figure from Lewis, 1980)
glycogen stores to produce elevated levels of circulating glucose. It is suggested that both these mechanisms come into play and produce the characteristic hyperglycaemia of diabetes.

1.4b The Adrenal Glucocorticoids

The adrenal glands, situated just above the kidneys, are divided into two regions, the medulla and the cortex, of which the latter is the most important secretor of a range of steroids which control salt and water balance and also metabolism of carbohydrates, fats, and proteins. The metabolism of the carbohydrates plays a crucial role in maintaining blood glucose levels and so these steroids feature in diabetes. The steroids, hydrocortisone (cortisol), corticosterone & aldosterone, all have a basic cyclopentanoperhydrophenanthrene ring structure with a two carbon side chain at position C17. In addition they all feature a ketol grouping at position C20, with an oxygen atom at C3 position (see figure #12).

Although the action of these steroids overlap, aldosterone is principally a mineralocorticoid, concerned with salt and water metabolism, hydrocortisone is a glucocorticoid, controlling carbohydrate metabolism, whilst corticosterone functions in both arenas (Currie et al, 1962). Hydrocorti and corticosterone are biosynthesized in the fasciculata and reticularis zones of the adrenal cortex and their secretion is governed by the peptide adrenocorticotrophic hormone (ACTH) which is secreted by the anterior pituitary gland in response to circulating levels of the steroids (Brandon, 1962; Myles & Daly, 1974; Ganong et al, 1966).
FIGURE #12
THE STRUCTURES OF ADRENOCORTICAL STEROIDS

Hydrocortisone
(Cortisol, 17-hydroxycorticosterone)

Corticosterone

Aldehyde form

Aldosterone

Hemi-acetal

(Figure from Lewis, 1980)
The first 24 residues are identical in ACTH preparations from several species. A synthetic polypeptide containing the first 24 residues of ACTH has biological activity in vivo similar to that of the isolated hormone. Thus, residues 25 to 33 are not essential for hormonal action. However, these residues are significant for the species immunological specificity of the hormone. Removal of a few residues from the amino-terminal end of ACTH destroys biological activity.

**CODE FOR AMINO ACIDS**

- Ala - Alanine
- Asp - Aspartic acid
- Glu - Glutamic acid
- Ile - Isoleucine
- Met - Methionine
- Ser - Serine
- Tyr - Tyrosine

- Arg - Arginine
- Cys - Cysteine
- Gln - Glutamine
- Leu - Leucine
- Phe - Phenylalanine
- Thr - Threonine
- Val - Valine
- Asn - Asparagine
- Gly - Glycine
- His - Histidine
- Lys - Lysine
- Pro - Proline
- Trp - Tryptophan

(Figure from Lewis, 1980)
Though the steroids have a large spectrum of activity the following list only seeks to deal with their glucocorticoid behaviour (Leung & Munk, 1975; Slater, 1965).

(i) Increase gluconeogenesis, the formation of glucose from amino acids.
(ii) Increase the breakdown of proteins to amino acids to promote the above-mentioned cycle.
(iii) Directly inhibit the action of insulin and thus prevent glucose utilisation.
(iv) Increase the mobilization of fats.
(v) Increase the deposition of glycogen in the liver.

1.4c Adrenocorticotropic Hormone (ACTH)
The adrenocorticotropic hormone (ACTH or corticotrophin) is released by the anterior pituitary gland in response to either the nervous system or circulating levels of corticosteroids in a negative feedback system. It is also released at times of extreme mental and physical stress such as haemorrhaging, burning, severe cold, and pain. It consists of thirty-nine amino acid residues (see figure #13) and it is deactivated if a single amino acid is removed from the NH₂-terminal end of the molecule. Its primary action is to stimulate the secretion of the aforementioned glucocorticoids from the adrenal cortex, but it also acts to elevate blood glucose levels (Currie et al, 1962; Nyles & Daly, 1974).

1.4e Regulatory Peptides
Many regulatory peptides have a profound effect on either increasing or decreasing food intake and blood glucose concentration in experimental animals (see figure #14, William & Bloom, 1989), but whether this is...
FIGURE #14
EFFECTS OF CERTAIN PEPTIDES

EFFECT ON BLOOD GLUCOSE CONCENTRATION

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Site of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperglycaemia</td>
<td>Bombesin</td>
</tr>
<tr>
<td></td>
<td>β-Endorphin</td>
</tr>
<tr>
<td></td>
<td>Thyrotrophin</td>
</tr>
<tr>
<td></td>
<td>Releasing Hormone</td>
</tr>
<tr>
<td></td>
<td>Neurotensin</td>
</tr>
<tr>
<td></td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td></td>
<td>Somatostatin</td>
</tr>
<tr>
<td>Hypoglycaemia</td>
<td>Somatostatin</td>
</tr>
<tr>
<td></td>
<td>Neurotensin</td>
</tr>
<tr>
<td>No Effect</td>
<td></td>
</tr>
</tbody>
</table>

SOME EXPERIMENTAL ACTIONS OF NEUROPEPTIDE Y

<table>
<thead>
<tr>
<th>Action</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central Action</td>
<td></td>
</tr>
<tr>
<td>Hyperphagia</td>
<td>PVN, VMH, LHA</td>
</tr>
<tr>
<td>Polydipsia</td>
<td>PVN, VMH</td>
</tr>
<tr>
<td>LH secretion stimulated &amp; inhibited (depends on oestrogen status)</td>
<td>ICV</td>
</tr>
<tr>
<td>TSH secretion inhibited</td>
<td>? arcuate nucleus</td>
</tr>
<tr>
<td>GH secretion inhibited</td>
<td></td>
</tr>
<tr>
<td>Prolactin secretion inhibited</td>
<td></td>
</tr>
<tr>
<td>Vasopressin release stimulated</td>
<td>SGN</td>
</tr>
<tr>
<td>Insulin release stimulated</td>
<td>ICV</td>
</tr>
<tr>
<td>Hypotension</td>
<td>ICV</td>
</tr>
<tr>
<td>Bradycardia</td>
<td></td>
</tr>
<tr>
<td>Bradypnoea</td>
<td></td>
</tr>
<tr>
<td>sedative</td>
<td>ICV</td>
</tr>
<tr>
<td>libido inhibition</td>
<td></td>
</tr>
<tr>
<td>Peripheral Action</td>
<td></td>
</tr>
<tr>
<td>Vasoconstriction</td>
<td>IV</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
</tr>
</tbody>
</table>

ICV, intracerebroventricular (3rd & 4th ventricle); VMH, ventromedial hypothalamic nucleus; SGN supraoptic nucleus; IV, intravenous; LHA, lateral hypothalamic area; & PVN, paraventricular nucleus.

(Figure from William & Bloom, 1989).
their true physiological role remains unclear.

Somatostatin is a well established regulatory peptide, which occurs in 2 forms, containing 14 and 28 amino acid residues (see figure #15; Pradayrol et al, 1980).

Somatostatin-14 is found in D-cells of the pancreatic islets and in the nerves supplying the stomach. Somatostatin-28 occurs in gut mucosa cells and in the retina; whilst both forms occur abundantly in the brain (Polak & Bloom, 1986; Ishikawa et al, 1987; Reichlin, 1983). Somatostatin release from pancreatic D-cells is stimulated by many factors including high glucose, glucagon, and ketone body concentrations, and also insulin deficiency (Ipp et al, 1977; Vasada et al, 1981).

Somatostatin has a wide spectrum of biological activity, including inhibition of growth hormone, insulin, and glucagon (Brazeau et al, 1979). It directly blocks nutrient transport across the gut mucosa, reduces exocrine gut secretions, inhibits the release of various gut peptides, and decreases gut motility and splanchnic blood flow (Adrian et al, 1981; Krejs et al, 1980; Bloom et al, 1974; Boden et al, 1975; Price et al, 1985; see figure #15).

In experimental animals somatostatin levels, both circulating and D-cell content, are elevated. Both increased turnover and reduced clearance further contribute to elevate circulating levels (Makino et al, 1977; Patel et al, 1978; Patel & Weir, 1976; Hellman & Petersson, 1963; Hara et al, 1979; Trimble & Gerber, 1981; Ruggere & Patel, 1984; Schusdziarra et al, 1981). In diabetic patients somatostatin levels are increased in both insulin-dependent and -independent diabetes mellitus (Williams & Bloom, 1989).

Native somatostatin and its analogues have been used to reduce the elevated levels of growth hormone prevalent in diabetes mellitus
FIGURE #15

SOMATOSTATIN: STRUCTURE & EFFECTS


Somatostatin

D-PHE-CYS-PHE-PHE-[D-TRP]-LYS-THR-CYS-THR-OH

Octeotide (SMS 201-995)

(See figure #1 for Amino Acid code)
(From Davies, Turner, Alberti, & Johnston, 1989)

EFFECTS

Endocrine actions
(inhibition)

Pituitary: growth hormone
TSH
ACTH

Pancreas: insulin
glucagon
cellular somatostatin

Gut: gastrin
gastric inhibitory peptide
enteroglucagon
cholecystokinin
secretin
motilin

Exocrine actions
(inhibition)

Salivary glands
Stomach: acid, pepsin, intrinsic factor
Pancreas: bicarbonate, enzymes
Colonic fluid

Other gastrointestinal actions
Reduces gut motility (stomach, intestine,
gall bladder)
Reduces splanchnic blood flow
Reduces intestinal absorption

CNS actions
Inhibits (or stimulates) feeding
Inhibits centrally-mediated hyperglycemia
Analgesia

(From Williams & Bloom, 1989)

(35)
Octreotide is an analogue of somatostatin (Sandostatin/SMS 201-995, Sandoz Pharmaceutical) currently being evaluated for its potency. It has a duration time of 6-8hrs when administered subcutaneously. Compared to somatostatin this analogue has equipotency against insulin, but is x11 more potent against glucagon and x45 more potent against growth hormone secretion (Davies et al, 1989; see figure #15).

Neuropeptide Y is a 36 amino acid residue peptide which is structurally related to pancreatic polypeptide and is named for the tyrosine residue ('Y') at either end of the molecule (William & Bloom, 1989). It is widely distributed throughout the CNS, but is particularly high in the hypothalamus, cortex, basal ganglia, and limbic system (De Quidt & Emson, 1986; Allen et al, 1982).

It's primary action is to greatly stimulate appetite, and negate the effect of strong appetite suppressants such as cholecystokinin (William & Bloom, 1989). It increases appetite for carbohydrate-rich foods, stimulates exploratory food-seeking behaviour, whilst suppressing other forms of activity, such as sex (Clark et al, 1984; Stanley et al, 1985 & 1986; Marley et al, 1987; Steinman et al, 1987).

Neuropeptide Y has powerful, and generally inhibitory, effects on anterior pituitary hormone secretion (William & Bloom, 1989). Its injection into the third ventricle of the hypothalamus blocks release of growth hormone, thyroid stimulating hormone (TSH), prolactin, and under some circumstances, LH (McDonald et al, 1985; Harfstrand et al, 1987 & 1986; Kalra & Kalra, 1986). In experimental diabetic animals elevated levels of the peptide have been found, up to x5 greater than the control animals (Pierson et al, 1988; see figure #14).
It seems therefore that the neuropeptide Y has a homeostatic role, coming into play when insulin levels fall in the diabetic state, to maintain body weight and energy.

It is obvious that much work remains to be carried out to elucidate the role and/or relevance of this and other peptides to diabetes.

1.4f Growth Hormone

Growth hormone is released by the anterior pituitary gland in response to the Growth Hormone Releasing Factor secreted by the hypothalamus and it is species specific.

The hormone acts primarily on bones rapidly promoting growth and consequently there is a rise in plasma levels of alkaline phosphatase and inorganic phosphate. It is therefore not surprising that an excess or deficiency leads to gigantism and dwarfism respectively (Harris; 1955).

In addition it utilises fat stores and there is a concurrent increase in blood glucose levels (Glick et al, 1965).

1.4g Adrenaline

Adrenaline, in addition to being a neurotransmitter, is also present in the adrenal medulla and is released by extreme emotional or physical stress and hence fits its classical description of a 'fight or flight' hormone.

It is biosynthesized from both tyrosine and phenylalanine via a very well understood pathway. Phenylalanine is converted to tyrosine and thence to 3-(3,4-dihydroxyphenyl) alanine or DOPA. DOPA then undergoes decarboxylation and forms 2-(3,4-dihydroxyphenyl)-ethylamine or dopamine, the precursor to noradrenaline; which is itself the precursor for adrenaline (Blascko, 1939). (see figure #16).
FIGURE #16
THE BIOSYNTHESIS OF NORADRENALINE
AND ADRENALINE

Tyrosine \( \text{HO-C}_6\text{H}_4-\text{CH}_2-\text{CH-NH}_2 \)
\( \text{COOH} \)
\[ \text{tyrosine hydroxylase} \]
\[ \text{L-DOPA} \]
\( \text{(HO)}_2-\text{C}_6\text{H}_3-\text{CH}_2-\text{CH-NH}_2 \)
\( \text{COOH} \)
\[ \text{L-DOPA decarboxylase} \]
\[ \text{Dopamine} \]
\( \text{(HO)}_2-\text{C}_6\text{H}_4-\text{CH}_2-\text{CH}_2-\text{NH}_2 \)
\[ \text{dopamine-\(\beta\)-hydroxylase} \]
\[ \text{Noradrenaline} \]
\( \text{(HO)}_2-\text{C}_6\text{H}_4-\text{CH}_2-\text{CH-NH}_2 \)
\( \text{OH} \)
\[ \text{phenylethanolamine-N-methyltransferase} \]
\[ \text{Adrenaline} \]
\( \text{(HO)}_2-\text{C}_6\text{H}_4-\text{CH}_2-\text{CH-NH-CH}_3 \)
\( \text{OH} \)

(-) Tyrosine is taken up by the chromaffin cells of the adrenal medulla. The conversion of noradrenaline to adrenaline only occurs in the chromaffin cells of the adrenal medulae which store adrenaline.
(Figure from Day, 1979)

(38)
Adrenaline has a wide range of effects within the body, this includes the cardiovascular system, the respiratory system, smooth muscle tissue, the nervous system, exocrine glands, and also body metabolism. It's glucocactivity closely resembles glucagon and both it and noradrenaline prevent glucagon-stimulated insulin secretion (Currie et al, 1962).

Adrenaline acts directly to increase blood glucose levels by breaking down glycogen in the liver and it's secretion is also triggered off by falling blood glucose levels. It also has a strong stimulating effect on fat mobilization leading to increased levels of circulating fatty acids and glycerol. Both of these actions are initiated by the activation of phosphorylase and lipase via the adenyl cyclase-cyclic AMP link.

Adrenaline acts on two different types of receptors \( \alpha \) and \( \beta \), which are further subdivided into \( \alpha_1, \alpha_2 \) and \( \beta_1, \beta_2 \). Each of this quartet of receptors elicits a different cell response, but it is mainly the \( \beta \) receptors, together with some \( \alpha_2 \) activity, which control metabolic activity. \( \beta_1 \) causes lipolysis whilst \( \beta_2 \) is responsible for the breakdown of liver glycogen (Lewis, 1980).

### 1.5 TREATMENT

#### 1.5a Insulin Injection Therapy

A normal person maintains a circulating plasma glucose level that fluctuates between 60 and 160 mg/dl during the fasting and fed states (Arky, 1979), and the body easily restores glucose levels. In diabetes mellitus the pancreas is unable to cope with the high levels of glucose following a meal. Before insulin is used to control hyperglycaemia in either juvenile or maturity onset diabetes mellitus a controlled carbohydrate diet is essential. For patients suffering from juvenile onset diabetes a controlled carbohydrate diet is supplemented with
insulin injections. In obese patients suffering from maturity onset diabetes mellitus weight reduction is a first priority followed by a controlled carbohydrate diet, and if necessary oral hypoglycaemics; if all these prove inadequate only then is insulin recommended (Oakley et al, 1975).

Since insulin is broken down in the gut it is administered via a subcutaneous injection. The different preparations available on the market vary in their time of onset of action and in their duration of action; the onset time is directly related to the crystal size.

The relatively quick onset preparations are:

(i) Soluble Insulin - An aqueous solution of insulin crystals with an onset time of 1 hour or less and a duration of nearly 6 hours.

(ii) Globin Zinc Insulin - A solution of insulin globin and zinc with an onset time of 2 hours and a duration of 24 hours or less.

(iii) Isophane Insulin - A modified protamine zinc insulin solution with an action spectrum similar to the previous one.

(iv) Lente Insulin - Zinc insulin in an acetate buffer, pH 7.2-7.5, with an onset time of 2 hours and a duration of nearly 14 hours.

(v) Semilente Insulin - Amorphous zinc insulin in an acetate buffer, with an onset time of 2 hours and a duration of 10 hours.

The slow onset and longer acting preparations are:

(i) Protamine Zinc Insulin - Insulinh/protamine complex with a trace of zinc; onset time of 6-12 hours and duration of 48hrs.

(ii) Insulin Ultralente - A suspension of the crystalline form of zinc insulin with a onset time of 5 hours and a duration of 36 hours.

Any treatment involves tailoring the requirements of the patient to the preparations available. Rarely is only one preparation used, more usually a mixture of both the quick onset/short duration and slow
onset/longer acting preparations is used (Turner & Richens, 1978).
There are however side effects associated with the use of insulin preparations. In some patients tumours develop at the injection site whilst in others atrophication of the subcutaneous fat occurs at these sites leading to unsightly hollows, though this side-effect has been known to be reversible in some. Some patients are allergic to all forms of insulin whilst some are only allergic to protamine zinc insulin preparations. Most of these problems are largely due to impurities present in insulin and since the introduction of purer animal and synthetic human insulins, they have become much less common. Continued use of insulin preparations can lead to tolerance, so diabetics require ever higher doses of insulin.

1.5b Alternatives to Insulin Injections
Efforts recently have been directed towards finding a replacement, for diabetics, for the daily injections of insulin. The alternatives are islets of Langerhans transplant, whole pancreas transplant, artificial pancreases including full-mechanical models and hybrid models, and insulin pump implantation. Diet and oral hypoglycaemic drugs are also options in less severe maturity onset diabetes.

Islets of Langerhans have recently been successfully implanted into diabetic patients by Norman Kneteman of the University of Alberta (Dayton, 1989). Pancreas implants have several disadvantages; patients requiring transplants are usually too weak to cope with the major surgery involved, they must also take immunosuppressants to overcome rejection and the implant must be carried out within hours of the organ becoming available (Dayton, 1989).

Islet transplant in comparison is more simple and safe, they suffer from rejection, and they can be
frozen and stored indefinitely (Rajotte, 1989). Initially the islets are isolated and purified from a human pancreas, about 2-3 g's, then they are inserted through a catheter into the portal vein from where they enter the liver and start to produce insulin (Kneteman et al, 1989; Dayton, 1989). Two patients who received transplants are, after six months, producing enough insulin to significantly reduce their reliance on injected insulin (Dayton, 1989).

Once the method has been fully developed it offers the possibility of transplants completely freeing diabetics from their daily chore of insulin injections.

The development of artificial pancreases has been slow and problematic. There are currently two types undergoing development; a full-mechanical model comprising glucose sensor, insulin reservoir, and insulin pump, and a hybrid model consisting of living insulin-secreting tissue in a man-made chamber.

In a hybrid artificial pancreas living insulin-secreting tissue is placed in a chamber composed of semi-permeable membrane shaped either as hollow fibres or simply as a box. Modern polymer chemistry offers the possibility of encapsulating individual islets in an inert polymer and implanting them (Friedman, 1989). The semi-permeable membrane allows the passive transfer of plasma and tissue solutes, both in and out, so that sufficient nutrition is provided for the cells (Altman et al, 1986; Aebischer et al, 1986).

Initial work shows that normalization of blood glucose levels, in experimental diabetic animals, was achieved with a hybrid artificial pancreas containing either isolated islets or foetal pancreas, with the implant maintaining activity from a few days up to nearly a month (Friedman, 1989).
The search for a full-mechanical artificial pancreas has been going on for over two decades (Kadish, 1964), and the old external cumbersome models have given way to smaller implantable models (Pfeiffer, 1989). But the major problem of an implantable neutral glucose sensor has hampered progress.

Currently a glucose sensor, 6cm long and with a 2mm cross-sectional diameter, is being evaluated by Pfeiffer and colleagues (Pfeiffer, 1989). The sensor, an amperometric electrode, works on the electrical energy generated by the following chemical reaction:

\[ \text{Glucose} + \text{O}_2 \rightarrow \text{Gluconic acid} + \text{H}_2\text{O}_2 \]

The current flow, caused by the transfer of electrons, is proportional to the concentration of glucose (Pfeiffer, 1989).

The sensor has been tested in experimental animals where it successfully monitored blood glucose levels, its response to changes in blood sugar levels following an injection of either glucose or insulin was only 5-15 minutes (Pfeiffer, 1989).

There still remains a large amount of work to be carried out before either of these systems can be considered a serious alternative to insulin injections.

Continuous subcutaneous insulin infusion (CSII) either by external or internal insulin pumps has been slowly developing over the years. There are three components to the pump, insulin, pump device, and catheter. The insulin used is buffered, neutral and human, such as U-100 insulin (Hoechst). The main problem has been with the catheters which have suffered blockages, inner lumen fibrinous and cellular material deposition, fibrous granuloma at the tip, and full or partial catheter encapsulation by omentum. Other problems concern the pump device itself which causes local irritation leading from pain to
infections and ulcers. Also the inherent task of refilling the reservoir, presents further problems. Considering these shortcomings it seems unlikely that implantable insulin pumps will gain wide currency (Selam, 1988).

The obvious solution to pancreatic failure would appear to be whole pancreas transplants but these have had mixed results. Between December 17, 1966, and June 30, 1988, 1549 pancreas transplants were reported to the International Pancreas Transplant Registry. The overall one year actuarial graft function was 42%, whilst recipient survival rate was much higher at 80%. The longest surviving graft is over ten years, but the encouraging fact is that 441 transplants have survived for over a year and 597 grafts are still functioning to date. Failure of the transplants are due to rejection, primary thrombosis, local infections, and other immunological complications; whilst some patients (145) died with a functioning transplant (Sutherland & Moudry, 1989). But no matter how successful the survival rate donors will still remain the limiting factor; though recent advances in genetically engineering animals whose organs are compatible with humans may offer an alternative.

A more 'physiological' administration of insulin providing continuous control of plasma glucose concentration might be particularly beneficial to diabetics who, because of inherent instability or loss of symptoms of hypoglycaemia or hyperglycaemia, have difficulty controlling their blood glucose concentrations. It might also reduce the incidence of diabetic complications. Unregulated acute hypoglycaemia or hyperglycaemia would be particularly harmful, as susceptible tissues would be seriously damaged. Rather than insulin injections into the arm, delivery of it into the hepatic portal vein, as in normal physiology, may be more beneficial.
It is possible that one, or a combination, of the alternatives under development will offer a realistic choice to diabetics suffering daily injections.

1.5e Oral Hypoglycaemica

There are two main classes of oral hypoglycaemica, the sulphonylureas and the biguanides.

The SULPHONYLUREAS were first developed from carbutamide which was found by Franks & Fuchs (1955) to produce hypoglycaemia. They can only work if there is some functioning islets of Langerhans, since these compounds stimulate the release of insulin. In addition to this they sometimes reduce the production of glucose from the liver.

Chlorpropamide, a long acting hypoglycaemic, occasionally has a fatal side-effect; it continues to stimulate insulin secretion at night when no food is being taken so that severe glucose depletion occurs leading to coma and finally death, though this is a problem which may also arise with the long acting insulin preparations. Apart from this some patients, =20%, suffer nausea and flushing if chlorpropamide is taken in conjunction with alcohol. A very small number of patients suffer skin rashes, gastrointestinal problems and fatigue (Slater, 1961; Lewis, 1980; see figure #17).

Phenformin was the first BIGUANIDE found and this was shortly followed by metformin. These class of drugs have a different mechanism of operation than the sulphonylureas; whereas the latter can cause death in healthy people the former have no effect.

The biguanides act in three different ways:

(i) Increase insulin-facilitated transport of glucose into cells.
(ii) Greatly decrease glucose absorption from the gut.
**FIGURE #17**

**ORAL HYPOGLYCAEMICS FOR THE TREATMENT OF DIABETES MELLITUS**

**THE SULPHONYLUREAS**

<table>
<thead>
<tr>
<th>Name</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Dose/day (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolbutamide</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;-</td>
<td>-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.5-3</td>
</tr>
<tr>
<td>Carbutamide</td>
<td>NH&lt;sub&gt;2&lt;/sub&gt;-</td>
<td>&quot; &quot;</td>
<td>NOT USED</td>
</tr>
<tr>
<td>Chlorpropamide</td>
<td>Cl</td>
<td>&quot; &quot;</td>
<td>0.1-0.4</td>
</tr>
<tr>
<td>Acetohexamide</td>
<td>C&lt;sub&gt;3&lt;/sub&gt;H&lt;sub&gt;7&lt;/sub&gt;C(O)-</td>
<td>C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;11&lt;/sub&gt;-</td>
<td>0.5-15</td>
</tr>
<tr>
<td>Tolazamide</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;-</td>
<td>-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.2-1</td>
</tr>
</tbody>
</table>

**THE BIGUANIDES**

<table>
<thead>
<tr>
<th>Name</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Dose/day (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metformin</td>
<td>H</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;CH&lt;sub&gt;2&lt;/sub&gt;-</td>
<td>1.5-3g's</td>
</tr>
<tr>
<td>Phenformin</td>
<td>H</td>
<td>-(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;11&lt;/sub&gt;</td>
<td>50-150mg's</td>
</tr>
<tr>
<td>Proguanil</td>
<td>-CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-C&lt;sub&gt;6&lt;/sub&gt;H&lt;sub&gt;4&lt;/sub&gt;-Cl</td>
<td></td>
</tr>
</tbody>
</table>

*When R is a substituent group.*

(Figure from Lewis, 1980)
(iii) Decrease glucose production in the liver from glycogen.

Metformin has the unpleasant side-effects of gastrointestinal upset, by inhibiting metabolism of lactic acid, leading to anorexia, nausea and vomiting. Phenformin can cause lactic acidemia in some patients suffering renal and cardiac problems. Phenformin is also known to interact with ethanol and this problem has now made the drug obsolete. Both drugs, with prolonged use, can interfere with the proper absorption of B₁₂. Also metformin is not broken down by the body whereas phenformin is (see figure #17).

1.6 COMPLICATIONS OF DIABETES

In many diabetic patients conventional insulin treatment does not prevent the onset of a range of vascular and nervous complications. The onset and rate of progression of these diabetic complications which include neuropathy, cardiomyopathy, cataracts, corneal epitheliopathy, and vascular damage leading to problems such as nephropathy and retinopathy, appear to be dependent upon both the duration and the severity of the disease. Surveys have shown that the prevalence of complications is much greater in poorly controlled diabetes (Kadar et al, 1985; Brownlee & Cerami, 1981) but severity of the disease has not been effectively linked to complications, although this may, in part be due to difficulties in assessing control. However most studies suggest that duration of diabetes is an important factor.

1.6a NEUROPATHY

In the peripheral nerve of the diabetic axon dwindling and segmental demyelination are associated with a very high prevalence of motor, sensory and autonomic dysfunction (Brownlee & Cerami, 1981; Vinegrad et
These basic dysfunctions lead to a range of clinical symptoms. Commonest among these is a loss of sensory function in the extremities which contribute, with vascular problems, to a propensity among diabetics for foot-related problems. Diabetics are particularly susceptible to gangrene and related infections, together with anesthesia to pin pricks below ankles and absent toe proprioception. Constant burning and aching of feet, intermittent sharp shooting pains in the legs and proximal leg weakness are all common symptoms in diabetes (Jaspan et al, 1986). Motor deficit manifests itself by a weakness of hip flexors and knee extensors, together with an absence of knee and ankle jerk reflexes.

Pain in the extremities in diabetics may be associated with hyperaemia which, in turn, may reflect reduced sympathetically mediated vasoconstriction. In diabetics noradrenaline uptake 1 blockers, such as amitriptyline and desipramine, are effective in reducing both pain and hyperaemia (Max et al, 1991).

Loss of autonomic control of the heart is a common feature in diabetics leading to arrhythmias. Also in diabetics peripheral sweating may be absent, whilst there may be an inappropriately profuse upper body sweating. Neuropathic dysfunction in diabetics is indicated by reduced testicular sensation, impotence whose physiological rather than psychological cause may be confirmed by the absence of nocturnal penile tumescence, atony of the bladder, and urine retention (Jaspan et al, 1986).

The entire gastrointestinal tract may also suffer severe autonomic disorder leading to reduced peristalsis and dilation of the oesophagus, delayed gastric emptying, disordered small bowel movement,

(48)
and atony of the large bowel which occasionally results in gross
dilation of the colon (Brownlee & Cerami, 1981; Goldstein et al, 1973;

Diabetic neuropathy is visibly manifested by structural alterations of
neurones. Reduced conduction velocity is thought to result from
diminished internodal resistance in myelinated neurons leading to
reduced current density at the nodes and delayed excitation (Brownlee &
are linked to decreases in the number of intramembranous particles on
both the inner and outer myelin surface. Together with this, endoneurial
oedema occurs, leading to shrinkage of axons and Schwann cells. The
permeability of nodal gap substance is also increased (Brownlee &
Cerami, 1981). In patients with diabetic neuropathy, loss of large and
small myelinated and unmyelinated fibers is evident early in the
disease. All of these neuronal structural dysfunctions are present in
both diabetic patients and experimental animals (Brownlee & Cerami,

1.6b VASCULOPATHY

Peripheral vascular disease is commonly associated with diabetes
mellitus in humans (Brody & Dixon, 1964). Vascular complications such as
hypertension, atherosclerosis and thrombosis are prevalent in diabetic
patients (Wakabayashi et al, 1987).

Atherosclerosis, caused by a deposition of hard yellow plaques of lipoid
material in the arteries and a thickening of the media, is believed to
be related to changes in the vascular endothelium (Wakabayashi et al,
1987). The vascular endothelium, an important regulator of vascular
tonus (Marks, 1971), is particularly susceptible to a range of agonists,
in addition it forms a barrier between the blood and the underlying vessel wall. Damage to the endothelium may be the primary event leading to the development of atherosclerosis. Diabetics also suffer increased atheromata in medium and large arteries which makes coronary arterial disease and stroke twice as common. Symptomatic peripheral arterial disease is three to four times more common among diabetics (Paley et al, 1961; Brownlee & Cerami, 1981; Brown et al, 1976; Gregerson, 1968; Eliasson, 1964; Schmidt, 1985).

Atherosclerotic vascular disease leads to myocardial infarcts, strokes and gangrene in diabetic patients (Chobanian et al, 1982). In addition diabetics are prone to hyperlipoproteinemia, obesity and hypercoagulability of blood (Chobanian et al, 1982). Vascular changes in nervous tissue may underlie or contribute to the problem of neuropathy (Brownlee & Cerami, 1981, see below).

In the eye a large number of dysfunctions begin to appear in patients with diabetes. Retinal capillary damage leading to oedema together with new vessel formation and loss of intramural pericytes and microaneurysms cause severe problems for many diabetics (Caird et al, 1969; Davis et al, 1969; Smith & Becker, 1976; Caird et al, 1964). In addition to this there appear areas of non-perfusion, haemorrhaging becomes common place, coupled with exudates into the vitreous humour (Brownlee & Cerami, 1982; Cogan & Kuwabara, 1963; Ashtom, 1963; Kohner et al, 1973; Ticho & Patz, 1973). A combination of these complications to make blindness twenty times more common among diabetics; and cataracts five times more common (Brownlee & Cerami, 1981).

Diabetics suffer complications in the kidney leading to gross problems nearly as frequently as they suffer blindness. Patients very often
develop basement membrane thickening in the glomerulus, but the first sign of nephropathy is a small increase in urinary albumin excretion (microalbuminuria), in addition there may be an initial increase in glomerular filtration rate (GFR) (Østerby, 1972; Watkins et al, 1972; Lundbaek & Østerby, 1976; Westberg & Michael, 1973). Nephropathy progresses to proteinuria and a declining GFR which combine to make renal failure seventeen times more common in diabetic patients than in the general population (Clarke et al, 1979; Brownlee & Cerami, 1981; Beiswenger, 1975).

Gangrene leading to amputation is at least five times more likely among diabetics (Brownlee & Cerami, 1981; Bell, 1957). Concurrent with this a wide range of debilitating symptoms such as diarrhoea (or constipation), loss of sensation, pain, ulcers and postural hypotension may also begin to appear. Atherosclerotic heart disease, due to vasculopathy, is prevalent in diabetics, though there is some regional variation with Europe and America having a higher rate than Japan (Winegrad et al, 1979; Blackard et al, 1965).

1.6c Cardiomyopathy

Diabetes is believed to be associated with specific cardiomyopathy (Ahmed et al, 1975; Hamby et al, 1974; Regan et al, 1974; Rubler et al, 1972; Senekiratne, 1977). Diabetes expresses itself in the heart with cardiac hypertrophy, congestive cardiac failure and paroxysmal nocturnal dyspnoea (Das et al, 1987).

Studies have shown impaired myocardial performance in the hearts of streptozotocin-induced diabetic rats. Abnormalities include increased time-to-peak isometric, decreased velocity of isotonic shortening, delayed rates of relaxation, decreased left ventricular pressure

(51)
development and diminished rates of left ventricular pressure decline (Fein et al, 1980; Penpargkul et al, 1980). Insulin treatment fails to correct these abnormalities (Malhotra et al, 1981).

Reduced ATPase activity of actomyosin, myosin and actin-activated myosin have been found in streptozotocin-induced diabetic rats (Malhotra et al, 1981). It has been proposed that ATPase activity is closely related to the contractile functions of skeletal muscle (Scheuer & Bhan, 1979) and is mediated by alterations in the isoenzyme of myosin (Flink et al, 1979; Hoh et al, 1977; Lompre et al, 1979).

It has been proposed that the early changes in the nervous system and the vasculature (see above) contribute to cardiac problems. Reduced blood supply to the myocardium due to vasculapathy is believed to result in angina. Sensory neuropathy may allow diabetic patients to overstress the heart without the resulting pain, which would normally act as a warning.

Recent studies have shown that the myocardium is itself directly damaged by diabetes. Contraction and relaxation times are slowed in the diabetic state, without any evidence of impaired nervous and vascular functions (Reddy & Khan, 1988). Prolongation of the total contractile phase would, presumably, reduce the periods of relaxation during which most myocardial blood flow takes place. Reduced blood flow would contribute to the increased incidence of and sudden death due to arrhythmias in diabetic patients (Reddy & Khan, 1988).
POSSIBLE MECHANISMS OF DIABETIC COMPLICATIONS

1.7a Proposed Mechanisms

(i) The Sorbitol (Polyol) Pathway

The sorbitol/polyol pathway has been suggested as one possible explanation for several complications which occur in diabetes. Many of the cell types which are most vulnerable to damage by diabetes, e.g. neurones and vascular endothelial cell, are capable of insulin-independent glucose transport, this leads to an increase in the intracellular glucose concentration during periods of hyperglycaemia. Intracellular glucose can suffer two fates, either glycolysis which requires phosphorylation by the enzyme hexokinase to glucose-6-phosphate or conversion to fructose and then glycolysis following phosphorylation by the enzyme fructokinase (Dvořák, 1978).

The conversion of glucose to fructose proceeds via the polyol pathway through the intermediate alcohol sorbitol under the action of two enzymes. Initially aldose reductase utilizes NADPH to reduce the aldehyde form of glucose to its corresponding sugar alcohol, sorbitol.

\[ \text{High } Km \text{ High } V_{\text{max}} \]

\[ \text{GLUCOSE + NADPH} \rightarrow \text{SORBITOL + NADP}^- \]

In the second step sorbitol dehydrogenase utilizes NAD\(^-\) to oxidise the alcohol sorbitol to the sugar fructose.

\[ \text{Low } Km \text{ Low } V_{\text{max}} \]

\[ \text{SORBITOL + NADP}^- \rightarrow \text{FRUCTOSE + NADPH} \]

Under normal physiological conditions available glucose is rapidly phosphorylated by hexokinase rather than being converted to sorbitol, since glucose has a greater affinity for hexokinase than aldose reductase. In diabetes hyperglycaemia raises intracellular glucose concentrations in susceptible cells and causes saturation of the
hexokinase, itself reduced in diabetes, so that the alternative pathway comes into play; resulting in the production of a significant amount of sorbitol. The polyol pathway functions unevenly however, sorbitol is produced more rapidly than it is converted to fructose, so that an accumulation of the alcohol occurs (Kador et al, 1983). This accumulation of sorbitol inside affected cells is further increased by its polar nature, since its polarity prevents membrane penetration and subsequent removal through diffusion. The intracellular sorbitol level increases to such an extent in some cells e.g. those of the lens in the eye, that an hyperosmotic effect occurs. This involves an infusion of fluid to counteract the resulting osmotic gradient. This influx of fluid may e.g. by damaging membrane permeability, contribute to cell pathology (Kinoshita, 1974; Ward et al, 1972; Gabbay, 1975; Palanco et al, 1977). It has been suggested that these permeability changes contribute significantly to diabetic cataract. Sorbitol accumulation in cells other than those of the lens may be insufficient to produce such gross osmotic effects but may cause damage by other mechanisms e.g. via a depletion of myo-inositol (Kador et al, 1985).

Due to the sorbitol pathway dysfunction in the diabetic peripheral nerve the levels of myo-inositol, fructose and sorbitol are all altered (Gillon et al, 1983). The sorbitol-producing enzyme aldose reductase can be inhibited and this brings about restoration of the levels of these compounds whilst simultaneously preventing the fall in sodium-potassium ATPase activity associated with diabetic neuropathy (Chandler & Miller, 1986). The effectiveness of these inhibitors has lead to their development as a new class of drugs and many are undergoing clinical trials.
Aldose and aldehyde reductases are members of a group of enzymes known collectively as the aldo-keto reductases. The enzymes are monomeric, primarily NADPH-dependent oxidoreductases with a broad substrate specificity for aldehydes (Sarges & Peterson, 1986).

There are three aldo-keto reductases present in animal tissue (Paulsen, 1979):

(i) **ALR$_1$**, a high Km enzyme, called Aldose Reductase.

(ii) **ALR$_2$**, a low Km reductase, possibly involved in diabetic neuropathy.

(iii) **ALR$_3$**, the only ketone reductase & called carbonyl reductase.

Aldose reductase contains sulphhydryl groups and it is postulated to exist in a globular form. Three distinct enzyme regions have been identified; a substrate region, a nucleotide, and an inhibitor site. Studies have revealed a nucleophile residue, possibly tyrosine, is present at the inhibitor site (Sarges & Peterson, 1986).

The aldose reductase inhibitors reversibly interact with aldose reductase at a common site independent of either the substrate or nucleotide cofactor fold. The inhibition which results is, therefore, non-competitive inhibition of the enzyme and is in part related to the lipid solubility of these inhibitors; increased inhibition has been observed with the introduction of hydrophobic substituents (Flynn, 1986; Kador et al, 1986). This is not surprising since they must cross the cell membrane to reach their target. In addition studies reveal that inhibitory activity is also increased in the presence of selective phenolic groups. The development of sterically constrained spirohydantoin aldose reductase inhibitors has also revealed that the enzyme can stereochemically differentiate between inhibitors; showing that inhibitory activity resided mainly with the S-enantiomer. Aldose reductases.
reductase inhibitors usually contain a group, such as carbonyl, thiocarbonyl etc. which is capable of undergoing reversible nucleophilic attack (Flynn, 1986; Srivastava et al, 1986; Pitts et al, 1986; Jaspan et al, 1986).

The implication of the polyol pathway in the onset of non-insulin treatable complications, which has been suspected for over a decade, has been supported by a vast range of experimental and clinical data (see figure #18). Briefly aldose reductase inhibitors prevent sorbitol accumulation in the diabetic peripheral nerve, retina, lens, kidneys, microvasculature and red blood cells. In clinical trials aldose reductase inhibitors have been reported to alleviate a variety of autonomic and somatic neuropathic symptoms.

Aldose reductase inhibitors prevent the build-up of sorbitol and fructose in the diabetic nerve and concurrently raise the level of myo-inositol. Restoration of myo-inositol levels is believed by some to be the mechanism by which aldose reductase inhibitors restore sodium-potassium ATPase activity. There are a range of inhibitors undergoing clinical evaluation (see figure #19).

(i) Myo-Inositol & the Phosphoinositides in Diabetes

Inositol (hexahydroxycyclohexane) is an isomer of glucose which has 7 optically inactive and one pair of optically active stereoisomers. The optically inactive form myo-inositol (see figure #13) is the one required by the body. Myo-inositol is present in high concentrations in human milk, in vegetables and meat; cereals have the hexaphosphate form, phytic acid, which is readily absorbed (Lewis, 1980).

Deficiency of myo-inositol is difficult to demonstrate since the body readily manufactures it, but it has been demonstrated (Eagle et al, (56))
FIGURE #18
EFFECTS OF ALDOSE REDUCTASE INHIBITORS IN ANIMAL MODELS

PERIPHERAL NERVE
Prevents changes in sorbitol, fructose, sodium-potassium ATPase, and myo-inositol levels in the sciatic nerve in diabetic rats. It also prevents or reverses changes in nerve conduction velocity and orthograde axonal transport. (Greene, 1986; Williams, Tomlinson & Robinson, 1986; Chandler & Miller; 1986)

RETINA
Prevents polyol accumulation in retinal capillaries and human retinoblastoma cell line.
In diabetic animals, prevents changes in retinal sorbitol, myo-inositol, sodium-potassium, and associated retinal electrical activity. Prevents capillary basement membrane-thickening in galactosemic rats. (Williamson et al; 1986)

LENS
Normalizes sorbitol, fructose, sodium-potassium ATPase activity, glutathione, and myo-inositol. Prevents and reverses sugar cataracts. Diminishes cataract formation in animal senile cataract model. (Kador, Akagi & Kinoshita; 1986)

KIDNEY
Prevents changes in sorbitol and sodium-potassium ATPase activity in diabetic rats.
Diminishes proteinuria in diabetic rats. (Beyer-Mears, 1986; Cohen, 1986)

RED BLOOD CELLS
Normalizes red blood cell sorbitol levels in diabetic rats. Prevents decrease in red blood cell deformation in diabetic rats. (Peterson, Page, Just, Aldinger & Malone; 1986)
FIGURE #19

ALDOSE REDUCTASE INHIBITORS
CURRENTLY BEING CLINICALLY TESTED.

(ponalrestat (ICI 128,436)

(epalrestat (Ono)

(sorbinil (Pfizer)

(tolrestat (Ayerst)

(Figures from: Stirling, 1988)
1957) that eighteen human cell lines all required it for growth.

Several observations have linked linked diabetic neuropathy to a reduction in the normal ninety-fold nerve to plasma myo-inositol concentration gradient (Mayhew et al, 1983; Winegrad & Greene, 1976; Gillon et al, 1983).

Tissue myo-inositol content is reduced by 20-30% in the nerves of diabetic patients (Hale et al, 1987). Acute experimental diabetes in the rat reduces nerve conduction velocity and nerve myo-inositol content by ~25% without changing plasma myo-inositol levels. In addition certain other abnormalities are present in the nerve, namely an increase in the levels of glucose, fructose, and sorbitol (Mayhew et al, 1983; Dyck et al, 1980; Tomlinson et al, 1984).

Insulin replacement that normalizes nerve conduction velocity in the diabetic rat also prevents the fall in myo-inositol nerve content.

Pharmacological elevation of plasma myo-inositol content by oral supplementation prevents or reverses both the reduced nerve myo-inositol content and motor nerve conduction velocity in the diabetic rat without affecting hyperglycemia or the raised nerve glucose, fructose or sorbitol levels (Greene et al, 1975).

These observations suggest that alterations in nerve myo-inositol content impairs nerve impulse conduction and other nerve functions.

Recent work has demonstrated a link between reduced myo-inositol content, the activity of the nerve Mg²⁺-dependent sodium potassium stimulated adenosine triphosphatase ("sodium-potassium ATPase), and plasmalemmal sodium gradient (Greene, 1986; Greene & Lattimer, 1982 & 1983).

A reduction of sodium-potassium ATPase activity in the diabetic nerve was postulated since the composite resting nerve energy utilization,
based on sodium-potassium ATPase activity, was reduced in experimental diabetes. The membrane-bound phosphoinositides have been implicated in membrane-bound sodium-potassium ATPase function in some mammalian tissue (Greene & Lattimer, 1985).

Myo-inositol uptake from plasma into tissue occurs by two transport systems, but mainly by a sodium and energy-dependent saturable transport system, which is responsible for at least 95% of the uptake, is the mechanism central in understanding diabetic neuropathy. The carrier present in this system has an affinity for myo-inositol 500X greater than glucose; but in hyperglycaemia the raised blood glucose level reduces myo-inositol uptake by 30% due to competitive inhibition. The hyperglycaemia undoubtedly inhibits myo-inositol uptake since in vivo equivalent amounts of fructose and mannitol have no effect (Greene & Lattimer, 1983). This primary uptake system probably contributes to the establishment and/or maintenance of the normal tissue to plasma myo-inositol concentration gradient (Greene & Lattimer, 1982; Gillon et al, 1983a & 1983b; ). Thus competitive inhibition of the carrier by glucose would seem to provide an explanation for the low levels of myo-inositol in, for example peripheral nerve, which has been observed in experimental diabetes. Though this mechanism doesn't explain normalization of myo-inositol levels by aldose reductase inhibitors. It would appear that raised intracellular sorbitol concentrations contribute to impaired cellular accumulation of myo-inositol.

Recent data has suggested that diabetes significantly decreases resting energy utilization by peripheral nerve in vitro (Greene & Lattimer, 1984). As nearly half of the steady-state energy utilization occurs via the sodium-potassium ATPase system, any reduction in utilization could reflect a corresponding reduction in sodium-potassium ATPase activity.
Since peripheral nerve myo-inositol uptake is driven by the sodium gradient, established by the sodium-potassium ATPase, any reduction in the ATPase activity would seriously impair myo-inositol uptake (Greene & Lattimer, 1985; Mayer & Tomlinson, 1983). These interactions, it is proposed, thus form a self-reinforcing cyclic metabolic derangement involving myo-inositol and sodium-potassium ATPase (see figure #20).

Myo-inositol is a substrate for the synthesis of phosphatidylinositol and the higher phosphoinositides, which are endogenous regulators of microsomal sodium-potassium ATPase. Any reduction in myo-inositol uptake, as outlined above, is likely to reduce the turnover of the phospholipids. Studies have shown a depletion in the myo-inositol pool utilized for the synthesis of phosphatidylinositol in sciatic nerve from streptozotocin-induced diabetic rats (Zhu & Eichberg, 1990). The phospholipids and sodium-potassium ATPase are both membrane bound, suggesting that since both are reduced in diabetes they may be linked in some way (Bell et al, 1982; Berti-Mattera et al, 1985a).

Myo-inositol is required for the synthesis of phosphatidylinositol, which is a parent compound to two important intracellular regulators, inositol 1,4,5-trisphosphate (IP$_3$) and diacylglycerol (DG). The normal cycle of phosphatidylinositol and the higher phosphoinositides synthesis and metabolism is shown in figure #21 (Natarajan et al, 1982).

Any reduction in the level of myo-inositol would tend to reduce the turnover of the phospholipids. This has been confirmed by the measurement of phospholipids in the nerve of the diabetic rat. The levels of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) have all been found to be reduced; as well as the activity of CDP-diacylglycerol and
FIGURE #20
PROPOSED CYCLE TO SHOW THE
CAUSE OF DIABETIC NEUROPATHY

HYPERGLYCAEMIA

\[ \text{HYPERGLYCAEMIA} \]

\[ \rightarrow \text{COMPETITIVE INHIBITION} \]

\[ \rightarrow \text{INCREASED SORBITOL LEVELS} \]

\[ \rightarrow \text{DECREASE IN SODIUM-DEPENDENT MYO-INOSITOL (MI) UPTAKE} \]

\[ \rightarrow \text{DECREASE IN } \text{Na}^{+}-\text{K}^{+} \text{ ATPase ACTIVITY} \]

\[ \rightarrow \text{DECREASED MI TISSUE CONTENT} \]

\[ \rightarrow \text{DECREASE IN MEMBRANE PHOSPHOINOSITIDE CONTENT} \]

This leads to decreased motor nerve conduction velocity and sodium-dependent amino acid uptake.

Proposed scheme interrelating glucose-mediated competitive inhibition of sodium-dependent myo-inositol (MI) uptake, phosphoinositide metabolism, and sodium-potassium ATPase activity in the diabetic nerve.

(Figure summarised from Greene, 1986)
FIGURE #21

THE SYNTHESIS AND METABOLISM OF THE PHOSPHOINOSITIDES.

FORMATION OF PHOSPHATIDYLINOSITOL

Glucose
↓
PA ------------------------> CDP-DG --------------------- PI

↓
DG

PG

ROLE OF PHOSPHATIDYLINOSITOL

Inositol
↓
PI KINASE
PI
↓
PIP Phosphomonoesterase
PIP
↓
PIP Phosphomonoesterase
PIP2

↓
PPIP2 Phosphomonoesterase
PPIP2 Phosphodiesterase
↓
DG + IP  <------------------------ DG + IP2  <------------------------ DG + IP3

EXPLANATION OF SYMBOLS.


(Figure summarised from Greene & Lattimer, 1983)
PIP kinase (Natarajan et al, 1982). The reduction of PI is important since it also reduces levels of sodium-potassium ATPase, which in turn reduces the level of sodium-dependent myo-inositol uptake (Gillon & Hawthorne, 1983; Berti-Mattera et al, 1985b). Measurements have shown raised levels of fructose and sorbitol concurrent with reductions in myo-inositol and sodium-potassium ATPase activity in the nerve of diabetics and experimental diabetic animals respectively, which would seem to support this proposal (Greene & Lattimer, 1985).

However, the above mechanism has been questioned by a recent observation that restoring myo-inositol levels is not necessary to restore functional sodium-potassium ATPase activity (Calcutt et al, 1990). The sorbitol pathway hypothesis, to explain the various dysfunctions occurring in the diabetic state, has also been contradicted by the finding of raised ouabain-sensitive sodium-potassium ATPase activities in the sciatic nerves of galactosaemic rats; which like diabetic rats have reduced levels of nerve myo-inositol (Lambourne, 1989). In addition to this, the observation that ouabain-sensitive sodium-potassium ATPase activities are depressed in several skeletal and cardiac muscles, has also cast doubt on the sorbitol proposal (Kjeldsen, 1987). Since glucose transport into skeletal muscle cells is generally insulin dependent, it seems unlikely that sufficiently high intracellular glucose concentrations occur to activate the polyol pathway. It is possible that intracellular glucose concentration is raised sufficiently if in conjunction hexokinase activity is sufficiently depressed.

This theory has been put forward to explain the many dysfunctions occurring in diabetes. It has gained so much currency that a whole range of aldose reductase inhibitors have been developed and are undergoing
clinical trials. These drugs inhibit the enzyme which converts glucose to sorbitol, and thus seek to prevent the accumulation of sorbitol and the fall in myo-inositol concentrations (Chandler & Miller, 1986). The results of clinical trials to date have, however, been disappointing (Florkowski et al, 1990). It has been suggested that the early changes occurring in diabetes are reversible whilst the later ones are not, so that aldose reductase inhibitors may only be of use prophylactically. However such a use poses severe practical and ethical difficulties, these will be discussed later.

(iii) Hypothyroidism

In consideration of the above findings an alternative suggestion has been made that the depression in sodium-potassium ATPase activities may be caused by hypothyroidism.

The thyroid gland releases two main hormones, thyroxine (T\(_4\)) & triiodothyronine (T\(_3\)), which are important regulators of body metabolism; in addition it also secretes thyrocalcitonin which acts in concert with parathyroid hormone to control calcium metabolism.

Thyroxine and T\(_3\) control body metabolism in a positive/negative feedback mechanism. They regulate energy metabolism at both the cellular and subcellular levels, and so control body growth (Lewis, 1980).

The thyroid hormones are synthesized from dietary iodide and tyrosine in the thyroid gland. Iodide ions are actively taken up by the follicle cells of the thyroid glands and oxidated to form iodine.

\[
21^- \rightarrow I_2
\]

Thyroglobulin, a complex glycoprotein, is synthesized in the follicle cells where it also iodinated, and during this process tyrosine residues of thyroglobulin are iodinated to give two forms.

(65)
Tyrosine + I$_2$ $\xrightarrow{\text{-----------}}$ 3-Iodotyrosine + 3,5-Diiodotyrosine

The resulting two forms undergo a further reaction to give the final hormones (Thomas & Kawhinney, 1973).

3-Iodotyrosine + 3,5,3'-'Triiodothyronine (T$_3$)

$\xrightarrow{\text{------------------------}}$ +

3,5-Diiodotyrosine + 3,5,3',5'-'Tetraiodothyronine (T$_4$)

The iodothyroglobulin complex forms a colloid, containing the various iodinated forms, and is stored in the follicles of the thyroid gland. The hormones are stored here as a colloid until required; in which case the colloid is taken back up by the epithelial cells. Here thyroglobulin is hydrolysed liberating T$_3$ and T$_4$ which are secreted into the bloodstream. 3-Iodotyrosine, 3,5-iodotyrosine, and other amino acids which are also liberated by the hydrolysis of thyroglobulin are reused.

Two tissues are involved in the control of the release of the thyroid hormones, the hypothalamus and the anterior pituitary gland. Of these the latter is the simplest and merely requires the pituitary gland to monitor circulating hormone levels and if these should fall it secretes Thyroid Stimulating Hormone (TSH or thyrotrophin) which stimulates thyroid hormone secretion (Kaloaf & Sadock, 1963; Lewis, 1980).

The hypothalamus controls the secretion of the anterior pituitary gland by secreting Thyrotrphin Releasing Factor into the portal system of blood vessels which supply the anterior pituitary gland.

Though both thyroxine and T$_3$ have the same action, there is a time difference in their action (see figure #22); T$_3$ acts immediately whereas there is a delay before thyroxine produces a response. The tetraiodoform undergoes conversion to the more active triiodo form in the liver. Both hormones act on the mitochondria, the power house of the cell, to increase metabolic activity. In addition they also sensitise cells to
FIGURE #22

SCHEMATIC REPRESENTATION OF THE SECRETION OF THE THYROID HORMONES

HYPOTHALAMUS

↓

TRF
(THYROTROPIN RELEASING FACTOR)

↓

ANTERIOR PITUITARY GLAND

↓

TSH
(THYROID STIMULATING HORMONE)

↓

THE THYROID GLAND

THYROCALCITONIN
(Works with parathormone to control Ca⁺⁺ metabolism)

THYROXINE
(T₄)

TRIODOTHYRONINE
(T₃)

Both control the metabolism of the body by acting on the mitochondria

The hypothalamus constantly monitors the circulating levels of the hormone, and if these should stray from designated parameters it responds accordingly.

(Figure from Lewis, 1980)
adrenaline and noradrenaline by either increasing adenyl cyclase synthesis or by producing transitory catecholamine receptors (Means et al, 196; Lewis, 1980).

Experimental diabetic rats have been found to be hypothyroid (Ganguly et al, 1987; Goyal et al, 1987); a condition which is known to affect sodium-potassium ATPase activity. This activity has been normalised by the administration of triiodothyronine (T$_3$) (Norgaard et al, 1988). It has been proposed that altered vasculature reactivity is mediated by diabetes-induced hypothyroidism, at least in the early period of diabetes (Saunders et al, 1978; Pittman et al, 1979; Dillman, 1982; Sundaresan et al, 1984; Jop, 1987).

Various studies have found a decrease in vascular responsiveness in experimental animals (Turlapaty et al, 1980; Pfaffman et al, 1982 & 1983; ----; Oyama et al, 1986) which has been normalized by insulin treatment (Pfaffman et al, 1983; Alexander & Oake, 1977; Macleod, 1985). Decreased responsiveness to isoproterenol in 8 weeks diabetic rats was restored by thyroxine treatment (Takiguchi et al, 1989). However thyroxine treatment failed to normalize responses to norepinephrine, 5-hydroxytryptamine and acetylcholine in 12 weeks diabetic rats (Takiguchi et al, 1989), suggesting that altered responsiveness in early period of diabetes may be the result of hypothyroidism whilst in later periods of diabetes other factors may be dominated e.g. hypoinsulinemia or hyperglycaemia. It seems insulin treatment is mediated through thyroid hormone levels; decreased myocardial β-adrenoreceptors in diabetes are mediated by hypothyroidism and insulin is effective in restoring β-adrenoreceptor numbers in the myocardium only if the thyroid is present (Sunderesan et al, 1984).
It is evident that early altered reactivity of the rat vasculature is linked partly to thyroid hormone deficiency but this does not lead to neuropathy. It may be that hypothyroidism is the trigger which initiates the changes in the vasculature observed in diabetics, however there is little evidence to support this contention.

Diabetes mellitus is associated with cardiomyopathy in humans (Kannel & McGee, 1979) and in experimental animals (Garber & Neely, 1983; Pemparkgul et al, 1980), with hypertension exacerbating the condition (Factor et al, 1981). Studies suggest that pressure load-induced left ventricular hypertrophy increases the susceptibility of myocardium to diabetes-related derangements (Fein et al, 1984; Rodgers, 1986; Rodrigues & McNeil, 1986). Hypothyroidism is closely associated with diabetes (Pittman et al, 1979) and has an equivalent effect on cardiac metabolism and function. Both conditions impair myocardial contractility, depress myosin adenosine triphosphate (ATPase) activity and sarcoplasmic reticular Ca$^{2+}$ uptake and reduce sensitivity to adrenergic agonists (Dhalla et al, 1985; Morkin et al, 1983; Rodgers et al, 1986; Bilezikian & Loeb, 1983). Treatment with T3, at a higher dosage than normal, of the diabetic spontaneously hypertensive rat was effective in preventing the depressions in measurements of heart function, including pressure generation, rates of contraction, and stroke work (Davidoff & Rodgers, 1990). It is argued that dosage adequate for therapy of hypothyroidism may not correct the hypothyroid state induced by diabetes since sensitivity to T3 maybe reduced in the latter (Davidoff & Rodgers, 1990). Further studies found myocardial dysfunction in diabetic rat heart including depressed myosin ATPase activity and reduced calcium uptake in the sarcoplasmic reticulum (Buccino et al, 1967; Ciaraldi & Marrinetti, 1977; Garber et
al, 1983). These defects are normalised in diabetic rats by the joint administration of T3 and palmoxinate (Tahilani & McNeil, 1985). The latter is a fatty acid analog which has been reported to decrease circulating glucose levels in diabetic rats, by inhibiting the excess fatty acid metabolism present in diabetes (Turner & Richens, 1978).

In diabetes, not only are serum levels depressed, but there is also tissue resistance. Diabetes impairs peripheral T4 to T3 conversion, tissue T3 uptake and both affinity and density of nuclear T3 receptors (Bagchi, 1982; Cantanni et al, 1988).

Diabetic cardiac hypertrophy and hypothyroidism leads to a shift from the predominant V, to V, myosin enzyme in rat ventricular muscle (Garber et al, 1983; Morkin et al, 1983; Dillman, 1982; Malhotra et al, 1981; Mercadier et al, 1981; Schaffer et al, 1989). T3 may be effective in shifting the ratio of myosin isoenzyme activity in favour of V, (Davidoff & Rodgers, 1990). Treatment with T3 also corrected the bradycardia of diabetic spontaneously hypertensive rats (Davidoff & Rodgers, 1990). Impaired thyroid function among diabetics may be as high as 30% (Ganguly et al, 1983) and T3 supplementation, with sufficient doses, may offer an effective treatment.

It is clear that T3 is strongly implicated in the depression of skeletal and cardiac muscle ATPase, and the sorbitol pathway, in some way, in nerve dysfunction and vasculopathy, but their interrelationship, if any, remains to be elucidated.

(iv) Protein Glycosylation

The non-enzymatic glycosylation of proteins is another mechanism proposed to account for the occurrence of both nervous and vascular diabetic complications.
Normally the free amino groups of protein lysine residues form a Schiff's base with the aldehyde groups of sugars, and the resulting compound rearranges to form a stable Amadori end-product.

In uncontrolled diabetics the increased sugar levels, due to hyperglycaemia, lead to an increase in the proportion of amino groups being glycosylated. One protein readily undergoing this reaction is haemoglobin; indeed the degree of haemoglobin glycosylation is an effective indicator of the degree and severity of diabetes (see figure #23).

Intra and extracellular proteins also readily undergo glycosylation (McVarry et al, 1979). Glycosylation of albumin leads to an increase in the rate of leakage from microvasculature, possibly contributing to the initiation of microangiopathy (Sampietro et al, 1987). Glycosylation of fibrinogen also occurs and may affect the rate at which fibrin is cleared by plasmin (Brownlee et al, 1983). Intracellular proteins, present in tissues permeable to glucose, also suffer the same fate (Vlassara et al, 1981; Vogt et al, 1982). As in the sorbitol hypothesis those cells likely to be most affected are those displaying insulin-independent glucose transport and which, therefore, are likely to experience high intracellular glucose concentrations in the presence of hyperglycaemia.

This process in particular occurs in the diabetic rat nerve where myelin, myelin-associated proteins and nerve tubulin are all affected; and which in turn mitigate the axonal transport of proteins (Williams et al, 1982). This effect appears to be marginal since tubules are involved in fast axonal transport whereas it is the slow transport which is deficient in the diabetic state (Williams et al, 1982). This is underlined by the observation that repeated injections of glycosylated
FIGURE #23

PROPOSED MECHANISMS TO EXPLAIN THE LATE COMPLICATIONS IN DIABETES

NON-ENZYMATIC GLYCOSYLATION

\[
\begin{align*}
\text{H} & \quad \text{C} \quad \text{O} \\
& + \quad \text{H}_2\text{N} \\
\text{(CHOH)}_n & \quad \text{CH}_3\text{OH} \\
\text{SCHIFF'S BASE} & \\
\text{NH} & \quad \text{CH} \\
\text{CH}_2 & \\
\text{C=} & \quad \text{O} \\
\text{(CHOH)}_n & \quad \text{CH}_2\text{OH} \\
\text{AMADORI PRODUCT} & \\
\text{NH} & \\
\text{CH}_2 & \\
\text{C=} & \\
\text{(CHOH)}_n & \\
\text{CH}_2\text{OH} & \\
\text{AMINOGUANIDINE} & \\
\text{NH} & \\
\text{CH}_2 & \\
\text{C=} & \\
\text{(CHOH)}_n & \\
\text{CH}_2\text{OH} & \quad \text{H}_2\text{N}-\text{NH}-\text{C}-\text{NH}_2 \\
\text{AUTOXIDATION OF SUGARS} & \\
\text{H} & \quad \text{H} \\
\text{O} & \quad \text{O} \\
\text{R-CH-CH} & \quad \text{R-C=CH} \\
+ & \quad \text{H}_2\text{O}_2 \\
(Both figures from Stirling, 1988)
proteins into normal rats produced no discernable change in the nerves (McVarry et al, 1980). Excess fructose produced by aldose reductase, from raised levels of sorbitol in hyperglycaemia, is more efficient than glucose at glycosylating proteins. Aldose reductase inhibitors have been reported to reduce glycosylation of proteins and this may be by reducing synthesis of fructose rather than lowering sorbitol levels (Monnier et al, 1991).

It has been shown that Amadori products undergo a further irreversible slow reaction leading to crosslinks in long half-life proteins (Brownlee et al, 1984), which in turn inhibit the proteolytic degradation and turnover of the protein. This has led to the suggestion that this effect is responsible for the known thickening of the basement membranes of capillaries which reduces oxygen tensions in the nerve (Reiser, 1990). The reduced oxygen tension consequently inhibits all energy-dependent processes perhaps resulting in the type of neuropathy prevalent in the diabetic state. It has been demonstrated that keeping diabetic rats in a hyperbaric oxygen restores nerve conduction velocities to normal in these animals (Low et al, 1986).

Nitric oxide has been recently identified as an endothelium derived relaxing factor (EDRF), an important mediator of smooth muscle responsiveness and resting vascular tone (Furchgott, 1984). EDRF is produced by endothelial cells, in response to agonists such as acetylcholine, and is responsible for activating smooth muscle responses by increasing intracellular levels of cyclic guanosine monophosphate (Ignarro et al, 1987; Palmer et al, 1987). However to initiate a response nitric oxide must traverse subendothelial collagen, and this protein accumulates, due to glycosylating modifications, over time (Eucala et al, 1991; Tanzer et al, 1982; Monnier et al, 1984). It has
been demonstrated that oxidising moieties of AGE products react with and quench nitric oxide activity in both vivo and vitro; and impairment of endothelium-dependent relaxation correlates directly to formation of AGE products (Bucala et al, 1991).

Treatment with aminoguanidine, a potent inhibitor of advanced glycosylation, prevented nitric oxide quenching and restored vasodilatory function (Bucala et al, 1991). Vasodilatory dysfunction appears to plateau after 2 months of diabetes, and it may be that other haemodynamic factors, e.g. prostaglandins, catecholamines, and electrolytes, intervene to prevent further impairment of the vasculature (Bucala et al, 1991; Tesfariariam et al, 1990; Weidmann et al, 1985 & 1979). Impaired nitric oxide function is not ameliorated by insulin administration in diabetic rats (Bucala et al, 1991).

This recent data provides a key link between protein glycosylation and endothelium dysfunction, however the role of other factors remains to be established and anti-glycosylation treatment has failed to be as effective as originally thought.

Evidence against glycosylation as a mechanism of neuropathy includes the fact that aspirin, a blocker in the formation of Schiff’s bases, has not been shown to alleviate diabetic neuropathy (Yue et al, 1984); although a long term effect by aspirin may be possible. Also the time difference, between the relatively quick on-set of slowed axonal transport and nerve conduction nerve velocity in diabetic animals and the slower production of advanced glycosylation end-products, suggests that glycosylation doesn’t produce these changes. The decreases in nerve conduction velocity and axonal transport may not however be directly related to diabetic neuropathy.

(74)
(v) Excess Free Radicals

The production of superoxide radicals, which are known to cause tissue damage, by the autoxidation of sugars (see figure #23) has been suggested to explain the neuropathy and vasculopathy occurring in the diabetic state.

Monosaccharides, in the presence of oxygen to form α-oxaldehydes and superoxide ions; and their production is increased in diabetes due to hyperglycaemia (Thornbally & Stern, 1984) The appearance of these superoxide radicals triggers off the body's immune response and are quickly detoxified by the enzyme superoxide dismutase or NADPH (Schaunstein et al, 1977). The α-oxaldehydes, which can cause protein crosslinks, are similarly detoxified by the glyoxalase pathway which utilizes glutathione (Carrol et al, 1986).

Ascorbic acid is a prime natural scavenger of free radicals in the body (Levine, 1986) and this vitamin is known to be deficient in diabetes, both in plasma and tissue (Som et al, 1981).

Free radical scavenger treatment fails to prevent the development of characteristic diabetic dysfunction such as reduced nerve conduction velocity, however, free radicals may participate in a more insidious manner (Stribling, 198). Alpha-oxaldehydes, produced by autoxidation of sugars, cause protein crosslinks and the formation of AGE-type products in proteins (Schaunstein et al, 1977). As previously outlined AGE products are believed to be responsible for quenching nitric oxide released from endothelium cells, and α-oxaldehydes may also contribute to the quenching effect. In addition it is significant that the time course of quenching product formation is similar to that which has been observed for the formation of free radicals (Namikawa & Hayashi, 1981).
It may be that excess free radicals interfere with the free release of nitric oxide by endothelium cells and induce inhibition of the vasodilatory effect in the diabetic state, since however there is a plateausing of this effect (Bucala et al, 1991), it is reasonable to suggest this is not the sole mechanism responsible for development of diabetic vasculopathy.

The significance of autoxidation may be marginal since free radicals scavengers do not prevent the development of tissue damage in the diabetic state, except that is in the lens, where low levels of reduced glutathione and elevated levels of oxidised glutathione have been found. However they are normal in the sciatic nerve of the streptozotocin-diabetic rats (Carrol et al, 1986). Some distortion would be expected in these levels, to account for the numerous dysfunctions occurring in the diabetic nerve. It would seem therefore that the body’s defence mechanisms can easily cope with the production of any free radicals by autoxidation.

(vi) Ganglioside Depletion

Gangliosides have attracted considerable attention since their neuritogenic and neuronotrophic properties were discovered. Extraneural tissues and cells contain very small amounts of gangliosides (Brunnergraber, 1979; Wiegandt, 1982 Ledeen, 1983) whereas neuronal plasma membrane has an estimated 10% of its total lipid in the form of sialoglycolipid (Ledeen, 1978) and nearly 20% in the outer leaflet of the bilayer. Also the majority of sialic acid is lipid bound rather protein bound.

Gangliosides play an important role in neuronal differentiation and this has been supported by studies which showed expression of GM1 on
maturing postmitotic cerebellar granule cell neurons and growing neurites and a preponderance of complex gangliosides in neurons undergoing neuritogenesis and synaptogenesis (Willinger, 1981).

Exogenously applied gangliosides are found to have neuritogenic and possibly neuronotrophic properties, influencing neuronal differentiation in vitro nerve regeneration in vivo (Ledeen, 1984).

It has been established that ganglioside structure is important, and that gangliosides differ in their ability to affect the various phases of neurite formation e.g. initiation, elongation and branching (Leskawa & Hogan, 1983; Ledeen, 1984); the asialo derivatives, neutral glycolipid, sulphatide and free sialic acid all proved ineffective. Also certain lines of cells, PC12 pheochromocytoma cells, failed to respond sufficiently to either gangliosides or nerve growth factor singly, but neuritic growth was promoted when both were administered jointly in the presence of high serum levels (Ferrari et al., 1983; Roisen et al., 1984; Katoh-Semba et al., 1984).

In vivo studies have demonstrated the beneficial effects of exogenous gangliosides in accelerating nervous system repair. Enhanced recovery of denervated cat nictitating membrane due to exogenous gangliosides has been found, for both pre- and postganglionic denervation, suggesting the effectiveness of this treatment for both cholinergic and adrenergic neurons (Ceccarelli et al., 1976). Serotonergic neurons are also reported to be regenerated by ganglioside treatment (Jonsson et al., 1983). In the extensor digitorum longus muscle of the rat exogenous gangliosides significantly increased polyneuronal innervation in the early stages of regeneration by stimulating sprouting of the dendrites (Gorio et al., 1980). Dosage is an
important feature since high levels of ganglioside produce an inhibitory effect on the recovery time for the startle reaction in the optic system of the goldfish (Grafstein et al, 1982). Axonal transport also appears to be increased by ganglioside application (Sbasehni-Agler, 1984). It would seem that these neuritogenic and neurotrophic are directly linked to gangliosides as antibodies to mixed gangliosides or GM1 retarded regeneration of axons in vivo (Gorio et al, 1980).

Ganglioside in addition promotes microvilli formation on the surface of the axon and tubulin mRNA (Spero & Rosien, 1984; Rybak et al, 1983); dibutyl cAMP has the same effect. This is not surprising as gangliosides increase intracellular levels of cAMP. It has been proposed that gangliosides affect the sprouting site of axons by possibly changing membrane ion permeability to increase Ca$^{2+}$ influx, so that it influences the membrane cytoskeletal relationship which might initiate neurite formation (Gorio et al, 1983).

In the diabetic state multiple dysfunctions occur in the axons, among these is decreased slow axonal transport of the G1 and G2 molecular forms of acetylcholinesterase and depressed nerve conduction velocity. Daily ganglioside injections to mutant diabetic mouse C57BL/ks (db/db) normalized these dysfunctions and also corrected defects in auditory perception, myelin particle density and axonal morphometry (Gorio et al, 1980). However in the early stages insulin was capable of improving nerve conduction velocity whilst gangliosides were ineffective. Ganglioside treatment was also observed to normalize axonal atrophy, sciatic nerve conduction velocity and auditory response in alloxan-induced diabetic rats (Gorio et al, 1984).
It has been proposed that gangliosides normalize these dysfunctions by stimulating regeneration, or alternatively, by stimulation of membrane enzymes to reconstitute a proper ionic distribution inside and outside the neuron (Gorio et al, 198.). This proposal has been supported by the recent observation that ganglioside treatment in human diabetics increased activity of Na⁺K⁺ ATPase which had been previously decreased by 53% (Della-Crippa et al, 1990).

It may be that ion channel irregularities in neurones are responsible for the reduced Na⁺K⁺ ATPase activity in the diabetic state rather than a failure of secondary-messenger phosphoinositides due to myo-inositol depletion. This is supported by evidence that restoration of myo-inositol levels is not necessary for normalization of Na⁺K⁺ ATPase activity in streptozotocin-induced diabetic rats and galactose-fed mice (Yeh et al, 1987; Calcutt et al, 1990).

(vii) Anoxia

It has been proposed that anoxia is the central defect in diabetic neuropathy.

Reduced oxygen tension in the nerve has been observed in the diabetic state (Brownlee et al, 1984). Also, the organic phosphate 2,3-DPG, believed to play an important regulatory role in oxygen exchange by hemoglobin, has been found to be reduced in diabetic patients and this may decrease oxygen delivery to critical tissue (Brownlee & Cerami, 1981). Furthermore, Low et al (1985) have reported that maintaining diabetic rats in hyperbaric oxygen restores nerve conduction velocity to normal in diabetic rats. The role of anoxia in the diabetic state is being currently evaluated and further work should reveal its significance in the onset of neuropathy in diabetes.
Eicosanoid Metabolism

Patients with diabetes mellitus have an excess activation of platelets, it is believed this, together with synthesis irregularities, may contribute to vasculopathy and cardiomyopathy in diabetes (Mustard & Packham, 1984; Tomasi et al, 1986).

In diabetes certain vascular dysfunctions occur, platelet release of thromboxane A₂ (TXA₂) is increased, endothelial prostaglandin (PGI₂) synthesis is decreased, and platelet sensitivity to PGI₂ is decreased (Halushka et al, 1981a & 1981b; Butkus et al, 1980; Gerard et al, 1980; Schernthner et al, 1981). Previously the ratio between platelet derived TXA₂ (pro-coagulant) and endothelial synthesized PGI₂ (anti-coagulant) was believed to determine the aggregation of platelets; but however it is now acknowledged that other factors play a crucial part (Porta et al, 1987). Endothelial cells also synthesize and release TXA₂, von Willebrand Factor (vWF) (Tschapp et al, 1984), platelet activating factor (PAF) (Ingerman-Wajenski et al, 1981; Camussi et al, 1983; Prescott et al, 1984), antithrombin III (Busch & Owen, 1982; Chan & Chan, 1981), and thrombomodulin (Eisman & Owen, 1981).

Studies have shown elevated plasma levels of vWF, a platelet-subendothelial adhesive factor, in diabetic patients with microangiopathy, closely related to the duration of the disease (Bensoussan et al, 1975; Coller et al, 1978; Porta et al, 1981). It appears that the endothelium increases synthesis and release of this factor in diabetes, but its effect, in vitro, on platelet aggregation is minimal (Porta et al, 1987).

Vitamin E significantly decreases platelet sensitivity to the aggregation effects of ADP and collagen (Karpen et al, 1981; Steiner, 1983) and is responsible for decreasing stimulated thromboxane release.
Normally membrane phospholipids release arachidonic acid which is converted to prostaglandin endoperoxides, which in turn transforms to thromboxane and prostacyclin (Moore, 1985). Certain studies suggest that vitamin E reduces stimulated thromboxane release by reducing phospholipase A2 activity, the enzyme responsible for arachidonic acid release from membrane phospholipids (Karpen et al., 1981; Steiner, 1983; Hamelin & Chan, 1983; Pritchard et al., 1982). Vitamin E supplementation was effective in normalizing platelet TXA2 production and PGI2 in diabetic animals (Karpen et al., 1982; Gilbe t et al., 1983) and patients (Gisenger et al., 1988).

The beneficial effects of vitamin E supplementation suggests that TXA2 and PGI2 synthesis plays an important role in platelet aggregation, however whether thrombus or clot formation is ultimately responsible for vasculopathy in the diabetic state remains to be clarified.

Prostaglandins are synthesized in cardiac tissue and have potent effects on contractility, heart rate and coronary vessel tone (Limas et al., 1973; Malik & McGiff, 1976). Studies have shown increased arachidonic acid promoted formation of PGE2 and PGE1 in perfused hearts from diabetic rats (Rosen & Schror, 1980). Increased conversion of microsomal arachidonic acid in diabetic rats (Durante et al., 1989) induces elevated levels of synthesis of prostaglandins. Increased PG production does not however, lead to any increase in their catabolism (Durante et al., 1989). PGI2 is the major prostanoid formed in both control and diabetic rat cardiac homogenates. It is suggested that PGI2 is predominantly formed by vascular arteries, an effect which is enhanced in diabetes (Durante et al., 1989). Increased PG levels in diabetes would have a detrimental effect on the heart rate, increasing the cardiac work load and possibly causing further complications.
Further work should establish which element is the keystone of prostaglandin synthesis dysfunction and offer the possibility of useful therapies.

(ix) Vitamin C Depletion

Hyperglycaemia leads to ascorbic acid depletion and this has been proposed as a cofactor in the accumulation of sorbitol in insulin-independent cells, such as the endothelium, which has been proposed as a possible cause of vasculopathy in diabetics.

Ascorbic acid concentration and turnover is decreased in the plasma and tissue of diabetics (Yew, 1983; Chen et al., 1983). Ascorbic acid uptake is inhibited by glucose in vitro, this may be due to similarities in structure between L-ascorbic acid and D-glucose so that the ascorbic acid carrier is inhibited by glucose. This effect is exaggerated in hyperglycaemia, and ascorbic acids reduced form, dehydroascorbic acid, is inhibited in endothelial cells and erythrocytes (Kapeghian & Verlangier, 1984; Mann & Newton, 1975). Insulin actively promotes the uptake of ascorbic acid, thus in uncontrolled diabetes ascorbic acid uptake is doubly reduced due to hypoinsulinaemia and hyperglycaemia (Verlangier & Sestito, 1981; Berhanu & Olefsky, 1981).

Ascorbic acid is essential in affecting collagen biosynthesis and posttranslational modification by maintaining the enzyme prolyl hydroxylase (PRLase) which catalyses the hydroxylation of proline to form hydroxyproline, an amino acid required for the stability of the collagen molecule (Barnes, 1976; Myllyla et al., 1978; Berg & Prockop, 1973) PRLase activity has been observed to be decreased in the tissue of diabetic animals (Ramamurthy et al., 1985; Yue et al., 1987) and
appears to be strongly linked to ascorbic acid depletion. Ascorbic acid supplementation and tolerastat (an aldose reductase inhibitor) treatment in diabetic rats both prevented the previously observed decrease in PRLase (McLennan et al., 1988). Tolerastat appears to be linked to ascorbic acid levels, since treatment with this ARI increases ascorbic acid plasma levels (McLennan et al., 1988). A possible mode of action is that tolerastat increases tissue concentration of reduced glutathione, which is required for the recycling of dehydroascorbate to ascorbic acid, and it is ascorbic acid in conjunction with an ARI which reduce cell sorbitol accumulation. If so, it may be beneficial to combine ARI therapy with ascorbic acid supplementation.

Abnormal sorbitol accumulation in erythrocytes is normalized by ascorbic acid supplementation in both vivo and vitro (Vinson et al., 1989). It has been proposed that ascorbic acid consumes NADPH indirectly through the glutathione redox couple (Varma et al., 1979), thus depleting NADPH for the conversion of glucose to sorbitol (see above).

Ascorbic acid is undoubtedly involved with regulating cell sorbitol levels, and may be an important cofactor in the treatment of diabetes, wider clinical trials should be initiated to evaluate the efficacy of this treatment; since large ascorbate doses taken chronically appear to be free from ill effects, ethical problems in such trials would be minimal.

(x) Blood Rheological Changes

Diabetics have rheological abnormalities including an increase in whole blood viscosity, plasma viscosity and red cell aggregation and a
reduction in red cell deformability (Barnes, 1988), and it has been proposed that this is responsible for reduced flow and sludging of blood within capillaries in patients suffering vasculopathy (Simpson, 1988; Macrury et al, 1991).

Patients with microvascular complications have more rheological abnormalities than diabetics lacking vasculopathy (Lowe et al, 1986; Hill et al, 1982). Patients with and without diabetic neuropathy both had similar impairment of red blood cell deformability and increased blood viscosity, suggesting that abnormal rheology is not directly responsible for endoneurial hypoxia, it may be a contributing factor (Macrury et al, 1991). Reduced microvessel radi coupled with abnormal rheology may seriously reduce vital blood flow and thus initiate vascular dysfunction (Macrury et al, 1991).

Decreased red cell deformability in diabetic patients has been linked to increased platelet aggregation, due to increased synthesis of the coagulant ADP by red cells (Juhan et al, 1982). Deformability of red cells is essential in the microvasculature, any reduction in deformability hinders the cells passage across nutritive capillaries leading to narrowing of these capillaries and also reducing blood flow; these effects may combine to cause ischaemic organ damage (Macrury et al, 1991). White blood cell deformability is similarly reduced in diabetes (Ernst & Matrai, 1986) leading to blocking of the microvasculature and thus reducing blood flow and increasing viscosity (Ernst et al, 1987). In addition white and red blood cells show an increased tendency to adhere to the endothelial surface where they not only reduce blood flow but also damage the endothelium by releasing toxic oxygen compounds and proteolytic enzymes (Macrury et al, 1991; Chien, 1985; Vautier et al, 1986). Lack of red cell deformability
could also physically damage endothelial cells.

It appears that changes in blood rheology may be a contributing factor to the development of vasculopathy, they are certainly important in causing hypoxia, and it is only in conjunction with metabolic abnormalities that they cause dysfunction in the vasculature.

(ii) Blood Lipid Changes

Essential fatty acid synthesis and metabolism is abnormal in vascular tissue in diabetic patients and animals (Horrobin, 1988; Rot et al, 1983) and it is believed that this alters the prostacyclin and thromboxane A2 levels leading to vasculopathy (Koncada & Vane, 1979).

Fatty acid composition is significantly affected in the phospholipids of experimentally-induced diabetic animals in a variety of tissue, such as erythrocytes, liver, heart and kidneys (Eck et al, 1979; Faas & Carter, 1980 & 1983; Holman et al, 1983; Huang et al, 1984; Dang et al, 1988).

Certain desaturase enzymes are essential for the formation of arachidonic acid, palmitoleic acid, eicosapentaenic acid (EPA) and docosapentaenic acid (DHA). $\Delta^9$-desaturase converts saturated fatty acids to unsaturated ones, $\Delta^9$-desaturase converts linolenic acid to $\gamma$-linolenic acid, $\Delta^9$-desaturase converts dihomo-$\gamma$-linolenic acid to arachadonic acid and eicosatetraenoic acid to EPA, and $\Delta^6$-desaturase converts EPA to DHA. Dysfunction of fatty acid metabolism is probably linked to decreased activities of these desaturases, $\Delta^{9}$, $\Delta^{9}$ and $\Delta^{9}$ (Eck et al, 1979; Faas & Carter, 1980; Holman et al, 1983; Dang et al, 1988).

Relative levels of arachidonic acid are decreased whilst $\omega$-3 fatty acid content is increased in the tissue phospholipids of diabetic rats.
Holman et al, 1983). Reduced arachidonic acid levels would seriously compromise the formation of prostaglandins and subsequently impair the normal rheological functions of the vasculature. Insulin therapy is effective in normalizing Δ⁶ and Δ⁶-desaturase activities and fatty acid composition, but fails to correct reduced arachidonic acid levels (Faas & Carter, 1983; Dang et al, 1988). Essential fatty acid supplementation has been successfully used to counteract the effects of diabetes. Treatment with evening primrose (Oenothera biennis) oil, rich in γ-linolenic acid, was effective in improving reduced nerve conduction velocity in diabetic rats and increasing endonurial capillary density (Tomlinson et al, 1989; Julu, 1990; Cameron et al, 1991). It may be that increased arachidonic acid levels lead to elevated phosphoinositide turnover and/or enhanced diacylglycerol stimulation of protein kinase C, thus restoring Na⁺K⁺- ATPase activity and improving nerve conduction velocity.

γ-Linolenic acid is reduced in diabetes because of a deficit in linolenic acid 6 desaturase (Poisson, 1985), evening primrose oil treatment could correct this by allowing formation of prostaglandin E₃ (PGE₃) from dihomo-γ-linolenic acid and prostacyclin and PGE₂ via arachidonic acid (Cameron et al, 1991). PGE₁, PGE₂ and prostacyclin analogues have all been reported to be effective in improving blood flow and nerve conduction velocity in diabetic rats (Yasuda et al, 1989; Ito et al, 1990).

Ω-3 fatty acid supplementation, specifically eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), has been reported to have dramatic effect on vascular function, they not only modify platelet function and eicosanoid synthesis but also affect many other mechanisms which may be involved in the pathogenesis of vasculopathy (Axelrod, 1989;
Leaf & Weber, 1988; Von Schacky, 1986). 0-3 fatty acids inhibit arachidonic acid synthesis, decrease platelet production of thromboxane A₂, increase platelet production of thromboxane A₃, increase synthesis of prostaglandin I₃ (PGI₃), decrease platelet aggregation, decrease plasma lipoprotein levels, increase erythrocyte deformability, decrease blood viscosity, decrease blood pressure and increase thrombolytic activity (Leaf & Weber, 1988; Von Schacky, 1986; Herald & Kinsella, 1986). However in diabetics they have certain deleterious effects, namely increased plasma glucose levels, increased glycosylation of haemoglobin, and elevated plasma cholesterol levels (Axelrod, 1989); though there is some evidence to suggest that dosage may be the contributing factor (Axelrod, 1989).

Abnormal fatty acid metabolism is an acknowledged defect in diabetes and its consequences may seriously impair normal vasculature function. Dietary fatty acid supplementation offers a useful method to correct the dysfunctions of the vasculature, but this must be balanced against its side effects, and studies to establish correct dosage should provide useful information for later therapeutic trials.

(xii) Increased Blood Fibrinogen

Blood clotting factors play an important role in blood viscosity, platelet aggregation and blood clotting, any alteration in these factors would have serious implications for vasculature function. Fibrinogen, a primary clotting factor, is increased in diabetes and which may result in hypercoagulability of the blood, so contributing to dysfunction of the vasculature (Kannel et al, 1987).

Studies have shown that levels of circulating fibrinogen are closely related to the development of strokes, cardiac disease, and
cardiovascular mortality (Meade et al., 1980; Wilhelmsen et al., 1984).

Raised fibrinogen levels increased the risk of strokes and myocardial infarction, even when other factors, such as blood pressure, serum cholesterol level and smoking, had been eliminated. Hyperlipoproteinaemia, a common complaint in diabetes, also increased levels of fibrinogen (Lowe et al., 1979).

Atherosclerotic cardiovascular disease is linked to increased fibrinogen levels by its effect on hypercoagulability, blood viscosity, red cell deformability and haemorheology, especially in people with impaired circulation due to hyperlipidaemia, hypertension and glucose intolerance (Kannel et al., 1987). It is probable that fibrinogen is involved in fibrin accumulation and affects the binding of low-density lipoproteins (Lowe et al., 1979; Smith et al., 1979).

Fibrinogen levels can be reduced by weight reduction (a common feature of maturity-onset diabetes), cigarette smoking abatement, bloodletting, glucose control and reduction of hypertension (Kannel et al., 1987). Treatment with aspirin and pentoxifylline has been suggested to counteract certain effects of fibrinogen, such as increased blood viscosity, platelet aggregation and red blood cell rigidity (Kannel et al., 1987).

Fibrinogen levels have important ramifications for the vasculature and are probably a cofactor for dysfunctions to normal blood flow. However, whether controlling fibrinogen levels or the underlying factors which give rise to elevated fibrinogen offers the best course of treatment for diabetic vasculopathy remains to be elucidated.
(xiii) Hypertension

Patients with diabetes mellitus often have hypertension and it is believed that this may play an important role in the development of vasculopathy (Marks, 1971; Chriestlieb, 1973).

Angiotensin-converting enzyme (ACE), responsible for converting angiotensin I to angiotensin II, is synthesized by vascular endothelium and has a potent effect on blood pressure (Caldwell et al., 1976; Dorer et al., 1974). Levels of ACE have been reported to be increased in diabetic patients (Schmitz et al., 1985) and it may be that elevated ACE levels are responsible for producing hypertension. However, another study failed to confirm these observations (Porta et al., 1987) and it appears that this effect is limited to a minor proportion of patients suffering microangiopathy. It is probably a manifestation of vascular dysfunction rather than a cause of it.

It seems more probable that hypertension is a clinical symptom of various changes occurring in the vasculature, such as increased platelet aggregation, decreased red cell deformability, and increased blood viscosity (Porta et al., 1987). Treatment to reduce hypertension fails to tackle the basic dysfunctions inherent in diabetic vasculopathy; insulin successfully reduces hypertension but endothelium irregularities still persist in diabetes (Alexander & Oake, 1977). Hypertension undoubtedly contributes to vasculopathy, but it is not the only factor.

(xiv) Sympathetic Neuropathy

Diabetic patients and experimental animals are abnormally sensitive to circulating vasoconstrictors, such as catecholamines, and this may be
caused by impairment in function of the sympathetic fibres which innervate the microvasculature, so that a process of denervation sensitization occurs (Brody & Dixon, 1964), though this would only explain increased sensitivity to catecholamines and the profound neuropathy in diabetic patients.

Alloxan-diabetic rats were found to be supersensitive to intra-arterial injections of epinephrine, norepinephrine and synthetic angiotensin (Brody & Dixon, 1964). Increased vascular reactivity to K⁺, serotonin and prostaglandins has been reported in alloxan-diabetic rabbits and streptozotocin-diabetic rats (Agrawal et al, 1985; Cseuz et al, 1973; Scarborough & Carrier, 1984; Macleod & McNeill, 1985).

Sympathetic nervous function abnormalities have been observed in diabetic patients (Low et al, 1975) and experimentally diabetic rats (Yoshida et al, 1987; Ganguly et al, 1987). Decreased nerve conduction, slowed axonal transport, axon dwindling and segmental demyelination are all common features of diabetic neuropathy (Brownlee & Cerami, 1981; Vingrad et al, 1979; Thomas & Lascelles, 1964; Dyck et al, 1971). It is believed these neuronal dysfunctions affect normal catecholamine turnover and lead to irregularities in the responses of the vasculature. Increase in peripheral blood flow, often accompanied by chronic pain, frequently occurs in diabetics. Turnover of catecholamines seems to be implicated in this since noradrenaline uptake 1 blockers, such as desipramine, were effective in preventing these symptoms (Max et al, 1991).

Increased sensitivity to catecholamines appears to be linked to decreased sympathetic activity in the diabetic state, whether this is wholly responsible for vasculopathy is doubtful, more likely it is a contributing factor.
Alterations in nerve growth factor, an important peptide involved in the development, maintenance and regeneration of neurones (Levi-Montalcini & Calissano, 1979; Levi-Montalcini, 1987; Mobley et al, 1977; Calissano et al, 1984), have been postulated as underlying diabetic neuropathy (Mobley et al, 1977).

Studies have shown that alloxan and streptozotocin-induced diabetes mellitus is associated with neuropathy, and with impairment of nerve growth factor transport in nerve fibers (Longo, 1985; Schmidt, 1985; Schmidt & Yip, 1985). In addition endogenous levels of nerve growth factor are altered in rodents during streptozotocin-induced diabetes both in nerve growth factor producing target organs (Hellweg & Hartung, 1990; Kasayama & Oka, 1989) and in neuronal structures known to transport nerve growth factor or to be nerve growth factor dependent (Hellweg & Hartung, 1990).

Nerve growth factor producing tubulary cells of the submaxillary gland show vacuolization and rupture of granules from streptozotocin-induced diabetic rats (Camacho et al, 1982). Studies in diabetic patients, suffering neuropathy, have found decreased nerve growth factor levels, and the severity of neuropathy, as assessed by reductions in motor nerve conduction velocity, is directly linked to low nerve growth factor levels (Faradj & Sotelo, 1990).

Insulin has many functional and chemical similarities with nerve growth factor (Frazier et al, 1972), such as physiological activity, and molecular structure (Frazier et al, 1972; Frazier et al, 1973). It may be that in diabetes both peptides are similarly affected since, it is argued, diabetic complications are not common when diabetes mellitus is secondary to other diseases like chronic pancreatitis.
In painful diabetic neuropathy increased numbers of small unmyelinated axons have been found (Brown et al, 1976), suggesting that abnormal regeneration could be due to low nerve growth factor levels. The primary target cells for nerve growth factor are sensory and sympathetic neurons (Levi-Montalcini & Calissano, 1979; Levi-Montalcini, 1987; Mobley et al, 1977), and since diabetic neuropathy is predominantly mixed or sensory (Brown & Asbury, 1984; Adams & Victor, 1985), there would appear to be a correlation. Insulin has trophic properties (Frazier et al, 1972), it induces neuronal differentiation, and hypoinsulinaemia during unregulated diabetes could be a factor in the development of neuropathy (Sotello et al, 1980).

Reduced nerve growth factor levels have been normalized in the sciatic nerve, submandibular gland and iris of streptozotocin-diabetic rats by allotransplantation of insulin-producing islets (Hellweg et al, 1991). Daily injections of insulin were also successful in normalizing nerve growth factor levels (Kasayama & Oka, 1989), this suggests that nerve growth factor is not important in diabetes as properly regulated insulin therapy would presumably correct any defect in nerve growth factor levels.

It is clear that nerve growth factor is involved, in some capacity, in diabetes; only further studies, such as nerve growth factor measurement in diabetics without neuropathy, in nerve biopsy, in insulin-dependent diabetics, and in other neuropathies of toxic and nutritional origin, can reveal its true...
1.7b Summary

The difficulty of assigning any single clinical symptom in diabetes, to one of the three groups outlined above, is aptly demonstrated in the 'diabetic foot'. In diabetes ulceration of the foot is fifteen times more common possibly leading to hospitalization and amputation (Boulton, 1990). The cause was originally believed to be vascular in genesis. Evidence of microvascular abnormalities and reduced blood flow all seemed to support this contention (Timperley et al, 1985).

However evidence for a neuropathic factor, both somatic and sympathetic, in the aetiology of foot ulceration was found in studies which showed that canine hind paw ulceration only occurred if both sciatic and sympathetic nerves were transected (Nielubowicz et al, 1974). Various clinical studies showed 90% (combined figure) of diabetic ulceration implicated neuropathy as the aetiological factor (Boulton, 1990).

Inability to assign clinical symptoms to a single group makes the development of effective treatment even more difficult.

The above mechanisms are not mutually exclusive, it is not a case of one or the other to explain diabetic complications. Several mechanisms may contribute partially or wholly to cause the dysfunctions observed in diabetes. Some of these mechanisms may interact, in as yet unknown manner, to produce diabetic complications. The restoration of Na⁺K⁺-ATPase activity in diabetes by the administration of gangliosides, β₃, myo-inositol and an aldose reductase inhibitor, suggests possibly that several different mechanisms are responsible. It is likely that only when investigators study a number of mechanisms in conjunction that a true picture of diabetic complications will emerge.

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Various mechanisms have been proposed as being responsible for diabetic complications. Mechanisms such as sorbitol accumulation, myo-inositol and phosphoinositide depletion, hypothyroidism, eicosanoid metabolic dysfunction, non-enzymatic protein glycosylation, excess free radicals, ganglioside depletion, anoxia, vitamin C depletion, hypertension, reduced nerve growth factor, blood lipid, rheological and fibrinogen changes, and sympathetic neuropathy have all been investigated, to a lesser or greater degree, as the possible cause of diabetic complications. The reversal of certain dysfunctions, e.g. sorbitol accumulation by aldose reductase inhibitors, myo-inositol depletion by oral supplementation, hypothyroidism by triiodothyronine treatment, and prostacyclin and thromboxane normalization by ω-3 fatty acids or thromboxane antagonists, requires them to be fully tested for efficacy. If they have therapeutic value these treatments may then be introduced to patients suffering diabetic complications.

Studies to investigate the effects of diabetes and possible therapies can only occur on a suitable model, susceptible to all the symptoms prevalent in humans, which will reveal the full interplay of different factors. The interrelationship between vasculopathy, cardiomyopathy and neuropathy creates problems, as no single model suffers the full range of defects detected in diabetic patients; though even diabetics vary in their susceptibility to complications. Various animal models have been used for the complications occurring in the diabetic state.

The spontaneously diabetic Chinese hamster develops autonomic neuropathy with bladder and bowel dysfunction but appears to be relatively resistant to the development of arterial disease and is of such small size that studies of various cardiovascular risk factors,
such as plasma lipoproteins, coagulation factors, and blood pressure, are impractical (McCombs et al, 1974; Dail et al, 1977; Bryan et al, 1977; Diani et al, 1978). Similar problems affect obese KK, ob/ob and db/db mice; they also fail to develop cardiovascular complications (Chobanian et al, 1974; Coleman, 1978; Camerini-Davalos et al, 1970).

The Wistar BB rat develops hyperglycaemia, hypoinsulinaemia, platelet prostaglandin synthesis dysfunction, and endothelium arachidonic acid depression, but it is resistant to the development of atherosclerosis (Nakhooda et al, 1976; Subbiah & Dietemeyer, 1980). Increases in plasma levels of cholesterol, triglycerides and free fatty acids have been found in the obese Zucker rat, but it fails to develop vasculopathy (Haudenschild et al, 1981; Schonfeld et al, 1974; Subbiah & Connelly, 1976). Arterial disease is present in obese hypertensive rats, together with hyperglycaemia, marked hyperinsulinaemia, and hyperplastic pancreatic islets (Okamoto, 1972; Koletsky, 1973).

Rats can be made chemically diabetic by injection with alloxan or streptozotocin. Autonomic nervous dysfunctions have not been found in these animals (Still et al, 1964). Diabetic rats do exhibit hyperlipoproteinaemia, hypercholesterolaemia, and hypertriglyceridaemia, though evidence for macrovascular disease is limited and they appear to be resistant to atherosclerotic vascular disease (Urrutia et al, 1962; Wolinsky et al, 1978; Schein et al, 1971; Bar-On, et al, 1976; Christlieb, 1973). Rats offer an opportunity to study the effects of diabetes on lipid metabolism, though its metabolism in rat differs from that in man. Biochemical differences, rats lack cholesterol ester transfer protein, mean that data from rat model studies should be
treated carefully (Chobanian et al, 1982).

Alloxan treatment can be used to make rabbits diabetic and they tolerate its effects remarkably well, provided severe ketosis and dehydration is avoided (Kushner et al, 1969). Macrovascular disease can be induced in rabbits with cholesterol feeding following which atherosclerosis successfully develops (Duff & McMillan, 1949; Duff et al, 1954; Duff & Payne, 1950). Rabbits suffer hyperlipidaemia, increased phospholipid serum levels, reduced hexokinase activity, and hypertriglyceridaemia (Pierce, 1952; Mulcahy & Winegrad, 1962; Morrison et al, 1972). However they fail to develop hypertension, a drawback to investigating multiple risk factors in diabetes (Clements et al, 1969; Morrison et al, 1972).

Yucatan miniature swine with reduced glucose tolerance develop atherosclerosis spontaneously with involvement of both the coronary and cerebral circulations. In addition, the serum lipoproteins of swine appear to resemble those in man, and the animals are of sufficient size to permit extensive metabolic investigations (Philips et al, 1979).

Several species of monkeys develop a range of dysfunctions which mirror human diabetic complications. Spontaneously and chemically-induced diabetes in black celebes macaques, squirrel monkeys, cynomolgus macaques, and obese rhesus monkeys leads to increased plasma triglycerides, elevated pre-beta lipoproteins, increased nonesterified fatty acids, development of atherosclerosis and marked ketonuria and glucosuria (Howard, 1979; Howard & Palotay, 1979; Davidson et al, 1967; Ausman & Gallina, 1978; Gallina & Ausman, 1979; Lehner et al, 1972; Bellinger & Bullock, 1980; Hamilton et al, 1972; Lockwood et al, 1979).
The neonatal lamb, with alloxan-induced diabetes, has been used to study cardiac dysfunction. Though myocardial uptake of glucose and fatty acids was reduced, left ventricular performance was not affected (Lee & Downing, 1979). Since ventricular performance impairment is a primary cardiac defect in diabetes, this model offers limited scope for the assessment of cardiomyopathy.

Alluxan-induced diabetes in dog leads to glucose intolerance and reduced cardiac stroke volume associated with reduced end-diastolic volume and high end-diastolic pressure (Regan et al, 1974; Hiader et al, 1977).

The partial susceptibility of various species to diabetic complications requires workers to be particularly thoughtful when selecting the model of diabetes. Comparison of data among different species leads to difficulties, and fragmentation of the literature, about a specific diabetic defect, among species hinders understanding. Monkeys, swine and dogs offer useful models for studying vasculopathy and cardiomyopathy, but financial constraints limit their extensive use. Whilst rodents may be financially more viable, their relevance to human diabetes makes interpretation difficult. Rabbits offer a medium course, they are susceptible to most of the defects of human diabetes and readily available.

There is a need for a suitable model of diabetes, one that is consistent and financially viable.
There is a need for a reliable animal model for diabetic complications to evaluate mechanisms and possible therapies. The large number of diabetics in population and the frequency at which they develop complications make the market for any effective therapy a very large one, particularly if prophylactic therapy proved necessary. Using human subjects as test subjects for understanding the disease has several disadvantages. Diabetic complications develop very slowly and inconsistently, therefore large trials over several years would be required to evaluate the various changes occurring. There would also be ethical problems as subjects would be given treatment for problems they wouldn't develop. Many experiments involving nerve tissue wouldn't be feasible with human subjects; experimental animal models would appear to overcome these disadvantages.

Various abnormalities have been reversed by a wide range of therapies e.g. aldose reductase inhibitors, myo-inositol supplementation, T3 treatment, ganglioside supplementation, aspirin, ω-3 fatty acids, and of course insulin (see above for more detail). Aldose reductase inhibitors have been the only class of drugs to have undergone wide clinical assessment, though with mixed results. Reversibility of some possible mechanisms, implicated in diabetic complications, by various treatments offers potentially useful therapies. However their assessment can initially only occur in an effective diabetic model, the development of which must be a priority.

Current models of diabetes assess neuropathy by evaluating changes in nerve conduction velocity and axonal transport. Whether these observed changes are indeed central to diabetic neuropathy or merely side-effects of other, as yet, unrecognised changes is the main fault with
the current models. This failure to appreciate alternatives and explore other possibilities in the current models means that perhaps the true cause of diabetic complications still eludes workers in this field. A good model should allow other possibilities to be tested.

A wide range of aldose reductase inhibitors are being tested, but their effectiveness has been patchy. It may be that early changes leading to diabetic neuropathy do involve the polyol pathway but in which case trials should involve patients before neuropathic symptoms occur, perhaps from the time of diagnosis of diabetes and following up the patients for many years. Aldose reductase inhibitors, or other potential treatment, in this way would pose very severe ethical and practical (including cost) problems.

The aims of the project are:

(1) To examine autonomic and vascular function in the streptozotocin-induced diabetic rat with the primary purpose of identifying more appropriate animal models of diabetic complications.

(2) To investigate the effects of an aldose reductase inhibitor, dietary myo-inositol supplementation and triiodothyronine (T₃) on diabetes induced changes.

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CHAPTER TWO
METHODS AND MATERIALS
2.1 EXPERIMENTAL ANIMALS

Rats for the study were obtained from Nottingham University, from its site at Sutton Bonnington.

Diabetes was induced in male Wistar rats, weighing between 200 - 250g, by a single injection of 1ml/Kg of a freshly prepared solution of streptozotocin (40 mg/ml pH 4.5 citrate buffer) via a tail vein. Control animals received an injection of just the citrate buffer, once again via the tail vein.

Three days after the injection a small drop of blood was obtained by puncturing the tail vein with a sterile needle. The blood glucose concentration was assessed using test strips (BM-test-BG; Boehringer Mannheim). All rats that had a blood glucose concentration of 22 mMol/l or above were assigned to the diabetic groups.

Aldose reductase inhibitor treated diabetic animals received a dose of 25 mg/Kg of 'ponalrestat', ICI 128436; 3-CA-bromo-2-fluorobenzyl-4-oxo-34 phthalazin-1-yl acetic acid; 2.5mg/Kg of a 10mg/ml solution in 1% Tween 80 orally once a day.

Myo-inositol treated diabetic animals received an oral dose of myo-inositol which was present in the drinking water (1 g/l solution). Observations showed that the diabetic rats (weight between 200-250 g) drank approximately 250 ml/day of water; so that the animals received a dose of 1 g/Kg/day. This dose has been reported to prevent diabetes induced falls in sciatic nerve myo-inositol content. The untreated diabetic and the control groups both received oral 1% Tween 80 only.

Triodothyronine (T3) diabetic animals received triiodothyronine, 25μg/Kg/day s.c. When the animals were to be sacrificed they were all first weighed. Then they were anaesthetised with sodium pentobarbitone.
Once the animals were fully unconscious a 0.1ml sample of blood was taken for glucose measurement.

Tissues required for study were then removed, described below. Finally, the animals were killed while still anaesthetised either by removal of the heart (where this was required) or by anaesthetic overdose.

2.2 ANOCOCCYGEUS METHODS

2.2a SENSITIVITY TO NORADRENALINE, PHENYLEPHRINE & CLONIDINE

The anococcygeus muscles were identified and removed as described by Gillespie (1972).

They were placed in ice-cold physiological saline solution aerated with a 95% O₂ - 5% CO₂ gaseous mixture. The composition of the saline was NaCl 143.3mM, KCl 15.9mM, MgSO₄ 1.2mM, CaCl₂ 2.6mM, KH₂PO₄ 2.2mM, NaHCO₃ 24.9mM, glucose 10mM, ascorbic acid 0.07mM, and E.D.T.A. 0.067mM. The solution was then adjusted to pH 7.4; and this was the composition of the saline used throughout the study.

A single anococcygeus muscle was suspended in a 50ml organ bath under a resting tension of 0.5g. The bathing fluid was physiological saline, maintained at 37°C, and aerated with the same gaseous mixture as before.

The muscle was suspended for 1 hour and allowed to reach a equilibrium state. Noradrenaline was then added cumulatively over a wide concentration range (0.1µM to 0.3mM). The tissue was then washed several times, reequilibrated and the above procedure repeated.
with phenylephrine.

Finally the tissue was rewashed, reequilibrated, and in the presence of prazosin (0.3µM) clonidine was added. The responses to the drugs were recorded on a Washington Chart recorder.

Following this procedure the tissue was removed from the organ bath, blotted dry and weighed.

2.2b NEUROVIAL 3[H]-NORADRENALINE OVERFLOW, BASAL & STIMULATED RELEASE AND THE EFFECTS OF UPTAKE 1 AND ALPHA-2 BLOCKADE

A single anococcygeus muscle was placed in 1ml physiological saline solution containing 1µCi 3H-noradrenaline/ml (total noradrenaline concentration was 0.614µM). After 1 hour incubation, at 37°C, the tissue was mounted between two platinum wire electrodes under 0.5g tension and superfused with physiological saline at 37°C at a rate of approximately 1ml/minute.

After 1 hour superfusion the tissue was stimulated with a train of 300 2ms pulses, supramaximal voltage, delivered at 5Hz. This stimulation was repeated at 1½ hrs., 2 hrs., and 2½ hrs., with two 4 minute samples of superfusate being collected each time. The first collection was immediately before stimulation and the second during the 1 minute period of stimulation plus a further 3 minutes.

Following the second of the two superfusate collections at 1½ hrs., a 10µM solution of desipramine was infused by means of a syringe pump at a rate of 20µl/min. into the superfusate to give a final concentration of 0.2µM. This was continued until the 2hrs. superfusates were collected.

Following this yohimbine, 50µM, was also superfused in, as well as the desipramine already present, to give a final yohimbine concentration
of 1μM. This infusion was continued until after 2 superfusate collections at 2hrs.

Once these had been collected the tissue was removed from the superfusion apparatus, blotted dry and weighed. The tissue were then solubilized by incubation at 50°C in Optisolve (LKB, Loughborough UK). Tissue and perfusate sample radioactivity was subsequently measured, after the addition of 10ml scintillation fluid (Ecoscint), in a LKB liquid scintillation counter. Prestimulation samples were taken to indicate basal 3H-NA release whilst stimulated release was estimated by subtracting these values from those obtained for the samples collected during and after stimulation. Stimulated 3H release was assumed to be 3H-NA and expressed as pg NA (Doggerell & Woodruff, 1978).

2.3 AORTIC RING METHOD

The thoracic aorta was excised, and excess connective tissue and fat removed.

The aorta was cut into rings approximately 5mm's in length and placed in ice-cold physiological saline solution aerated with a 95% O₂ & 5% CO₂ mixture. The composition of the physiological saline was same as in the previous anococcygeus muscle preparation.

During the removal of the aorta care was taken to avoid contact with the luminal surface so as to preserve the endothelial cells.

In one ring the endothelium was mechanically removed (Furchgott, 1983), whilst in the second the endothelium was undisturbed. The rings were suspended in 50ml organ baths containing physiological saline
solution maintained at 37°C and continuously aerated with the same
gaseous mixture as before.

A tension of 2g per ring was applied and the preparation was left in
the bath for 1 hour to allow for equilibrium.

The fluid in the organ baths was changed after 30 minutes during this
period.

Noradrenaline was then added cumulatively over a wide range, the final
organ bath concentrations varied from 10nM to 0.3mM.

Following this the tissue were flushed out twice with physiological
saline and the basal tension allowed to return to 2g.

After approximately 45 minutes 5-hydroxytryptamine (5-HT) was added
to the organ baths over a wide range, 10nM to 0.3mM; following which
the organ baths were once again flushed out and the tissue allowed to
return to their equilibrium state.

Following this a dose of desipramine (0.3μM) was added to the organ
baths and after a period of 20 minutes, 5-HT was once again added over
a wide range as before.

The responses to the drugs were recorded on a Washington Chart
recorder.

Finally the tissue was blotted dry and weighed.

The method was however modified in later experiments with only rubbed
aorta being used.
The oesophagus was removed according to established procedure (Bieger & Triggle, 1985), and placed in an ice-cold physiological saline solution which was aerated with a 95% O₂ / 5% CO₂ gaseous mixture. The excised oesophagus was pulled over a small plastic tube to facilitate the removal of the external layer. The outer layer was split length-wise and carefully removed whilst ensuring that the inner layer remained intact.

The muscularis mucosa was suspended in a 50ml organ bath, between 2 platinum electrodes, containing physiological saline maintained at 37°C and aerated with the same gaseous mixture as before. The composition of the saline was NaCl 143.3mM, KCl 15.9mM, MgSO₄ 1.2mM, CaCl₂ 2.6mM, KH₂PO₄ 2.2mM, NaHCO₃ 24.9mM, glucose 10mM, ascorbic acid 0.07mM, and E.D.T.A. 0.067mM; and this was the composition of the physiological saline throughout the study.

The tissue was allowed to equilibrate for 1 hour until a steady baseline had been achieved. After this period the tissue was stimulated for 10s by pulses of 0.5ms width, at a frequency of 30Hz., over a large voltage range until a supermaximal voltage was determined. At this supermaximal voltage and pulse width the frequency was varied from 1-1,000Hz., again using train durations for 10s. Following this atropine, 10μM, was added and the tissue restimulated as before.

After washing out the tissue was allowed to rest and recover for 40mins and a dose response curve to acetylcholine was obtained over a wide range, from 10nM to 1μM. Finally the tissue's length and weight were measured.
Animals were weighed, anaesthetised with sodium pentobarbitone (60mg/Kg i.p.) and a small section (8-10cm) of ileum just proximal to the ileocaecal valve and the oesophagus was removed and placed in ice-cold modified Krebs-Hensleit solution. The composition of the physiological saline was NaCl 143.3mM, KCl 15.9mM, MgSO₄ 1.2mM, CaCl₂ 2.6mM, KH₂PO₄ 2.2mM, NaHCO₃ 24.9mM, glucose 10mM, ascorbic acid 0.07mM, and E.D.T.A. 0.067mM; and this was the composition of the physiological saline throughout the study.

Innervated longitudinal muscle of the terminal ileum was isolated by first carefully pulling the excised section of ileum over a small glass rod (Paton & Aboo-Zar, 1967). Then the longitudinal muscle was gently separated from the underlying circular muscle using cotton wool swabs moistened with physiological saline. The tissue was frequently irrigated with physiological saline during this process.

The tissue was mounted in a 50ml organ bath, which was filled with the physiological saline maintained at 37°C and continuously aerated with 95% O₂ & 5% CO₂.

A tension of 1g was applied and the tissue left for 1 hour to allow it to equilibrate.

A dose response curve to acetylcholine was obtained over a wide range, from 30nM to 3mM. Following this the length of the tissue was measured whilst it was still suspended and then it was gently blotted dry and weighed, and the mean cross sectional area was calculated (assuming a tissue density of 1.05g/cm³).
The bladder, together with the urethra, was removed and placed in ice-cold modified Krebs solution aerated with a 95% O₂/ 5% CO₂ gaseous mixture. The composition of the physiological saline was NaCl 143.3mM, KCl 5.9mM, MgSO₄ 1.2mM, CaCl₂ 2.6mM, KH₂PO₄ 2.2mM, NaHCO₃ 24.9mM, and glucose 10mM. Excess tissue was removed and the lower bladder portion containing the urethra was cut off and opened out by cutting along one side, as described by Lincoln et al (1984).

The preparation was suspended in a 50ml organ bath containing the modified Krebs solution maintained at 37°C and aerated with the same gaseous mixture as before. Two platinum electrodes were placed on either side of the tissue, a tension of 1g was applied and the preparation was left for 1 hour until the tissue had reached a steady state had been achieved.

Following the resting period the preparation was field stimulated for 10secs duration, at a pulse width of 0.3ms, frequency 10Hz, over a large range of voltages (40-150 volts). After this the organ bath was washed out, the tissue allowed to recover for 30mins, and atropine, 3μM, was added to the organ bath. Twenty minutes after the addition of atropine the field stimulation was repeated.

After allowing the tissue to recover for 40mins carbachol was added non-cumulatively to the organ bath in concentrations varying from 1μM to 0.1mM.

Once the responses to carbachol had been elicited the organ bath was washed out twice and the tissue allowed to recover for 40 mins., and finally the tissue was gently blotted dry and weighed.
Radiochemical assays of choline acetyltransferase depend upon efficient isolation of acetylcholine from the incubation mixture without contamination from acetyl-CoA (Fonnum, 1975). This simple and efficient method uses the scintillation mixture as an extraction solvent and carries out the extraction in the scintillation vial. Acetylcholine is isolated by liquid cation exchange using acetonitrile in a toluene scintillation mixture. Labelled acetylcholine moves into the organic phase to be counted at high efficiency; whilst acetyl-CoA remains in the aqueous phase and is not counted, since water does not function as a scintillation solvent.

The method was as described by Dietz & Salvaterra (1980). Initially the tissue was homogenised in 10 volume of 0.025M sodium phosphate buffer and centrifuged for 0.5 hours using super-head at 6000 RPM. The buffer was prepared from 1M solutions of Na₂HPO₄ and NaH₂PO₄, mixed together and adjusted to pH 7.4.

Following centrifugation 5μl of the homogenate was added to 5μl of a specially prepared mixture. The mixture contained [¹⁴C] Acetyl CoA 0.4mM (= 5000 cpm/μM), NaCl 0.6M, sodium phosphate buffer 0.1M, choline bromide 16mM, E.D.T.A 34mM, eserine sulphate 0.2mM, and bovine serum albumin 10%. The resultant mixture was incubated in a vial at 37°C over a wide range of time periods.

Following incubation the contents of the vial were flushed out, using a syringe, with 5ml of 10mM sodium phosphate buffer. Next 10ml of toluene-based scintillation fluid was added followed by 2ml of sodium tetraphenylboron in acetonitrile (5mg/ml). The contents were gently shaken for approximately a minute and then counted for 10mins in a LKB (110)
Liquid Scintillation counter.
The procedure was modified as results obtained were very variable. Fresh tissue rather than frozen tissue was used. The length of incubation time was progressively increased from 15 mins (as recommended by Dietz & Salvaterra, 1980) to 21 hours. The amount of 'hot' ligand ([^14]C Acetyl CoA) was also progressively increased. All of the modifications were combined in different sequences to make the procedure work. The original experiment, with rat brain tissue, was successfully performed.

2.8 CHOLINE UPTAKE ASSAY

The procedure used was as described by Beach & Jenden (1980). Previously weighed rings of rat ileum were placed in separate vials containing Krebs solution, 1 μM choline chloride and 2 μCi/ml ([N-methyl-^14]H)choline chloride (Set #1). In parallel with this rings of rat ileum were placed in vials containing Krebs solution, 2 μCi/ml ([N-methyl-^14]H)choline chloride, and 0.1 mM choline chloride and treated in the same manner as the other samples (Set #2). The composition of the Krebs solution was NaCl 143.3 mM, KCl 15.9 mM, K2HPO4 1.2 mM, CaCl2 2.6 mM, KH2PO4 2.2 mM, NaHCO3 24.9 mM, and glucose 10 mM. Each sample was triplicated.

The mixture, containing the rings, was placed in a water bath and incubated at 37°C for varying lengths of time. Following this the rings were removed from the incubating mixture and placed in ice-cold Krebs for 20 mins. They were then gently blotted dry and placed in counting vials, each containing 0.5 ml of Optisolve, and incubated at
50°C. Following solubilization 5ml of scintillation fluid was added, and the vials were left to stand for 5mins and then counted in a LKB scintillation counter.

Since the expected difference in counts between samples (Set #1 high & Set #2 low), without & with excess choline, did not appear the procedure was modified. The length of incubation time was increased from 3mins to 3hrs. KCl was substituted in Set #2 for the high choline chloride concentration (1mM). Modified Krebs solution was used, LiCl instead of NaCl. The choline chloride was replaced, LiCl Krebs was used in Set #1 and NaCl Krebs in Set #2.

The Krebs solution was further modified, glucose was replaced by sucrose. Preincubation in Krebs together with and without KCl was carried out. Different tissue was used, instead of rat ileum rat muscle was used. All of the modifications were attempted singularly and in combinations.

2.9 MYO-INOSITOL, SORBITOL & FRUCTOSE ASSAY

The tissue, rat sciatic nerve, was homogenised in 1ml of distilled water containing α-methyl mannoside (30 μM/ml) as an internal standard. A mixture of external standards (30 μg/ml), myo-inositol, sorbitol, fructose, also containing α-methyl mannoside (30 μg/ml) was similarly treated in parallel (Malone, 1982; Mayer & Tomlinson, 1983).

The homogenised mixture was boiled in a water bath for 20mins, cooled, and then deproteinised by the addition of ZnSO₄ (0.2ml of 0.2M solution), and 10mins later Ba(OH)₂ (0.2ml of 0.2M solution). The
samples were centrifuged at 800 Xg for 10mins and then the supernatant was freeze dried overnight.

The samples were silyated under a mixture of pyridine, hexamethyldisilaze, and trimethylchlorosilane (10:2:1, V/V; 0.5ml). After 24hrs incubation at room temperature (22°C), distilled water (2ml) and cyclohexane (0.4ml spectroscopic grade) were added and the samples vortex mixed. After centrifugation at 800 Xg for 2mins the cyclohexane phase was separated off for chromatographic analysis.

The extracts were assayed in duplicate using a Pye Unicam with a column 3% SP2100 on gas chrom Q with mesh size 100/120 and fitted with a flame ionization detector.

The temperature of the column was 190°C when the sample was injected and the column was linked to a SP4200 intergrater to give better resolution of the peaks.

The area under the peaks was taken to be proportional to the amount of substance present in the samples and this was correlated against the external standards.

2.10 SODIUM POTASSIUM OUABAIN-
SENSITIVE ATPase ASSAY

The method used was a modification of the procedure described by Kjeldsen et al (1986). The [3H]ouabain obtained was supplied in a toluene/ethanol mixture which was immiscible with the buffer used. The [3H]ouabain was placed in a scintillation vial in a fume cupboard and a pipette fitted to an air pump was used to gently evaporate the solvents off.
The tissue was prewashed twice in ice-cold Tris sucrose buffer for 10 mins.
The composition of the Tris sucrose buffer was Tris Cl 10mM, sucrose 250mM,
MgSO₄ 3mM, and vanadate 1mM.
The tissue was placed in a vial containing the Tris sucrose buffer, 0.1μM
ouabain, and 0.18-0.21 μCi/ml [³H]ouabain. The mixture was incubated at
37°C for 3 hours. In parallel with this tissue was placed in the same
mixture as before except that the ouabain (untritiated) concentration was
increased from 0.1μM to 1μM. Each sample was performed in triplicate.
During the incubation period the mixture was replaced twice, after the
first and second hour.
Following incubation the tissue samples were washed twice (30 mins each) in
ice-cold Tris sucrose buffer, blotted gently dry and weighed. The samples
were then placed in counting vials together with 5ml of 5% trichloroacetic
acid and left at room temperature (23°C) for 24 hrs.
The samples were then counted in a LKB scintillation counter.

2.11 VAS DEFERENS PREPARATION

The vas deferens was excised, excess connective tissue was removed, and
placed in ice-cold modified Krebs-Hensleit solution aerated with a mixture
of 95% O₂ & 5% CO₂. The composition of the physiological saline was NaCl
143.3mM, KCl 15.9mM, MgSO₄ 1.2mM, CaCl₂ 2.6mM, KH₂PO₄ 2.2mM, NaHCO₃ 24.9mM,
glucose 10mM, ascorbic acid 0.07mM, and E.D.T.A. 0.067mM; and this was used
throughout the study.
The tissue was suspended in a 50ml organ bath which was filled with
modified Krebs-Hensleit solution, aerated with the same gaseous
(114)
mixture as above, and maintained at a constant 37°C.

A tension of 1g was applied, and the tissue was allowed to equilibrate for 1 hour until a steady baseline was obtained.

Following this noradrenaline was added non-cumulatively to the organ bath; the physiological saline was replaced after each dose and the tissue allowed to recover (=5-10mins). A dose-response curve over a wide range, 10nM to 0.3mM was obtained.

Finally the tissue was gently blotted dry and weighed.

2.12 MATERIALS

All radiochemicals used in the experiments were from Amersham International plc., Buckinghamshire, England.

The drugs were obtained from Sigma Chemical Corp., USA.

Reagents used in the investigation were ANALAR.

2.13 ANALYSIS OF RESULTS

The $E_{max}$, the maximum response of the tissue, and the EC$_{50}$, effective concentration required to produce 50% of the maximal response, were calculated using a programme written by Dr P D Lucas on a ZX Spectrum. Statistical analysis was carried out also using programmes written by Dr P D Lucas on a ZX Spectrum computer.

The significances of differences between groups were assessed initially by one-way analysis of variance and, where p values of less than 0.05 were obtained, individual groups were compared by Duncans multiple comparisons tests.

(115)
CHAPTER THREE
RESULTS ANALYSIS
1.0 RESULTS ANALYSIS

1 ANOCOCYGEUS MUSCLE PREPARATION

In the first 6-weeks group (table 1) diabetic animals and diabetic treated with myo-inositol (D+MI) had significantly elevated blood glucose levels (b.g.l.s) (p<.01 vs control), whilst the diabetic group alone had a significantly reduced body weight (p<.01). The D+MI also had reduced b.g.l.s (p<.02 vs diabetic) and increased body weight gain (p<.005 vs diabetic).

In the second 6-weeks group the diabetic and diabetic treated with an aldose reductase inhibitor (D+ARI) both had significantly b.g.l.s (p<.01) as well as significantly reduced body weight gains (p<.01).

In the third 6-weeks group the diabetic and diabetic treated with triiodothyronine (T₃) and D + ARI all had significantly elevated b.g.l.s (p<.01). In addition these three classes of animals all had significantly reduced body weight gains (p<.01).

In the 6-month diabetic animals (table 2) all groups, diabetic and D + MI & D + ARI, had significantly higher b.g.l.s (p<.01). As well as this, these three groups all had significantly reduced body weights (p<.01).

Compared to the diabetic the D+ARI group had lower b.g.l.s (p<.05) and increased body weight gain (p<.005) and the D+MI group had increased body weight gain (p<.05).

In the 1 year animals, the diabetic and D + ARI groups both had significantly elevated b.g.l.s (p<.01) and reduced weight gains (p<.01). Also the D+ARI group had reduced b.g.l.s (p<.02 vs diabetic).
# TABLE 1

**CONFIRMATION OF DIABETES**

**6 WEEKS ANIMALS**

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CONTROL, DIABETIC &amp; WI TREATED ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>4.34±0.75</td>
<td>+63.9±9.3</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>28.36±2.34 *a</td>
<td>-27.2±8.9 *a</td>
</tr>
<tr>
<td>DIABETIC + WI (6)</td>
<td>19.91±1.53 *E</td>
<td>+17.0±7.6 *g</td>
</tr>
</tbody>
</table>

|                     |                          |                             |
| **CONTROL, DIABETIC & ARI TREATED ANIMALS** |                          |                             |
| CONTROL (6)         | 3.86±1.53                | +55.2±25.62                 |
| DIABETIC (6)        | 27.65±4.30 *a            | +5.4±8.35 *b                |
| DIABETIC + ARI (6)  | 26.48±2.39 *a            | -14.3±19.53 *a              |

|                     |                          |                             |
| **CONTROL, DIABETIC & ARI & T<sub>3</sub> TREATED** |                          |                             |
| CONTROL (12)        | 4.79±3.72                | +41.6±7.65                  |
| DIABETIC (8)        | 25.93±1.93 *a            | -5.3±6.72 *a                |
| DIABETIC + ARI (7)  | 25.34±2.71 *a            | -0.3±8.38 *a                |
| DIABETIC + T<sub>3</sub> (9) | 26.69±1.27 *a | +4.6±12.99 *b              |

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by B p<.01, and significance of difference from the untreated diabetic by E p<.02 and G p<.005; when WI is myo-inositol, ARI is an aldose reductase inhibitor (ponalrestat) and T3 is triiodothyronine.
## TABLE 2

**CONFIRMATION OF DIABETES**

**6 MONTHS & 1 YEAR ANIMALS**

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 MONTHS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>7.32±1.33</td>
<td>+177±14.50</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>40.60±1.08$^a$</td>
<td>-34.80±9.01$^b$</td>
</tr>
<tr>
<td>DIABETIC+ARI (6)</td>
<td>29.93±4.19$^{bc}$</td>
<td>+39.80±17$^{cd}$</td>
</tr>
<tr>
<td>DIABETIC+MI (6)</td>
<td>38.53±2.90$^a$</td>
<td>+3.20±13.70$^{cd}$</td>
</tr>
<tr>
<td><strong>1 YEAR ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (12)</td>
<td>8.17±1.87</td>
<td>+174.22±25.60</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>38.66±2.44$^a$</td>
<td>+32.67±11.93$^b$</td>
</tr>
<tr>
<td>DIABETIC + ARI (8)</td>
<td>27.25±3.12$^{bc}$</td>
<td>+33.88±17.03$^b$</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of differences from the control values are indicated by $B$ p<.01 and significance of differences from the untreated diabetic by $D$ p<.05, $E$ p<.02, & $G$ p<.005; when MI is myo-inositol and ARI is an aldose reductase inhibitor ("ponalrestat").
In the first 6-weeks animals group, for responses to noradrenaline; (table 3) the diabetic animals had significantly elevated $E_{\text{max}}$'s ($p<.05$) together with a significantly higher $E_{\text{C}-50}$ range ($p<.05$). The $D + \text{MI}$ group had an $E_{\text{max}}$ significantly lower than the diabetic animals ($p<.05$), whilst the $E_{\text{C}-50}$ ranges were unchanged. There was no significant change in the maximum force of contraction in the diabetic and $D + \text{MI}$ groups compared to the control values.

**TABLE 3**

**6 WEEKS ANIMALS**

$E_{\text{max}}$'s, $E_{\text{C}-50}$ RANGES FOR RESPONSES TO NORADRENALINE & MAXIMUM FORCE OF CONTRACTION DUE TO FIELD STIMULATION

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (6)</th>
<th>DIABETIC (7)</th>
<th>DIABETIC + MI (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{C}-50}$ Ranges ($\mu$M)</td>
<td>0.50(0.1-1.5)</td>
<td>1.30(0.7-2.4)*</td>
<td>1.80(1.2-2.9)</td>
</tr>
<tr>
<td>$E_{\text{max}}$ (mg/ten. mg wet weight)</td>
<td>520±59</td>
<td>738±81*</td>
<td>505±350</td>
</tr>
<tr>
<td>Max Con. (mg/ten. mg wet weight)</td>
<td>259±39</td>
<td>240±32</td>
<td>191±52</td>
</tr>
</tbody>
</table>

Results are expressed for the $E_{\text{max}}$'s and maximum force of contraction as mean ± s.e. and for the $E_{\text{C}-50}$ ranges as geometric means with 95% confidence limits when the number of animals per group is in parenthesis. The significance of difference from the control values is indicated by A $p<.05$ and from the untreated diabetic values by D $p<.05$; when MI is myo-inositol.
GRAPH 1
Dose-response curves of rat anococcygeus muscle to Noradrenaline from 6-weeks STZ diabetic, control, and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO NORADRENALINE

Legend
- CONTROL
- DIABETIC
- DIABETIC + PROPRIOL

log [NORADRENALINE]
In the second 6-weeks animals group (table 4) responses to three exogenous drugs were evaluated, noradrenaline, phenylephrine and clonidine (in the presence of prazosin). For noradrenaline the EC$_{50}$ ranges of the diabetic and treated animals were unchanged. However the diabetic animals' Emax was significantly higher (p<.05) and the D + ARI group had a significantly lower Emax (p<.01 vs diabetic).

For phenylephrine once again the EC$_{50}$ ranges were unchanged whilst the diabetic Emax was significantly higher (p<.05). The D + ARI group Emax was significantly lower (p<.01 vs both Control & Diabetic).

For clonidine (in the presence of prazosin) the EC$_{50}$ ranges for the diabetic group was lower, whilst the D + ARI group was significantly lower (p<.01 vs the control group). The Emax for the diabetic group was significantly higher (p<.05) and the D + ARI groups Emax was significantly lower (p<.05 vs the diabetic group).

In the third 6-weeks animals group (table 5) there were no changes in responses to noradrenaline and phenylephrine, in both EC$_{50}$ ranges and Emax's; though the Emax's for the diabetic were higher they were not significantly so. For clonidine (in the presence of prazosin) the D + ARI group's EC$_{50}$ range was significantly different (p<.05 vs control & diabetic values). The diabetic Emax was significantly higher (p<.01), whilst the Emax's for the D + ARI and D + T$_3$ were significantly lower (both p<.01) compared to the diabetic value.
TABLE 4

6 WEEKS ANIMALS

Emax's & EC-50 RANGES

<table>
<thead>
<tr>
<th></th>
<th>EC-50 Ranges</th>
<th>Emax mg/t en.</th>
<th>Emax mg wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORADRENALINE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>3.8 (2.1-6.9) μM</td>
<td>822±54</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>3.8 (2.1-7.0) μM</td>
<td>1142±57A</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>6.3 (3.9-10.3) μM</td>
<td>740±47F</td>
<td></td>
</tr>
</tbody>
</table>

| PHENTYLEPHRINE |            |               |                   |
| CONTROL (6)        | 2.2 (1.4-3.3) μM | 773±58       |                   |
| DIABETIC (5)       | 1.8 (1.0-2.9) μM | 1199±89A     |                   |
| DIABETIC + ARI (6) | 1.7 (1.1-2.8) μM | 468±70EF     |                   |

| CLOMIDINE (+PRA2OSIN) |        |               |                   |
| CONTROL (6)           | 0.1 (0.0-0.1) μM | 714±43    |                   |
| DIABETIC (5)          | 52 (27-99) nM | 1077±97A   |                   |
| DIABETIC + ARI (6)    | 32 (17-59) nM | 617±66D   |                   |

Results for Emax are expressed as mean ± s.e. and for the EC-50 ranges as geometric means with 95% confidence limits, with the number per group in parenthesis. The significance of difference from the control mean values is indicated by A p<.05 & B p<.01 & from the untreated diabetic values is indicated by D p<.05 & F p<.01; when ARI is an aldose reductase inhibitor ("ponalrestat").

(124)
Dose-response curves of rat anococcygeus muscle to Noradrenaline from 6-weeks STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM, when D (p<0.05) indicates a significant difference from the untreated diabetic values.
RESPONSES TO NORADRENALINE
Dose-response curves of rat anococcygeus muscle to Phenylephrine from 6-weeks STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM, when D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO PHENYLEPHRINE
GRAPH 4

Dose-response curves of rat anococcygeus muscle to Clonidine in the presence of Prazosin from 6-weeks STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM.
RESPONSES TO CLONIDINE

Legend

- CONTROL
- DIABETIC
- DIABETIC + penalrestat

log [clonidine]
## TABLE 5

### 6 WEEKS ANIMALS

**Emax’s & EC-so RANGES**

<table>
<thead>
<tr>
<th></th>
<th>EC-so Ranges</th>
<th>Emax, mg/ten</th>
<th>Emax, mg wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NORADRENALINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>2.4(1.6-3.6) µM</td>
<td>398±38</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>1.2(0.4-3.1) µM</td>
<td>634±126</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>4.5(2.4-8.3) µM</td>
<td>341±92</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + T₃ (6)</td>
<td>4.3(2.4-7.6) µM</td>
<td>468±98</td>
<td></td>
</tr>
<tr>
<td><strong>PHENYLEPHRINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>7.4(2.5-21.4) µM</td>
<td>404±30</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>10.3(3.4-31.7) µM</td>
<td>651±127</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>3.9(2.0-7.5) µM</td>
<td>413±124</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + T₃ (6)</td>
<td>3.9(2.1-7.1) µM</td>
<td>426±127</td>
<td></td>
</tr>
<tr>
<td><strong>CLONIDINE (+PRAZOSIN)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>1.5(0.5-4.4) µM</td>
<td>288±27</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>1.0(0.2-4.6) µM</td>
<td>782±130⁹</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>11.5(10.2-12.9) µM&lt;sup&gt;μ&lt;/sup&gt;</td>
<td>331±125⁹</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + T₃ (6)</td>
<td>3.3(1.7-6.6) µM</td>
<td>154±10⁹</td>
<td></td>
</tr>
</tbody>
</table>

Results for Emax are expressed as mean ± s.e. and for the EC-so ranges as geometric means with 95% confidence limits, , with the number per group in parenthesis. The significance of difference from the control values by A p<.05 & B p<.01 and from the untreated diabetic values by D p<.05 & F p<.01; when ARI is an aldose reductase inhibitor (ponalrestat) and T₃ is tri-iodothyronine.
GRAPH 5

Dose-response curves of rat anococcygeus muscle to Noradrenaline from 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with triiodothyronine (T3). Each point represents an average of at least five observations and vertical lines denote SEM, when A (p<0.05) indicates a significant difference from the control values, and D (p<0.05) indicates a significant difference from the untreated diabetic values.
RESPONSES TO NORADRENALINE

log [Noradrenaline]
Dose-response curves of rat anococcygeus muscle to Phenylephrine from 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with triiodothyronine (T₃). Each point represents an average of at least five observations and vertical lines denote SEM.
RESPONSES TO PHENYLEPHRINE

Legend
- Central
- Etubatil
- Etubatil 200 x equivalent
- Etubatil 100 x equivalent
Graph 7

Dose-response curves of rat anococcygeus muscle to Clonidine in the presence of Prazosin from 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with triiodothyronine (T$_3$). Each point represents an average of at least five observations and vertical lines denote SEM, when A (p<.05) and B (p<.01) indicate a significant difference from the control values and D (p<.05) and F (p<.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO CLONIDINE

Legend
- Control
- Drug A
- Drug B
- Drug C

log [clonidine]
For the 6 month animals (table 6) the diabetic group's EC$_{50}$ ranges were significantly different (p<.01) from the control values. The D + ARI group was significantly different from the control (p<.05) and the diabetic values (p<.01). The D + MI group was also significantly different from the control (p<.01) and diabetic values (p<.01). The diabetic Emax was significantly higher (p<.05) than the control, whilst the D + ARI & D + MI groups had a significantly lower values (p<.05) compared to the diabetic.

**TABLE 6**

**6 MONTHS ANIMALS**

<table>
<thead>
<tr>
<th>Emax's &amp; EC$_{50}$ RANGES FOR RESPONSES TO NORADRENALINE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC$_{50}$ Ranges</strong></td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>CONTROL (6)</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
</tr>
</tbody>
</table>

Results are expressed for the Emax's as mean ± s.e. and for the EC$_{50}$ ranges as geometric means with 95% confidence limits with the number of animals per group in parenthesis. The significance of difference from the control values is indicated by A p<.05 & B p<.01 and from the untreated diabetic by D p<.05 F f p<.01; when MI is myo-inositol and ARI is an aldose reductase inhibitor ("ponalrestat").
Dose-response curves of rat anococcygeus muscle to Noradrenaline from 6-months STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values, and D (p<0.05) indicates a significant difference from the untreated diabetic values.
In the 1 year animals (table 7) sensitivity to noradrenaline, phenylephrine, and clonidine were all unchanged in the untreated diabetic and D + ARI groups compared to the control values. For noradrenaline the diabetic Emax was significantly lower (p<.01), whilst the D + ARI group animals were significantly lower (p<.01 vs diabetic values). For phenylephrine the diabetic Emax was significantly lower (p<.01); as was the D + ARI groups' Emax (p<.05) compared to the control values. For clonidine only the diabetic Emax value was significantly lower (p<.05).

**TABLE 7**

**1 YEAR ANIMALS**

<table>
<thead>
<tr>
<th></th>
<th>Emax</th>
<th>EC-50 Ranges</th>
<th>Emx</th>
<th>EC-50 Ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>NORADRENAline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>1.6</td>
<td>(1.5-1.7) μM</td>
<td>1.4</td>
<td>503±63 μM</td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>3.2</td>
<td>(2.3-4.4) μM</td>
<td>6.0</td>
<td>1584±60 μM</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>3.6</td>
<td>(2.0-6.3) μM</td>
<td>1.1</td>
<td>1295±25 μM</td>
</tr>
<tr>
<td>PHENYLEPHRIIIE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>2.5</td>
<td>(2.1-2.9) μM</td>
<td>2.8</td>
<td>498±36 μM</td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>2.8</td>
<td>(2.3-3.5) μM</td>
<td>4.0</td>
<td>775±49 μM</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>3.4</td>
<td>(3.0-4.0) μM</td>
<td>1.1</td>
<td>503±63 μM</td>
</tr>
<tr>
<td>CLONIDINE (+PRAZOSIN)</td>
<td>0.20</td>
<td>(0.10-0.35) μM</td>
<td>1.0</td>
<td>109±16 μM</td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>0.20</td>
<td>(0.10-0.35) μM</td>
<td>0.4</td>
<td>419±44 μM</td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>1.0</td>
<td>(0.7-1.5) μM</td>
<td>0.4</td>
<td>419±44 μM</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>0.4</td>
<td>(0.3-0.5) μM</td>
<td>1.0</td>
<td>109±16 μM</td>
</tr>
</tbody>
</table>

Results for Emax are expressed as mean ± s.e. and for the EC-50 ranges as geometric means with 95% confidence limits, with the number per group in parenthesis. The significance of difference from the control values is indicated by A p<.05 & B p<.01 and from the untreated diabetic values by F p<.01; when ARI is an aldose reductase inhibitor ("ponalrestat").

(134)
Dose-response curves of rat anococcygeus muscle to Noradrenaline from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values, and D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO NORADRENALINE

![Graph showing responses to noradrenaline with different conditions: Control, Diabete, and Diabete + Pandalrestat. The graph plots log [noradrenaline] against response magnitude.]

Legend:
- Control
- Diabete
- Diabete + Pandalrestat
GRAPH 10

Dose-response curves of rat anococcygeus muscle to Phenylephrine from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values, and D (p<0.05) indicates a significant difference from the untreated diabetic values.
RESPONSES TO PHENYLEPHRINE

log [phenylephrine]
GRAPH 11

Dose-response curves of rat anococcygeus muscle to Clonidine in the presence of Prazosin from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values.
RESPONSES TO CLONIDINE

Legend
- CONTROL
- DIABETIC
- DIABETIC + p450

![Diagram showing responses to clonidine with different symbols representing control, diabetic, and diabetic with p450. The x-axis is labeled as log [clonidine] and the y-axis ranges from 0 to 500. The graph includes error bars.]
In the anococcygeus muscle preparation (table 8) for the variable tension set-up diabetes was confirmed by the significantly elevated blood glucose levels (p<.01) and significantly reduced body weight gain (p<.01). At 1g tension the Emax's for the diabetic group were higher, but not significantly so, compared to the control group. At the optimum tension, which varied between 0.5 and 0.25g, the diabetic Emax's were once again higher but not significantly so. There was no change in sensitivity to noradrenaline in the diabetic groups for both the fixed (1g) and variable tension. Responses to field stimulation were similar in both the control and diabetic groups with no significant variation.

**TABLE 8**

**CONFIRMATION OF DIABETES**

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (6)</th>
<th>DIABETIC (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Glucose Level (mM)</td>
<td>6.92±1.15</td>
<td>22.61±8.36*</td>
</tr>
<tr>
<td>Change in Body Weight (g's)</td>
<td>+78.5±3.90</td>
<td>+17.56±11.99*</td>
</tr>
<tr>
<td>Emax (1g tension)</td>
<td>414±91</td>
<td>489±82</td>
</tr>
<tr>
<td>Emax (xg tension)</td>
<td>410±88</td>
<td>546±147</td>
</tr>
<tr>
<td>EC50 (1g Tension)</td>
<td>0.5(1.1-0.3)μM</td>
<td>1.1(3.2-0.4)μM</td>
</tr>
<tr>
<td>EC50 (xg Tension)</td>
<td>0.4(1.0-0.1)μM</td>
<td>1.0(2.4-5.6)μM</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by *p<.01*; when MI is myo-inositol, ARI is an aldose reductase inhibitor (ponalrestat). In the second phase of the experiment the tension (xg) was varied until the optimum tension was reached and a maximal response was obtained.

(138)
Dose-response curves of rat anococcygeus muscle at a fixed tension of 1g to Noradrenaline from 6-weeks STZ diabetic and control rats. Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO NORADRENALINE AT FIXED (1g) TENSION

![Graph showing responses to noradrenaline at fixed tension levels.](image)

Legend:
- **CONTROL (1g Tension)**
- **DIABETIC (1g Tension)**

The graph illustrates the responses to noradrenaline at a fixed tension level of 1 gram. The x-axis represents the logarithm of noradrenaline concentration, while the y-axis shows the tension response. The data points are accompanied by error bars indicating the variability in the responses.
GRAPH 13

Dose-response curves of rat anococcygeus muscle at an optimal tension to Noradrenaline from 6-weeks STZ diabetic and control rats. Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO NORADRENALINE AT OPTIMAL TENSION

Legend
- CONTROL (Vasodilated)
- DIABETIC (Vasodilated)

log [NORADRENALINE]
Response curves of rat anococcygeus muscle to field stimulation with varying frequency from 6-weeks STZ diabetic and control rats. Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO FIELD STIMULATION WITH VARYING FREQUENCY

Legend
- CONTROL
- DIABETIC

FREQUENCY [Hz]
In the tritiated noradrenaline overflow and stimulated release experiments in the anococcygeus muscle diabetes was confirmed in all three groups. In the first 6 week group (table 9) the diabetic and D + ARI groups all had significantly elevated blood glucose levels (p<.01) and reduced body weight gain (p<.01). This pattern of diabetes was also confirmed in the second six weeks animals with the diabetic, D + ARI and D + T3 groups all having significantly elevated blood glucose (p<.01) and reduced body weight gains (p<.01) compared to the control values. In the first 6-weeks group basal release of tritium and stimulated release (table 10), in the absence of drugs, were similar in all groups; namely control, diabetic & D + ARI. The addition of desipramine (0.2μM) elevated the tritium release due to stimulation to a markedly lesser degree in the diabetes group compared to the control values (p<.05). This effect was rectified in the D + ARI group which was significantly different from the diabetic values (p<.01). Further potentiation of release by the addition of yohimbine (1μM) was equivalent in all groups. Tissue 3H-NA uptake was similar in all three groups. In the second 6-weeks group (table 10) the same pattern of results were observed. The basal release and stimulated release, in the absence of drugs, was similar in all groups. Tritium release was potentiated by the addition of desipramine (0.2μM); but once again significantly to a lesser degree in the diabetic group compared to the control values (p<.05). Treatment with an aldose reductase inhibitor and triiodothyronine produced a significant positive effect (p<.05 vs the diabetic values); and restored values comparable to the control value. The addition of yohimbine (1μM) caused a potentiated release higher in the control group compared to the diabetic, but not significantly so.
In a further set of overflow and stimulated release of tritiated noradrenaline experiments in 6-months streptozotocin-induced diabetic rats, carried out by a co-researcher, there was no difference between basal and stimulated release both in the absence and presence of drugs, desipramine and yohimbine (unpublished data).

OVERFLOW & STIMULATED RELEASE OF $^{3}^{2}$[NA] BY ANOCOCGYGEUS MUSCLE CONFIRMATION OF DIABETES

TABLE 9

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Weeks Animals (1st Set)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>4.36±1.83</td>
<td>+63.9±22.8</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>28.39±5.72*</td>
<td>-27.2±21.8*</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>27.12±6.14*</td>
<td>-19.8±18.9*</td>
</tr>
</tbody>
</table>

| 6 Weeks Animals (2nd Set) |                          |                            |
| CONTROL (6)              | 6.91±1.24                | +78.5±13.9                 |
| DIABETIC (6)             | 22.64±8.42*              | +17.6±11.9*                |
| DIABETIC + ARI (6)       | 23.27±9.29*              | +10.1±13.5*                |
| DIABETIC + T₃ (6)        | 25.91±8.47*              | +30.6±12.4*                |

Results are expressed as mean ± s.d. with the number per group in parenthesis. The significance of difference from the control values is indicated by B p<0.01; when ARI is an aldose reductase inhibitor ('ponalrestat'), MI is myo-inositol and T₃ is triiodothyronine.
OVERFLOW & STIMULATED RELEASE OF $^{3}$[NA] BY ANOCOCYGEUS MUSCLE

STIMULATED $^{3}$[H] OVERFLOW (pmol/g)

TABLE 1A

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (6)</th>
<th>DIABETIC (6)</th>
<th>DIABETIC + ARI (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drugs</td>
<td>30.8±4.2</td>
<td>31.1±6.0</td>
<td>35.8±6.5</td>
</tr>
<tr>
<td>+ Desipramine</td>
<td>39.1±5.9</td>
<td>31.6±5.9</td>
<td>49.7±8.0</td>
</tr>
<tr>
<td>% Increase</td>
<td>26.6±4.7</td>
<td>2.8±4.0</td>
<td>40.1±7.2</td>
</tr>
<tr>
<td>+Des.+Yohimbine</td>
<td>58.4±9.5</td>
<td>44.9±7.4</td>
<td>69.4±10.4</td>
</tr>
<tr>
<td>% Increase</td>
<td>50.8±8.4</td>
<td>47.3±10.5</td>
<td>40.5±10.6</td>
</tr>
<tr>
<td>Tissue $^{3}$H-NA (nmol/g) @t=0</td>
<td>2.32±0.49</td>
<td>2.10±0.34</td>
<td>2.21±0.17</td>
</tr>
<tr>
<td>after superfusion</td>
<td>1.91±0.42</td>
<td>1.53±0.65</td>
<td>1.77±0.18</td>
</tr>
</tbody>
</table>

6 WEEKS ANIMALS (2nd Set)

<table>
<thead>
<tr>
<th></th>
<th>CONTROL (6)</th>
<th>DIABETIC (6)</th>
<th>DIABETIC+ARI (6)</th>
<th>DIABETIC+T3 (6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Drugs</td>
<td>61.3±16.7</td>
<td>61.4±17.6</td>
<td>64.7±22.6</td>
<td>55.9±22.7</td>
</tr>
<tr>
<td>+Desipramine</td>
<td>77.4±18.8</td>
<td>64.3±18.7</td>
<td>79.2±22.3</td>
<td>61.3±27.6</td>
</tr>
<tr>
<td>% Increase</td>
<td>35.2±22.2</td>
<td>10.1±19.5</td>
<td>38.3±22.3</td>
<td>30.7±11.7</td>
</tr>
<tr>
<td>+Des+Yohimbine</td>
<td>131.5±26.5</td>
<td>99.6±27.8</td>
<td>102.5±22.1</td>
<td>112.6±30.6</td>
</tr>
<tr>
<td>% Increase</td>
<td>96.8±29.3</td>
<td>36.6±16.3</td>
<td>60.8±17.9</td>
<td>45.8±14.7</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by $A_p<.05$ and from the untreated diabetic values by $D_p<.05$ & $F_p<.01$; when ARI is an aldose reductase inhibitor ('ponalrestat'), MI is myo-inositol. and T3 is triiodothyronine.

Tissue $^{3}$H-NA at time 0 i.e. tissue $^{3}$H-NA uptake was estimated by adding total basal $^{3}$H release (calculated, assuming 1st order kinetics) and the stimulated release to the $^{3}$H measured in the tissue after superfusion.
Effects of desipramine (0.2μM) and desipramine with yohimbine (1μM) on stimulated [³H]-Noradrenaline release in rat anococcygeus muscle from 6-weeks STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each bar represents the average of at least of six observations and vertical bars denote SEM.
EFFECTS OF DRUGS ON TRITIATED NORADRENALINE RELEASE

Legend

- NO DRUGS
- + Desipramine (0.2 μM)
- + Desipramine + Yohimbine (μ)

Control

Diabetic

Diabetic + Statil

H-NA Collected pg/gissue.
Per centage increase of stimulated \([^{3}H]\)-Noradrenaline release in rat anococcygeus muscle due to desipramine (0.2µM) and desipramine with yohimbine (1µM) from 6-weeks STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each bar represents the average of at least of six observations and vertical bars denote SEM, when A (p<0.05) indicates differences from the control values and D (p<0.05) indicates differences from the diabetic values.
INCREASE IN TRITIATED NORADRENALINE RELEASE DUE TO DRUGS

Legend
- CONTROL
- DIABETIC
- DIABETIC + penebritat
Effects of desipramine (0.2μM) and desipramine with yohimbine (1μM) on stimulated [$^3$H]-Noradrenaline release in rat anococcygeus muscle from 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat) and diabetic treated with triiodothyronine (T$_3$). Each bar represents the average of at least of six observations and vertical bars denote SEM.
EFFECTS OF DRUGS ON TRITIATED NORADRENALINE RELEASE

Legend
- CONTROL
- DIABETIC
- DIABETIC + Triiodothyronine
- DIABETIC + papaveretum
GRAPH 18

Percentage increase of stimulated [³H]-noradrenaline release in rat anococcygeus muscle due to desipramine (0.2μM) and desipramine with yohimbine (1μM) from 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat) and diabetic treated with triiodothyronine (T₃). Each bar represents the average of at least six observations and vertical bars denote SEM, when A (p<0.05) indicates differences from the control values and D (p<0.05) indicates differences from the diabetic values.
INCREASE IN TRITIATED NORADRENALINE RELEASE DUE TO DRUGS

**Legend**
- CONTROL
- DIABETIC
- DIABETIC + Triliodothyronine
- DIABETIC + propranolol
In the aortic ring experiments (table 11) diabetes was confirmed in the diabetic D + ARI and D + MI, in 6-week, 6-month, and 1 year animals, by the significantly elevated blood glucose levels ($p<.01$) and reduced body weight gains ($p<.01$).

### TABLE 11
CONFIRMATION OF DIABETES

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 WEEKS ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (8)</td>
<td>6.89±1.05</td>
<td>+60.48±9.56</td>
</tr>
<tr>
<td>DIABETIC (10)</td>
<td>26.48±2.21$^a$</td>
<td>−22.68±11.70$^a$</td>
</tr>
<tr>
<td>DIABETIC + MI (7)</td>
<td>30.51±2.65$^a$</td>
<td>−33.70±8.90$^a$</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>32.70±2.03$^a$</td>
<td>−27.90±13.70$^a$</td>
</tr>
<tr>
<td><strong>6 MONTHS ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>7.32±1.33</td>
<td>+177±14.5</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>40.60±1.08$^a$</td>
<td>−34.80±9.10$^a$</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>29.93±4.19$^a$</td>
<td>+39.80±17.90$^a$</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>33.53±2.90$^a$</td>
<td>+3.20±13.70$^a$</td>
</tr>
<tr>
<td><strong>1 YEAR ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (12)</td>
<td>8.17±1.87</td>
<td>+174.22±25.60</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>38.66±2.44$^a$</td>
<td>+32.67±11.93$^a$</td>
</tr>
<tr>
<td>DIABETIC + ARI (8)</td>
<td>27.25±3.12$^a$</td>
<td>+33.88±17.03$^a$</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e. with number per group in parentheses. The significance of difference from the control group values are indicated by $B p<0.01$ and from the untreated diabetic by $D p<.05$, $E p<.02$ & $G p<.005$; where MI is myo-inositol, and ARI is an aldose reductase inhibitor ("ponalrestat").

(149)
In the 6-week animals (table 12) there was no change in sensitivity and 
Emax's in response to noradrenaline in both rubbed and unrubbed aorta. 
Sensitivity to 5-hydroxytryptamine (5-HT) in the rubbed aorta in the 
diabetic, D + MI and D + ARI groups was significantly different from the 
control values (p<.01); whereas in the unrubbed aorta only the D + MI 
group was significantly different from the control values (p<.05). 
The Emax's for 5-HT, for both rubbed and unrubbed, were unchanged in the 
untreated diabetic and the treated groups. 
In the 6 month animals (table 13) only rubbed aorta was used. Sensitivity 
to noradrenaline and 5-HT, both in the absence and presence of desipramine, 
was measured. Diabetic and D + MI animals both had significantly increased 
sensitivity to noradrenaline, (p<.01 and p<.05 respectively), whilst the 
sensitivity of the D + ARI group was significantly restored (p<.05 vs the 
diabetic values). Emax's for the diabetic group were significantly 
increased (p<.05 vs control values), whereas those for the D + MI group 
were significantly reduced (p<.01 vs the diabetic values). The Emax's for 
the D + ARI group were significantly different (p<.05 vs control & p<.01 
vs diabetic). 
There was no change in sensitivity to 5-HT in the diabetic and treated 
groups in the absence of desipramine. The diabetic Emax was significantly 
higher (p<0.5), whilst the D + MI group Emax was significantly lower 
(p<0.5 Vs control and p<.01 Vs diabetic); as was the Emax for the D + ARI 
(p<.01 Vs both control & diabetic values). 
Sensitivity to 5-HT in the presence of desipramine was unchanged. Emax's 
however, for the diabetic group were significantly higher (p<.01) and for 
the D + MI group significantly lower (p<.01 Vs control & diabetic 
values). An interesting feature was that the responses for the D + ARI 
group were so reduced as to be unmeasureable.
### AORTIC RING SENSITIVITY TO DRUGS

#### TABLE 12

**6 WEEKS ANIMALS**

**Emax's & EC-50 Ranges.**

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>DIABETIC</th>
<th>DIABETIC+MI</th>
<th>DIABETIC+ARI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NORADRENALINE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubbed Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (mg/ten mg wet weight)</td>
<td>393±43 (8)</td>
<td>438±66 (6)</td>
<td>446±59 (6)</td>
<td>408±62 (6)</td>
</tr>
<tr>
<td>EC-50 (nM)</td>
<td>2.2(1.2-3.8)</td>
<td>2.6(1.2-5.5)</td>
<td>5.8(3.9-8.9)</td>
<td>3.4(1.8-6.3)</td>
</tr>
<tr>
<td>Unrubbed Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (mg/ten mg wet weight)</td>
<td>209±20 (8)</td>
<td>247±28 (7)</td>
<td>361±78 (7)</td>
<td>256±37 (6)</td>
</tr>
<tr>
<td>EC-50 (nM)</td>
<td>21(10-43)</td>
<td>15(4.6-50)</td>
<td>48(21-100)</td>
<td>24(7-49)</td>
</tr>
<tr>
<td><strong>5-HYDROXYTRYPTAMINE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rubbed Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (mg/ten mg wet weight)</td>
<td>303±38 (9)</td>
<td>358±52 (9)</td>
<td>350±53 (5)</td>
<td>363±59 (5)</td>
</tr>
<tr>
<td>EC-50 (μM)</td>
<td>0.9(0.1-0.7)</td>
<td>2.3(1.6-2.3)</td>
<td>4.1(2.2-7.7)</td>
<td>1.8(0.3-5.3)</td>
</tr>
<tr>
<td>Unrubbed Aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emax (mg/ten mg wet weight)</td>
<td>184±21 (9)</td>
<td>143±27 (9)</td>
<td>193±51 (7)</td>
<td>126±32 (6)</td>
</tr>
<tr>
<td>EC-50 (μM)</td>
<td>3.1(1.9-5.3)</td>
<td>6.7(3.5-13)</td>
<td>10.3(5.8-18)</td>
<td>5.6(3.9-19)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e., with the number per group in parenthesis. The significance of difference from the control values is indicated by A p<.05 & B p<.01 ; when MI is myo-inositol and ARI is an aldose reductase inhibitor ("ponalrestat")

(151)
### AORTIC RING SENSITIVITY TO DRUGS

**TABLE 13**

**6 MONTHS ANIMALS**

<table>
<thead>
<tr>
<th>Emax's &amp; EC-50 Ranges</th>
<th>NORADRENALINE</th>
<th>5-HYDROXYTRYPTAMINE</th>
<th>5-HYDROXYTRYPTAMINE (+ DESIPRAMINE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC-50</td>
<td>Emax</td>
<td>NORADRENALINE</td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>44 (15-100) nM</td>
<td>167±14</td>
<td>CONTROL (6)</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>3.5 (2.3-5.3) nM</td>
<td>243±43</td>
<td>DIABETIC (6)</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>8.7 (5.8-13) nM</td>
<td>79±5</td>
<td>DIABETIC + MI (6)</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>21 (9.8-48) nM</td>
<td>141±5</td>
<td>DIABETIC + ARI (6)</td>
</tr>
</tbody>
</table>

Results for Emax's are expressed as mean ± s.e. and for EC-50 Ranges as geometric means with 95% confidence limits, with the number per group in parenthesis. The significance of difference from the control values is indicated by **A p<.05 & B p<.01** and from the untreated diabetic values by **D p<.05 & F p<.01**; when **MI** is myo-inositol & ARI an aldose reductase inhibitor ("ponalrestat").

(152)
Dose-response curves of rubbed rat aortic rings to Noradrenaline from 6-months STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values, and D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO NORADRENALINE

Legend
- CONTROL
- DIABETIC
- DIABETIC + insulin
- DIABETIC + glarginestr.
Graph 20

Dose-response curves of rubbed rat aortic rings to 5-Hydroxytryptamine from 6-months STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values, and D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO 5-HYDROXYTRYPTAMINE IN THE ABSENCE OF DESIPRAMINE

Legend
- CONTROL
- DIABETIC
- DIABETIC + myo-inositol
- DIABETIC + probucol

log [5-HYDROXYTRYPTAMINE]
Dose-response curves of rubbed rat aortic rings to 5-Hydroxytryptamine in the presence of desipramine from 6-months' STZ diabetic, control, and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM, when A ($p<0.05$) and B ($p<0.01$) indicate a significant difference from the control values, and D ($p<0.05$) and F ($p<0.01$) indicate a significant difference from the untreated diabetic values.
RESPONSES TO 5-HYDROXYTRYPTAMINE IN THE PRESENCE OF DESIPRAMINE

![Graph showing responses to 5-hydroxytryptamine in the presence of desipramine. The graph plots log [5-hydroxytryptamine] against the response, with different symbols and lines representing control, diabetic, and diabetic + insulin groups. The legend is provided at the bottom of the graph.]
In the 1 year animals (table 14) the sensitivity to noradrenaline in the diabetic group was significantly higher compared to the control values (p<.05). The sensitivity to noradrenaline of the D + ARI group was significantly lower (p<.05 vs diabetic & p<.01 vs control). There was no change in the Emax's for the diabetic and D + ARI groups. Emax's and sensitivity to 5-HT, in the absence and presence of desipramine, were unchanged in the diabetic and D + ARI groups. An anomaly was the fact that the diabetic group's response was so low as to be unmeasurable.

**TABLE 14**

**1 YEAR ANIMALS**

<table>
<thead>
<tr>
<th>Emax's &amp; EC-50 Ranges</th>
<th>EC-50 Ranges</th>
<th>Emax mg/ten.</th>
<th>Emax mg wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NORADRENALINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>0.1(0.2-0.1) µM</td>
<td>228±54</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>1.2(3.8-0.3) µM</td>
<td>170±15</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>47(85-26) µM</td>
<td>204±36</td>
<td></td>
</tr>
<tr>
<td><strong>5-HYDROXYTRYPTAMINE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>1.6(3.8-0.7) µM</td>
<td>121±32</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>0.7(3.2-0.1) µM</td>
<td>110±22</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>0.4(1.1-0.1) µM</td>
<td>151±36</td>
<td></td>
</tr>
<tr>
<td><strong>5-HYDROXYTRYPTAMINE (+ DESIPRAMINE)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>0.5(1.7-0.1) µM</td>
<td>117±26</td>
<td></td>
</tr>
<tr>
<td>DIABETIC (5)</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>0.6(1.2-0.3) µM</td>
<td>139±21</td>
<td></td>
</tr>
</tbody>
</table>

Results for Emax's are expressed as mean ± s.e. and for EC-50 Ranges as geometric means with 95% confidence limits, with the number per group in parenthesis. The significance of difference from the control values is indicated by A p<.05 and from the untreated diabetic values by F p<.01; when MI is myo-inositol & ARI an aldose reductase inhibitor ("ponalrestat").

(156)
GRAPH 22

Dose-response curves of rubbed rat aortic rings to Noradrenaline from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO NORADRENALINE

---

**Legend**
- **CONTROL**
- **DIABETIC**
- **DIABETIC + pontrestat**

---

<table>
<thead>
<tr>
<th>log [noradrenaline]</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
<th>140</th>
<th>160</th>
<th>180</th>
<th>200</th>
<th>220</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>120</td>
<td>140</td>
<td>160</td>
<td>180</td>
<td>200</td>
<td>220</td>
</tr>
</tbody>
</table>
Dose-response curves of rubbed rat aortic rings to 5-Hydroxytryptamine from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM, when A (p<0.05) indicates a significant difference from the control values, and D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
Dose-response curves of rubbed rat aortic rings to 5-Hydroxytryptamine in the presence of desipramine from 1 year STZ diabetic and control animals. Each point represents an average of at least five observations and vertical lines denote SEM, when A (p<0.05) and B (p<0.01) indicate a significant difference from the control values.
RESPONSES TO 5-HT IN THE PRESENCE OF DESIPRAMINE

Legend

O Control

■ Diabetic papillae

log [5-hydroxytryptamine]
In the vas deferens preparation (table 15) diabetes was confirmed by the significantly elevated blood glucose levels \((p<.01)\) and reduced body weight gain \((p<.01)\) of the diabetic and treated groups, in both 6 month and 1 year animals.

TABLE 15

CONFIRMATION OF DIABETES

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 MONTHS ANIMALS: CONTROL, DIABETIC &amp; ARI &amp; MI TREATED</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>7.32±1.33</td>
<td>+177±14.5</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>40.60±1.08</td>
<td>-34.80±9.10</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>29.93±4.19</td>
<td>39.80±17.90</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>38.53±2.90</td>
<td>+3.20±13.70</td>
</tr>
<tr>
<td><strong>1 YEAR ANIMALS: CONTROL, DIABETIC &amp; ARI TREATED</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (12)</td>
<td>8.17±1.87</td>
<td>+174.22±25.60</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>38.66±2.44</td>
<td>+32.67±11.93</td>
</tr>
<tr>
<td>DIABETIC + ARI (8)</td>
<td>27.25±3.12</td>
<td>+33.88±17.03</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e. with number per group in parenthesis. The significance of difference from the control group values are indicated by B \(p<0.01\) and from the untreated diabetic values by D \(p<.05\), E \(p<.02\) & G \(p<.005\); where MI is myo-inositol, and ARI is an aldose reductase inhibitor ("ponalrestat").
Sensitivity to noradrenaline (table 16) was unchanged in both 6 month and 1 year animals. In the 6 month animals the Emax for the diabetic group was significantly higher (p<.05). The Emax for the D + XI group was significantly lower (p<.05 vs diabetic). There was no change in the Emax for the 1 year animals.

**TABLE 16**

**Emax's & EC-\(50\) RANGES**

<table>
<thead>
<tr>
<th>EC-(50) Ranges</th>
<th>Emax mg's/ten mg wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 MONTHS ANIMALS</strong></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>1.9(0.7-2.7)(\mu)M</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>3.3(2.4-4.5)(\mu)M</td>
</tr>
<tr>
<td>DIABETIC+XI (7)</td>
<td>2.5(0.4-15.0)(\mu)M</td>
</tr>
<tr>
<td>DIABETIC+ARI (6)</td>
<td>1.4(1.0-2.0)(\mu)M</td>
</tr>
<tr>
<td><strong>1 YEAR ANIMALS</strong></td>
<td></td>
</tr>
<tr>
<td>CONTROL (7)</td>
<td>1.4(0.7-2.7)(\mu)M</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>1.4(0.5-3.4)(\mu)M</td>
</tr>
<tr>
<td>DIABETIC+ARI (6)</td>
<td>1.2(0.4-3.4)(\mu)M</td>
</tr>
</tbody>
</table>

Results for Emax's are expressed as mean ± s.e. and for EC-\(50\) Ranges as geometric means with 95% confidence limits; with the number per group in parenthesis. The significance of difference from the control values is indicated by A p<.05 and from the untreated diabetic values by D p<.05; when XI is myo-inositol & ARI is an aldose reductase inhibitor ("ponalrestat").
Dose-response curves of rat vas deferens to noradrenaline from 6-months STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each point represents an average of at least six observations and vertical lines denote SEM, when A (p<0.05) indicates a significant difference from the control values, and D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO NORADRENALINE

(log [NORADRENALINE])

Legend
- CONTROL
- DIABETIC
- DIABETIC + noninsulin
- DIABETIC + pre-insulin
GRAPH 26

Dose-response curves of rat vas deferens to Noradrenaline from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least six observations and vertical lines denote SEM.
RESPONSES TO NORADRENALINE

Legend
- CONTROL
- DIABETIC
- DIABETIC + pentalrestat

Responses (mg tension/mg wet weight)

log [noradrenaline]

-8 -7 -6 -5 -4 -3
In the Na+–K+ ouabain-sensitive ATPase and triiodothyronine assay in the rat diaphragm the blood glucose and body weight data confirming diabetes are given in table 17.

Levels of triiodothyronine were measured (table 17) and found to be significantly reduced in the diabetic group and the diabetic treated with an aldose reductase inhibitor group (both were p<.01). Levels of the hormone were however restored in diabetic rats treated with triiodothyronine (p<.05).

Sodium-potassium ATPase levels were significantly reduced in the diabetic group compared to the control group values (p<.01). The enzyme levels were restored in the diabetic treated with an aldose reductase inhibitor group, but whilst higher than the diabetic group the levels were not restored to the control values. In the diabetic treated with triiodothyronine group the enzyme levels were significantly higher (p<.05) than the untreated diabetic group values.

<table>
<thead>
<tr>
<th>TABLE 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONFIRMATION OF DIABETES &amp; T₃ LEVELS</td>
</tr>
</tbody>
</table>

6 WEEKS ANIMALS

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
<th>T₃ Levels µg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (12)</td>
<td>4.79±3.72</td>
<td>+41.67±7.65</td>
<td>2.68±0.26</td>
</tr>
<tr>
<td>DIABETIC (8)</td>
<td>25.93±1.93</td>
<td>-5.33±6.72</td>
<td>0.64±0.34</td>
</tr>
<tr>
<td>DIABETIC + ARI (7)</td>
<td>25.34±2.71</td>
<td>-0.33±8.38</td>
<td>0.35±0.13</td>
</tr>
<tr>
<td>DIABETIC + T₃ (9)</td>
<td>26.69±1.27</td>
<td>+4.67±12.99</td>
<td>1.94±0.74</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the mean control values is indicated by B p<.01 and from the untreated diabetic values by D p<.05; when WI is myo-inositol, ARI is an aldose reductase inhibitor (ponalrestat) and T₃ is triiodothyronine.
Levels of ouabain-sensitive sodium-potassium ATPase in the rat diaphragm of 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with triiodothyronine T3. Each bar represents the average of at least seven observations, each observation being triplicated, and vertical lines denote SEM, when B (p<0.01) indicates significant difference from the control values and D (p<0.05) indicate significant difference from the diabetic values.
SODIUM–POTASSIUM ATPASE LEVELS

Legend
- CONTROL
- DIABETIC
- DIABETIC + penicillat
- DIABETIC + triiodothyronine
In the second sodium-potassium ATPase and triiodothyronine assay various tissues were used, namely the rat heart, superior cervical ganglion and sciatic nerve. Diabetes was confirmed in the untreated diabetic group, and diabetic treated with an aldose reductase inhibitor and triiodothyronine by the significantly elevated blood glucose levels (p<.01) and significantly reduced body weight gains (p<.01) (table 18).

Triiodothyronine levels (table 18) were found to be significantly reduced in the untreated diabetic group (p<.01) and in the diabetic treated with an aldose reductase inhibitor (p<.01). The levels of this hormone were restored in the diabetic rats treated with triiodothyronine (p<.01).

In the superior cervical ganglion and sciatic nerve insufficient number of samples were available to test for significance of difference from the control values (table 19).

In the superior cervical ganglion mean sodium-potassium ATPase levels for the diabetic group was, however, much lower than the control group. In the diabetic groups treated with an aldose reductase inhibitor and triiodothyronine the mean levels of the enzyme were higher but not restored to the control levels.

In the sciatic nerve the diabetic group mean level of enzyme was higher than the control. The mean levels of the diabetic groups treated with an aldose reductase inhibitor and triiodothyronine were similar to the control.

In the heart the sodium-potassium ATPase level of the diabetic group was significantly reduced compared to the control values (p<.05). The diabetics groups treated with an aldose reductase inhibitor and triiodothyronine groups both had significantly lower values than the control (both p<.05) whilst the DARI group had increased levels compared to the diabetic values (p<.05).
ASSAY OF \( \text{Na}^+\text{K}^+\) ATPase & \( \text{T}_3 \) LEVELS IN VARIOUS RAT TISSUES

TABLE 18
CONFIRMATION OF DIABETES & \( \text{T}_3 \) LEVELS
6 WEEKS ANIMALS

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
<th>( \text{T}_3 ) Levels ( \mu \text{g/l} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (6)</td>
<td>6.9±1.2</td>
<td>+78.5±3.9</td>
<td>1.95±0.12</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>22.6±8.4</td>
<td>+17.6±11.9</td>
<td>0.79±0.11</td>
</tr>
<tr>
<td>DIABETIC + ARI (7)</td>
<td>23.2±9.2</td>
<td>+10.1±13.5</td>
<td>0.91±0.07</td>
</tr>
<tr>
<td>DIABETIC + ( \text{T}_3 ) (6)</td>
<td>25.9±8.4</td>
<td>+30.6±12.4</td>
<td>2.26±0.73</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the mean control values is indicated by B \( p<.01 \) and from the untreated diabetic values by F \( p<.01 \); when MI is myo-inositol, ARI is an aldose reductase inhibitor (ponalrestat) and \( \text{T}_3 \) is triiodothyronine.

TABLE 19
\( \text{Na}^+\text{K}^+\) ATPase LEVELS
(pmollg wet weight)

<table>
<thead>
<tr>
<th></th>
<th>Superior Cervical Ganglion</th>
<th>Heart</th>
<th>Sciatic Nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>219±98</td>
<td>82±9</td>
<td>27±10</td>
</tr>
<tr>
<td>(5)</td>
<td>(9)</td>
<td>(6)</td>
<td></td>
</tr>
<tr>
<td>DIABETIC</td>
<td>79±22</td>
<td>15±7A</td>
<td>92±53</td>
</tr>
<tr>
<td>(4)</td>
<td>(5)</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ARI</td>
<td>121±37</td>
<td>45±9AD</td>
<td>32±12</td>
</tr>
<tr>
<td>(3)</td>
<td>(8)</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td>DIABETIC + ( \text{T}_3 )</td>
<td>149±58</td>
<td>39±13A</td>
<td>12±5</td>
</tr>
<tr>
<td>(3)</td>
<td>(5)</td>
<td>(4)</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by A \( p<.05 \) and from the untreated diabetic values by D \( p<.05 \); when ARI is an aldose reductase inhibitor (‘ponalrestat’) and \( \text{T}_3 \) is triiodothyronine.
In the oesophagus preparation diabetes was confirmed (table 20) by the significantly elevated blood glucose levels (p<.01) of the diabetic and D + MI groups and reduced body weight gain (p<.01) of the diabetic group in the 6-weeks animals. In the 6 month, and 1 year animals diabetes was confirmed by the significantly elevated blood glucose (p<.01) and reduced body weight gain (p<.01) of the diabetic and treated animals.

**TABLE 20**

**CONFIRMATION OF DIABETES**

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 WEEKS: CONTROL, DIABETIC &amp; MI TREATED.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (6)</td>
<td>4.34±0.75</td>
<td>+63.9±9.3</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>28.36±2.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-27.2±8.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DIABETIC+MI (6)</td>
<td>19.91±1.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>+17.0±7.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

6 MONTHS ANIMALS: CONTROL, DIABETIC & ARI & MI TREATED

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (6)</td>
<td>7.32±1.33</td>
<td>+177±14.5</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>40.60±1.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-34.80±9.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>29.93±4.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>+39.80±17.90&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>38.53±2.90&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>+3.20±13.70&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1 YEAR ANIMALS: CONTROL, DIABETIC & ARI TREATED

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (12)</td>
<td>8.17±1.87</td>
<td>+174.22±25.60</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>38.66±2.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+32.67±11.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DIABETIC + ARI (8)</td>
<td>27.25±3.12&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>+33.88±17.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as mean +/- s.e. with number per group in parenthesis. The significance of difference from the control group values are indicated by B p<0.01 and from the untreated diabetic values by D p<.05, E p<.02 & G p<.005; where MI is myo-inositol, and ARI is an aldose reductase inhibitor ("ponalrestat").

(168)
In the 6 weeks animals (table 21) sensitivity to acetylcholine was unchanged, whilst the Emax for the D + MI group was significantly lower (p<.01 vs diabetic).

In the 6 months animals only sensitivity to acetylcholine in the D + MI group was significantly different (p<.05 vs diabetic). The Emax's for the D + MI and D + ARI groups were significantly lower (p<.05 vs diabetic).

In the 1 year animals sensitivity to acetylcholine was unchanged, only the D + ARI group Emax was significantly lower (p<.05 vs diabetic).

**TABLE 21**

<table>
<thead>
<tr>
<th>Emax's &amp; ECso Ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EC-so Ranges</strong></td>
</tr>
<tr>
<td>6 WEEKS ANIMALS</td>
</tr>
<tr>
<td>CONTROL (7)</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
</tr>
<tr>
<td>6 MONTHS ANIMALS</td>
</tr>
<tr>
<td>CONTROL (6)</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
</tr>
<tr>
<td>DIABETIC + MI (5)</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
</tr>
<tr>
<td>1 YEAR ANIMALS</td>
</tr>
<tr>
<td>CONTROL (12)</td>
</tr>
<tr>
<td>DIABETIC (8)</td>
</tr>
<tr>
<td>DIABETIC + ARI (7)</td>
</tr>
</tbody>
</table>

Results for Emax's are expressed as mean ± s.e. and for the EC-so Ranges as geometric means with 95% confidence limits; with the number per group in parenthesis. The significance of difference from the untreated diabetic values is indicated by D p<.05 & F p<.01; when MI is myo-inositol and ARI an aldose reductase inhibitor ("ponalrestat").  
(169)
Dose-response curves of rat oesophageal muscularis mucosa to Acetylcholine from 6-weeks STZ diabetic, control, and diabetic treated with myo-inositol. Each point represents an average of at least five observations and vertical lines denote SEM, when D (p<0.05) and F (p<0.01) indicate a significant difference from the untreated diabetic values.
RESPONSES TO ACETYLCHOLINE

Legend
- CONTROL
○ DIABETIC
△ DIABETIC + myo-inositol

RESPONSE (mg tension/mg wet weight)

log (acetylcholine)
Dose-response curves of rat oesophageal muscularis mucosa to Acetylcholine from 6-months STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each point represents an average of at least five observations and vertical lines denote SEM.
RESPONSES TO ACETYLCOLINE

![Graph showing responses to acetylcholine](image-url)

**Legend**
- Control
- Diabetic
- Diabetic + propranolol
- Diabetic + ex-inhaler
Dose-response curves of rat oesophageal muscularis mucosa to Acetylcholine from 1 year STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least seven observations and vertical lines denote SEM, when A (p<0.05) indicates a significant difference from the control values, and D (p<0.05) indicates a significant difference from the untreated diabetic values.
RESPONSES TO ACETYLCHOLINE

Legend

○ CONTROL
■ DIABETIC
□ DIABETIC + pons trepat
Maximum force of contraction to field stimulation in 6-weeks animals was unchanged in both the presence and absence of atropine (table 22). There was no change in the maximum force of contraction in 6-months animals in the absence of atropine.

**TABLE 22**

**MAXIMUM FORCE OF CONTRACTION**

DUE TO FIELD STIMULATION

(mg tension/ mg wet weight)

<table>
<thead>
<tr>
<th></th>
<th>WITH ATROPINE</th>
<th>WITHOUT ATROPINE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6 WEEKS ANIMALS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL (7)</td>
<td>62.3±16.8</td>
<td>70.2±11.0</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>77.5±19.4</td>
<td>132.5±35.7</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>53.0±10.3</td>
<td>99.3±12.6</td>
</tr>
</tbody>
</table>

|                      |               |                  |
| **6 MONTHS ANIMALS** |               |                  |
| CONTROL (6)          | 42.4±10.4     |                  |
| DIABETIC (6)         | 32.5±6.9      |                  |
| DIABETIC + MI (5)    | 33.1±5.0      |                  |
| DIABETIC + ARI (6)   | 38.3±9.8      |                  |

Results are expressed as mean±s.e.; when MI is myo-inositol and ARI is an aldose reductase inhibitor, 'ponalrestat'.
LONGITUDINAL MUSCLE PREPARATION

In the 6-month diabetic longitudinal muscle preparation (table 23) diabetes was confirmed by the significantly elevated blood glucose levels (p<.01) and reduced body weight gain (p<.01).

The EC₅₀ ranges were unchanged (table 24), whilst the Emax’s for the diabetic and D + ARI groups were significantly lower (p<.01 vs control values).

**TABLE 23**
CONFIRMATION OF DIABETES
6 MONTH ANIMALS

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (6)</td>
<td>6.92±1.90</td>
<td>+171±28</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>35.62±6.40*</td>
<td>-38.3±12.6*</td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>36.94±6.70*</td>
<td>-20.2±10.3*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by B p<.01; when ARI is an aldose reductase inhibitor ("ponalrestat").

**TABLE 24**
Emax's & EC₅₀ Ranges

<table>
<thead>
<tr>
<th></th>
<th>Emax (mM/cm²)</th>
<th>EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (6)</td>
<td>1.6309±0.1715</td>
<td>4.7(2.3-9.2)</td>
</tr>
<tr>
<td>DIABETIC (6)</td>
<td>0.3492±0.0734*</td>
<td>12.0(2.4-58.8)</td>
</tr>
<tr>
<td>DIABETIC + ARI (5)</td>
<td>1.0949±0.1017*</td>
<td>9.7(3.0-30.9)</td>
</tr>
</tbody>
</table>

All results for Emax are expressed as mean ± s.e. and for EC₅₀ ranges as geometric means with 95% confidence limits. The significance of difference from the control values is indicated by B p<.01; when ARI is an aldose reductase inhibitor, "ponalrestat", and MI is myo-inositol.
Dose-response curves of rat terminal ileum longitudinal muscle to Acetylcholine from 6-months STZ diabetic, control, and diabetic treated with an aldose reductase inhibitor (ponalrestat). Each point represents an average of at least five observations and vertical lines denote SEM, when B (p<0.01) indicate a significant difference from the control values.
RESPONSES TO ACETYLCHOLINE

Legend
- Control
- Diabetic
- Diabetic + nonolrestat
CHOLINE ACETYLTRANSFERASE ASSAY (CHAT)

Diabetes in 6-weeks diabetic animals was confirmed (table 25) by the elevated blood glucose levels ($p<0.01$) and the reduced body weight gain ($p<0.01$).

**Table 25**

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (8)</td>
<td>6.89±1.05</td>
<td>+60.48±9.56</td>
</tr>
<tr>
<td>DIABETIC (10)</td>
<td>26.48±2.21$^a$</td>
<td>-22.68±11.7$^a$</td>
</tr>
<tr>
<td>DIABETIC + XI (7)</td>
<td>30.51±2.65$^a$</td>
<td>-33.70±8.90$^a$</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>32.70±2.03$^a$</td>
<td>-27.90±13.70$^a$</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with number per group in parenthesis. The significance of difference from the control group values are indicated by $B p<0.01$, where XI is myo-inositol, and ARI is an aldose reductase inhibitor ("ponalrestat").

There was no significant difference in levels of CHAT between the control group and the untreated diabetic group. Though mean levels of CHAT in both the XI and ARI treated groups were higher than the control and untreated diabetic groups, this mean elevation was not significant.

The levels of CHAT in control animals was measured in both the brain and the ileum and the mean level in the brain was found to be 100X greater than the mean level in the ileum.

**Table 26**

<table>
<thead>
<tr>
<th>Levels of Choline Acetyltransferase (µM/ g protein/ hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RAT ILEUM TISSUE</strong></td>
</tr>
<tr>
<td>CONTROL (8)</td>
</tr>
<tr>
<td>17.5±4.9</td>
</tr>
<tr>
<td><strong>CONTROL TISSUE</strong></td>
</tr>
<tr>
<td>Rat Ileum Tissue (3)</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis.

(176)
Diabetes was confirmed in 6-weeks diabetic rats (table 27) by the elevated blood glucose levels (p<.01) and the reduced body weight gain (p<.01).

**TABLE 27**

**CONFIRMATION OF DIABETES**

<table>
<thead>
<tr>
<th></th>
<th>Blood Glucose Level (mM)</th>
<th>Change in Body Weight (g's)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL (6)</td>
<td>4.34±0.75</td>
<td>+63.9±9.3</td>
</tr>
<tr>
<td>DIABETIC (7)</td>
<td>28.36±2.34*</td>
<td>-27.2±8.9*</td>
</tr>
<tr>
<td>DIABETIC + MI (6)</td>
<td>19.91±1.53**</td>
<td>+17.0±7.6**</td>
</tr>
<tr>
<td>DIABETIC + ARI (6)</td>
<td>32.62±0.86*</td>
<td>-19.7±7.7*</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± s.e. with the number per group in parenthesis. The significance of difference from the control values is indicated by E p<.01 and from the untreated diabetic values by E p<.02 & G p<.005; when MI is myo-inositol, ARI is an aldose reductase inhibitor (ponalrestat).

Levels of myo-inositol in untreated diabetic sciatic nerve were significantly reduced compared to the control values (p<.01). In the diabetic group treated with MI the level was significantly elevated compared to both the control and untreated diabetic group (both p<.01). Levels of myo-inositol were normalized in the ARI treated group and were significantly higher than the untreated diabetic group (p<.01).

Levels of sorbitol were significantly increased in the untreated diabetic group compared to the control values (p<.01). The MI treated group had elevated levels compared to the control (p<.01) and the untreated diabetic (p<.05) values. Levels of sorbitol were normalized in the ARI treated group and were significantly reduced compared to the untreated group (p<.01).

Levels of fructose were significantly elevated only in the untreated diabetic group compared to control values (p<.01). In the ARI treated group levels were significantly lower than the untreated diabetic group (p<.01).

Mean fructose levels in the D + MI group were lower, but not significant.

( 177 )
Levels of myo-inositol, sorbitol and fructose in the rat sciatic nerve of 6-weeks STZ diabetic, control, diabetic treated with an aldose reductase inhibitor (ponalrestat), and diabetic treated with myo-inositol. Each bar represents the average of at least six observations, each observation being triplicated, and vertical lines denote SEM, when B (p<0.01) indicates significant difference from the control values and D (p<0.05) and F (p<0.01) indicate significant difference from the diabetic values.
LEVELS OF MYO-INOSITOL, SORBITOL & FRUCTOSE

Legend
- CONTROL
- DIABETIC
- DIABETIC + myo-inositol
- DIABETIC + pentoestat
CHAPTER FOUR
4.1 DISCUSSION

The purpose of this study was to investigate the effects of different periods of streptozotocin-induced diabetes in the rat on nervous and vascular function using a range of tissues. The main aim was to establish whether neuropathy or vascular changes could be detected. Such changes might provide more appropriate models of diabetic complications than those currently in use.

Various mechanisms have been proposed, such as sorbitol accumulation, polyol pathway dysfunction, hypothyroidism, anoxia, blood lipid and fibrinogen changes, alterations in eicosanoid metabolism, sympathetic neuropathy, ganglioside depletion, vitamin C depletion, non-enzymatic protein glycosylation and excess free radicals, to explain the development of diabetic complications (as described in the introduction).

We decided to investigate the effects of dietary supplements of myo-inositol, an aldose reductase inhibitor (ponalrestat) and triiodothyronine to assess whether the proposed mechanisms, excess polyol pathway flux and hypothyroidism, were involved in any diabetes induced changes.

4.2 AORTA

Cardiovascular dysfunctions are more common among diabetics than the general public (Christlieb, 1973a,b). These dysfunctions, such as postural hypertension, disturbances in regional blood flow, and changes in elasticity of blood vessels, may be caused by changes in sensitivity to neurotransmitters in blood vessels (Christlieb et al., 1976; Weidmann
Many workers have tested this hypothesis by using various vascular preparations from rats with induced diabetes (Brody & Dixon, 1964; Foy & Lucas, 1976; Owen & Carrier, 1980; Cavaliere et al, 1980; Turlapaty et al, 1980; Pfaffman et al, 1982; Jackson & Carrier, 1981; MacLeod & McNeill, 1985). The aorta is a very reliable preparation as a model for measuring vascular responses in experimental diabetes, but it is not known how relevant these changes are to diabetic dysfunction. An advantage of the preparation is that it is larger than other arteries and is therefore easier to handle. It is usually prepared as helical strips or rings, though helical preparations usually damage the endothelium cells; in this particular study aortic rings were used.

The present study indicates little or no evidence for vascular changes in the streptozotocin-induced diabetic rat aorta.

In the six-weeks diabetic rat study, the untreated diabetic rubbed aorta was desensitised to 5-hydroxytryptamine (5-HT) compared to the control (p<.01). Treatment with ponarrestat and myo-inositol (MI) supplementation failed to rectify this abnormality; both of these groups remained desensitised (p<.01). In the unrubbed aorta only the diabetic treated with MI was desensitised (p<.05). Sensitivity to noradrenaline (NA) was unchanged in both rubbed and unrubbed aorta.

The variable duration of induced diabetes used by workers to represent the short-term state makes comparisons between studies difficult.

Workers found no change in sensitivity to a range of agonists, such as NA, 5-HT and KCl, in rats with 7 days induced diabetes; for both streptozotocin and alloxan induced animals (MacLeod & McNeill, 1985; Turlapaty et al, 1980). This short period of diabetes doesn't offer a valid comparison with the animals used in our study. Our data on changes
in sensitivity agreed with studies which found a reduction in sensitivity in 8-weeks alloxan induced diabetic rats (Turlapty et al, 1980; Pfaffman et al, 1982). Studies by these workers on 12-weeks alloxan induced diabetic rats showed similar reductions in sensitivity.

In the 6-months diabetic rat rubbed aorta sensitivity to NA was increased; this contradicted a study which showed no change in 6-months animals (MacLeod & McNeill, 1985). In 3-months diabetic animals they did however find an increase in sensitivity to NA with no alteration in the Emax's. Treatment of diabetic rats with MI produced no change in the altered sensitivity to NA, though it did lower the maximal response (p<.01 vs diabetic). Treatment with ponalrestat significantly decreased sensitivity to NA (p<.05 vs diabetic). The maximal response was significantly lowered (p<.01 vs diabetic), though it did not return to the control value (p<.05).

We did not detect a decrease in responsiveness to 5-HT as these workers did, but increases in maximal response did agree with this study. The maximal response of the untreated diabetic was increased (p<.05), whilst treatment with MI considerably reduced the maximal response (p<.05 vs control & p<.01 vs control) and ponalrestat treatment did not restore this response (p<.01 vs control).

Sensitivity to 5-HT in the presence of desipramine, a potent NA Uptake, blocker, was unchanged in the diabetic and MI treated animals. Maximal responses in the diabetic rat were increased (p<.01), and treatment with MI considerably lowered these responses (p<.01 vs diabetic & p<.01 vs control). We were unable to obtain any responses in diabetic animals treated with ponalrestat. It may be that ponalrestat was having an adverse effect; however further work was not carried out to verify this.

(182)
The increased Emax values for both NA and 5-HT in the 6 month study suggest a post-receptor change. The partial prevention by ARI/XI indicates an involvement of a polyol pathway dependent MI depletion. A measurement of sorbitol and MI in aortic smooth muscle might provide evidence to support this. A role for the polyol pathway in diabetes induced changes in smooth muscle, as well as in nervous tissues, would be of considerable interest. Diabetes induced changes in autonomic control of a tissue tend to be ascribed to nervous changes, they could equally be due to effector tissue changes. It has been pointed out that decreased responses in the ileum of streptozotocin-induced diabetic rats to acetylcholine could contribute to lack of proper gut muscle tone (Lucas & Sardar, 1991).

In the 1 year animals sensitivity to NA in the diabetic state was decreased whilst the maximal response was unchanged. This finding disagreed with earlier studies which showed supersensitivity to NA in the 1 year diabetic rat aorta (McLeod & McNeill, 1985); and to NA in the 42-43 weeks diabetic mesenteric arteries (Jackson & Carrier, 1981). We did not find an increase in sensitivity to 5-HT as other workers did (McLeod & McNeill, 1985).

The diabetic treated with ponalrestat was found to be supersensitive to NA with an unchanged maximal response. This indicated that decreased sensitivity was ameliorated by this treatment and that the polyol pathway was involved in decreasing sensitivity to NA in the 1 year animals.

Responses to 5-HT were unchanged in the diabetic and ponalrestat treated animals, this contrasted with a study which found 1 year streptozotocin diabetic rats supersensitive to 5-HT (McLeod & McNeill, 1985). The diabetic response to 5-HT, with desipramine, was completely (183)
absent, whereas it was present in the ponalrestat treated animals. The contradiction between our results of the 1 year induced diabetic animals with the previous studies could be due to differences in the severity of the induced diabetes, a common problem in this field. It may be related to the sex of the animals used, our study used male rats whereas McLeod and McNeill used female rats.

Our results show little evidence to suggest denervation is occurring in the diabetic rat aorta. Changes in sensitivity, to 5-HT in the 6 weeks animals and to NA in 6 months and 1 year animals, are probably due to smooth muscle changes. The degree and consistency of changes occurring in the rat aorta is difficult to assess.

Workers found increased maximal responses to NA and KCl in the 1 year rat only when they assumed a proportionate decrease in all the components of the diabetic aorta and calculated results by dividing the tension developed by the cross-sectional area (McLeod & McNeill 1985). It could be that the difference in results between the two studies was due to inconsistent smooth muscle changes occurring in the diabetic rat aorta.

It would appear from our study that the polyol pathway is not implicated directly in any changes occurring in the rat aorta in the early stages. It however, does play a role in the 1 year rat where ponalrestat was responsible for reversing diabetic-induced changes.

In the 6 weeks animals decreased sensitivity to 5-HT was not ameliorated by treatment with either MI or ponalrestat. In the 6 month animals sensitivity to NA was increased and treatment with MI had no effect on this change. Ponalrestat treatment was effective in restoring sensitivity, but sensitivity was still higher in these animals compared to the controls.
Decreased sensitivity to NAD in 1 year animals was reversed by treatment with ponalrestat producing EC₅₀ values of the same magnitude as those for the 6 month animals. Ponalrestat may have had a sensitising effect on the tissue, suggesting a mechanism not dependent on aldose reductase inhibition, in which case further investigation of a control + ponalrestat group would be beneficial.

The absence of responses to 5-HT with desipramine in the 1 year diabetic group may suggest total desensitisation, and the normal responses in the ponalrestat treated animals could indicate reversal of this desensitisation. However there was no difference in responses to 5-HT, without desipramine, in the diabetic state. It is worth noting though that 5-HT acts both directly on the aortic smooth muscle and indirectly via the release of catecholamines, the latter being prevented by desipramine. It seems that the direct effects were impaired in the diabetic treated animals but not in the ponalrestat treated animals.

A decrease in sensitivity to NA in control groups going from 6 months to 1 year animals, whereas there was no change to 5-HT. This concurs with other studies which found a nonspecific loss in the responsiveness of aortae to agonists with increasing age (Cohen & Berkowitz, 1976; McLeod & McNeill, 1985); the mechanisms for these changes have not been elucidated.

The changes in the rat aorta to external agonists are not progressive and postulating any mechanism is difficult. It may be that specific changes are occurring in the receptors for these agonists, or in the coupling of these receptors to the mobilisation of calcium ions. But these changes are not permanent as they vary between the different durations of diabetes investigated.

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In conclusion it would seem that vascular changes were seen but they didn't follow a clear progressive pattern. They don't appear to offer suitable models of diabetic neuropathy or vasculopathy.

4.3 THE ANOCOCCYGEUS MUSCLE

As described in the introduction, autonomic neuropathy frequently occurs in human diabetes (Clarke et al, 1979; Harati et al, 1987). The severity and degree of neuropathy depends on several factors, one of which is the duration of diabetes (Jeremy et al, 1986). Loss of autonomic function has been reported in sciatic nerves from long-term diabetic rats (Tomlinson & Yusof, 1983; Yagihashi & Simo, 1985; Schmidt & Plurad, 1986). Alterations in nervous tissue responses to agonists and field stimulation have been reported in short term diabetic studies. (Brody & Dixon, 1964; MacLeod & McNeill 1985; Scarborough & Carrier, 1984; Chang et al, 1986; Nowak et al, 1986).

Profound changes occur in tissue exhibiting sympathetic denervation, such as reduced responses to nervous stimulation and increased sensitivity to agonists, such as noradrenaline, either of these would be indicative of neuropathy (Scarborough & Carrier, 1984; Jackson & Carrier, 1981).

Increased sensitivity to NA in neuropathic tissue would most likely be due to a combination of receptor up regulation, i.e. increased receptor number, and reduced NA uptake by neurones (Brody & Dixon, 1964; Foy & Lucas, 1976; MacLeod & McNeill 1985; Chang et al, 1986; Nowak et al, 1986).

Several mechanisms have been proposed to explain the neuropathy prevalent in the diabetic state; we decided to investigate the involvement of two, the polynol pathway and hypothyroidism in diabetes induced changes. We examined the effects of different periods of
diabetes on tissue responses to electrical stimulation and to exogenous NA and the overflow and stimulated release \(^{3}H-NA\).

The tissues were considered suitable for a study of sympathetic innervation since they have a dense adrenergic innervation distributed throughout the muscle but apparently no cholinergic innervation, also the tissues exist as a pair and this offers certain advantages in some studies.

Sensitivity was decreased to NA and Emax values were increased in the 6 weeks untreated diabetic rats whilst the maximum force of contraction due to field stimulation was unchanged. However we were unable to show this defect when the experiment was repeated three times with NA and other agonists, phenylephrine and clonidine. Sensitivity to NA was increased in 6 months diabetic animals and treatment with XI and ponalrestat significantly desensitised the tissue but did not normalize it.

In the 1 year animals there was a mean decrease in sensitive to NA and phenylephrine from control to diabetic and ponalrestat treated diabetics, but this was not significant.

Failure to replicate the decrease in sensitive to NA in 6 weeks diabetic rats, even though this experiment was repeated three times, suggests the initial result was an aberration. Also there was no difference in the force of maximum contraction to field stimulation between control and diabetic animals. Any changes in the force of contraction are likely to reflect smooth muscle changes; changes in neuropathy should have a greater impact on sensitivity, in that it should have been increased.

The increase in sensitivity to NA in 6 months diabetic rats may indicate the presence of neuropathy, however treatment with XI and ponalrestat failed to normalise this defect. Also the failure to
detect neuropathy, i.e. increased sensitivity to NA, in 1 year animals indicates this effect is not progressive and is self-correcting. The mean decrease in sensitivity in 1 year animals may have been due to a reduction in receptor population. Studies have shown a reduced sensitivity to agonists following prolonged exposure and implicitly linked this to reduced receptor numbers (Vadlamundi & McNeill, 1983; Kofo-Abeyomi & Lucas, 1988), and this may have been responsible for the observed reduction in sensitivity to NA in 1 year diabetic rats. If so, then this is the opposite change to that expected to follow neuropathy.

Diabetes could have been responsible for the reduction in sensitivity to NA by increasing tissue exposure to NA. It has been proposed that excess flux through the polyol pathway, leading to accumulation of sorbitol within neurones, may cause a reduction in Na⁺-K⁺-ATPase activity, and this defect is responsible for subsequent cell damage (Greene et al, 1983; Greene, 1986). A depression of Na⁺-K⁺-ATPase activity in sympathetic nerve endings might be expected to affect Na⁺-gradient dependent transport, such as uptake, responsible for the uptake of NA back into neurones. Such an effect might contribute to increased NA turnover, which has been found in various tissue of short-term (6 weeks) diabetic rats (Lucas & Qirbi, 1989; Ganguly et al, 1987). The prolonged presence of extraneuronal NA would be likely to lead to desensitization.

Alternatively reduced sensitivity to NA may be due to a depression of post-receptor mechanisms, such as reduced secondary messengers, e.g. phosphoinositides. It has been proposed that diabetes is responsible for a reduction in phosphoinositides by causing a decrease in levels of myo-inositol, an essential substrate for the synthesis of
phosphatidylinositol. Studies have shown a reduction in the levels of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP₂), as well as reduced activity of CDP-diaclylglycerol and PIP kinase in the nerves of diabetic rats (Dyck et al, 1980; Tomlinson et al, 1984). Reduced myo-inositol levels leading to decreased turnover of phosphoinositides would produce a decrease in cell sensitivity to agonists, and this may be responsible for the mean reduction in sensitivity to NA in 1 year diabetic rats. A longer period of diabetes, such as 18 months, may establish whether the observed mean difference was significant or not. Whether this defect is mirrored in humans is not clearly established. Nerve tissue myo-inositol content is reduced by 20-30% in diabetic patients (Hale et al, 1987), but a recent study found increased membrane concentration of total phospholipids in the platelets of diabetic patients (Mazzanti et al, 1990). Studies have found reduced nerve myo-inositol in conjunction with unchanged plasma myo-inositol levels in diabetic rats (Dyck et al, 1980; Tomlinson et al, 1984). It may be that plasma levels of myo-inositol are elevated by diabetes since tissue levels are reduced and less myo-inositol is being taken up. In which case reduced neuronal phospholipids could coexist with increased platelet membrane phospholipids.

The data collected from 6 months animals suggests that maybe a significant change is occurring at this stage of diabetes. But the disappearance of these changes in 1 year diabetic animals implies that this change is not progressive and therefore its relevance to human diabetic neuropathy, which shows a consistent increase with the duration of the disease, is uncertain.

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We found that Emax's for untreated diabetic animals were consistently higher than the control values, in some cases significantly so. This data suggested that changes in the effector component of the responses was occurring in diabetic animals. Structural alterations in diabetic animals are well documented (Schmidt et al, 1983) and it could be that there are smooth muscle changes occurring in diabetes. It maybe that the diabetic animals preparations were closer to the optimum tension than the control animals. Since diabetic tissues are smaller, it maybe that subjecting them to the same total tension would have resulted in greater resting force/unit cross sectional area. Considering this we varied the tension used, so that responses at the optimum tension were recorded. However EC-so values for the diabetic animals were unchanged and Emax's at variable tension remained high, though not significantly so, compared to the control values. The lack of significance suggests that the differences previously observed were due to the diabetic tissues being close to their optimum tension.

The results of a previous study of NA overflow and stimulated release into sympathetic neurones of anococcygeus muscles and its inhibition by various blockers (Doggerell & Woodruff, 1978) found the ECso of desipramine to be about 10nM and suggested that 0.2 μM desipramine should produce near complete inhibition of Uptake1. The finding in the present study of a reduction in the ability of this concentration of desipramine to potentiate 3H overflow in the tissues from the untreated diabetic group suggests an impairment of this transport. The potentiation by transmural nerve stimulation of 3H-NA overflow in the control tissues but not those from the untreated diabetic group supports this conclusion.
Uptake, is dependent on the Na⁺ gradient produced by Na⁺K⁺ ATPase activity (Iversen, 1974). Amino acid uptake by cells is also dependent on a Na⁺ gradient maintained by Na⁺K⁺ ATPase activity (Schultz et al., 1966). This process has been found to be depressed in diabetic peripheral nerve, an effect which is prevented by aldose reductase inhibition or myo-inositol supplements (Greene & Lattimer, 1985) both of which have been found to prevent diabetes induced decreases in Na⁺K⁺ ATPase activity.

Decreases in the latter could, therefore, underlie the apparent depression of Uptake, observed in this study. Impaired uptake, would also be present if sympathetic nerve numbers were reduced in neuropathy, however, this is not consistent with the similar basal uptake of NA in diabetic compared to control tissues; also it is not consistent with unchanged responses to NA in 6 weeks ponalrestat treated diabetic rats. The effect of an aldose reductase inhibitor could be due to its prevention of Na⁺K⁺ ATPase activity depression.

The hypothyroidism hypothesis may be important since T₃ treatment also produced a significant increase in stimulated ³H-NA release when the experiment was repeated with an extra T₃ treated group. We have demonstrated that T₃ treatment is effective in elevating Na⁺K⁺ ATPase activity in skeletal muscle in 6 weeks diabetic rats. Therefore, the potentiation by hypothyroidism of noradrenaline turnover could be due to decreased Uptake, caused by reduced Na⁺K⁺ ATPase activity. The effectiveness of T₃ in increasing stimulated ³H-NA release could be that it is normalizing Na⁺K⁺ ATPase activity and so restoring Uptake.

The gradients of the regression lines, see appendix, were similar for each of the three groups of tissues. This may mean that basal ³H-NA turnover rates were not altered by diabetes or by the administration of
an aldose reductase inhibitor. The gradient may have been affected by the presence of desipramine at 2hr and by the presence of both desipramine and yohimbine at 2.5hr. However blockade of uptake, or of presynaptic \( \alpha_2 \) receptors per se is unlikely to have affected basal \(^3\)H release to any great extent since most of the latter probably represented \(^3\)H-NA metabolites rather than intact \(^3\)H-NAD. Marshall (1983) found only about 7% of basal \(^3\)H release from mouse vas deferens to represent intact \(^3\)H-NA. In contrast, during stimulation a figure of 35% was obtained. Langer (1970) reported 50% of \(^3\)H released from stimulated rat vas deferens being in the form of \(^3\)H-NA while Farnebo (1971) reported a figure of 80-90% using stimulated rat irises. Consequently any impairment of uptake, although unlikely to affect basal \(^3\)H release from isolated tissues loaded with \(^3\)H-NA, is likely to increase NA turnover during stimulation e.g. by normal sympathetic activity in vivo. A polyol pathway dependent depression of uptake, may, therefore, explain, at least in part, the increased in-vivo noradrenaline turnover in diabetic rats and its prevention by aldose reductase inhibition which were observed recently in our laboratory (Lucas & Qirbi, 1989).

\( \alpha_2 \) adrenoceptor mediated contractions of isolated rat aorta have been reported to be enhanced by diabetes (Scarborough & Carrier, 1984) while \( \alpha_2 \) receptor numbers on platelets from human diabetics have been reported to be decreased (Abraham et al, 1986). Noradrenaline, released by nerve stimulation is believed to inhibit further noradrenaline release via \( \alpha_2 \) receptors located on the sympathetic nerve terminals (Langer, 1981; Marshall, 1983). The present study found the potentiation by \( \alpha_2 \) blockade with yohimbine of \(^3\)H-NA release to be very similar in the three groups of tissues initially studied. In the repeat experiment,
whilst the sole effect of desipramine was confirmed, the mean stimulated release of \(^{3}H-NA\) in presence of both desipramine and yohimbine was lower in the diabetic group and not so low in the ARI and T\(_3\) treated groups; however these differences were not significant. The concentration of yohimbine used (10\(^{-8}\)M), it has an EC\(_{50}\) of less than 10\(^{-7}\), is likely to result in almost complete blockade of \(\alpha\) receptors. It seems that \(\alpha\) mediated inhibition of \(^{3}H-NA\) release by the sympathetic neurones of this tissue is unchanged by diabetes, and by ARI or T\(_3\) administration in the present study.

The implication of Na\(^{+}\)-K\(^{-}\) ATPase activity in both changes in sensitivity to NA and overflow and stimulated release of \(^{3}H-NA\) strongly suggests that its activity should be measured in neurones. However, though highly desirable, this was not practical since neuronal Na\(^{+}\)-K\(^{-}\) ATPase activity would be swamped by activity in smooth muscle because of the relative amounts of each tissue type in the anococygeus. Instead we chose to initially evaluate Na\(^{+}\)-K\(^{-}\) ATPase activity in other tissues, the diaphragm, and then in the superior cervical ganglion, the heart and the sciatic nerve. The results are discussed elsewhere.

It would appear that any anococygeus muscle changes occurring in the 6 months diabetic rats are not maintained, since they are not evident in the 1 year diabetic animals. The role of the polyol pathway and T\(_3\) is not significant in this tissue as treatment with MI, ponalrestat and T\(_3\) failed to reverse the changes occurring in the 6 weeks study.

There is no evidence of neuropathy occurring in this tissue after 6 weeks, 6 months and 1 year of experimental diabetes. Further work on \(^{3}H-NA\) uptake and release in 6 months diabetic rats by a colleague failed to find any changes. Any changes in the rat anococygeus muscle appear to be transient; they do not indicate diabetic neuropathy.

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4.4 VAS DEFERENS

The vas deferens of streptozotocin-diabetic rats were assessed for changes caused by diabetes. Changes in sensitivity to noradrenaline were evaluated after two periods of diabetes, 6 months and 1 year.

In the 6 months diabetic group there was no change in sensitivity to noradrenaline, the mean EC50 ranges were increased but not significantly so. This concurred with previous studies (Tomlinson & Yousuf, 1983) which found no changes in responses to sensitivity to noradrenaline and noradrenergic nerve stimulation in vas deferens from 7-8 months alloxan-induced diabetic rats. Similarly vas deferens from 5-5½ months genetically diabetic mice showed no change in sensitivity to noradrenaline and nerve stimulation when compared to age-matched controls (Wilson et al, 1982). The Emax's for the 6 months diabetic group were increased (p<.05) and myo-inositol was effective in normalizing them. There appears to be no obvious mechanism to explain why this normalization occurred. Most likely the diabetic vas deferens was, like the anococcygeous muscle, closer to the optimum tension than the control animals, however further experiments were not carried to validate this contention.

In the 1 year diabetic animals there was no change in sensitivity to noradrenaline, and Emax's for all three groups were unchanged.

It would appear there is no evidence to suggest any neuropathy is occurring in the rat vas deferens. Any changes, i.e. increased Emax for the 6 month diabetic group, are transient smooth muscle changes which are not present in the 1 year animals. This suggests that adrenergic denervation is not occurring in this tissue, even after 1 year, and it would not appear, therefore, to be a valid experimental model of diabetic neuropathy.

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4.5 PARASYMPATHETIC SYSTEM

Dysfunction of the autonomic nervous system in human diabetes leads to changes in the gastrointestinal tract. These alterations manifest themselves by reduced peristalsis and dilation of the oesophagus, delayed gastric emptying, disordered small intestine movement, clonic atony and delayed small intestinal transit (Clarke et al., 1979; Nelson et al., 1976; Schmidt et al., 1981; Scott & Ellis, 1980; Schmidt & Plurad, 1986; Schmidt & Scharp, 1982; Schmidt et al., 1983). Specific studies have shown adrenergic and cholinergic denervation of the colon (Schmidt et al., 1981), degenerative changes in VIPergic nerves in the ileum of the experimental diabetic rat (Loesch et al., 1986), together with reduced responses to cholinergic nerve stimulation in rat terminal ileum longitudinal muscle.

We attempted to evaluate these cholinergic changes in two innervated smooth muscle preparations, the terminal ileum longitudinal muscle and the oesophageal muscularis mucosa. Since the sorbitol/polyol pathway has been implicated in diabetic neuropathy, as outlined above, we studied the effects of short (6 weeks) and medium term (6 months) and long term (1 year) diabetes, in the case of the oesophagus, with or without treatment with an aldose reductase inhibitor, ponalrestat.

Previous studies of responses to transmural stimulation have supported the contention that diabetes causes a depression of cholinergic nerve function (Schmidt et al., 1981), though this study did not, however, examine responses to exogenous acetylcholine. The present study found responses to exogenous acetylcholine (ACh) in the terminal ileum longitudinal muscle to be depressed, to at least the same extent as were responses to nerve stimulation, in 6 weeks diabetic rats. These results were corroborated by the finding of a reduced sensitivity to ACh in
6 months diabetic rat. These results suggest that stimulated ACh release from nerves was not affected, but responses to released ACh may well have been reduced by diabetes.

If degeneration of the cholinergic nervous function was occurring then one would expect responses to electrical stimulation of cholinergic nerves to be depressed to a greater extent than those to exogenous ACh. Sensitivity to the latter is actually likely to be increased by compensatory mechanisms, e.g. increase in receptor number in response to reduced ACh release by cholinergic neurons.

Sensitivity should have been increased, rather than decreased. The reduction in sensitivity suggests that the depression of responses were due to post-synaptic changes rather than to depression of cholinergic innervation. Therefore the reduced responses to stimulation of the terminal ileum longitudinal muscle is unlikely to be an appropriate model of human diabetic neuropathy.

The post-synaptic changes, depression of smooth muscle responsiveness, may be significant; since if they are paralleled in human intestinal smooth muscle they would explain the reduced tone reported by some studies (Clarke et al, 1979). The mechanism of the reduction in responses of the terminal ileum longitudinal muscle to ACh is not revealed by the present study. Reduced sensitivity to cholinergic agonists has been previously observed in cardiac tissue similarly diabetic animals (Vadlamundi & McNeill 1983; Kofo-Abeyomi & Lucas, 1988). Binding tissues using muscarinic receptor ligand ³H-quinuclidinyl benzilate have revealed reductions in myocardial muscarinic receptor numbers in such animals (Kofo-Abeyomi & Lucas, 1987; Carrier & Aronstam, 1987). The pattern of changes in atrial cholinergic function may not be the same as that observed here in the terminal ileum.
longitudinal muscle, however, since untreated diabetes appeared to increase atrial responses to transmural cholinergic stimulation (Kofo-Abeyomi & Lucas, 1988).

The terminal ileum from the ponalrestat treated diabetic group produced mean responses to both transmural nerve stimulation and exogenous ACh which were significantly greater than those of preparations from the untreated diabetic group (Lucas & Sardar, 1991). It would appear that ponalrestat enhanced responses via its inhibition of sorbitol synthesis. The possibility that ponalrestat restored terminal ileum function by a mechanism independent of diabetes was investigated by examining the effects of ponalrestat in non-diabetic age-matched rats (Lucas & Sardar, 1991). No elevation of responses was seen suggesting that ponalrestat enhanced responses via its inhibition of sorbitol synthesis since the latter only occurs to excess in diabetic animals. The effect of ponalrestat in preventing a depression of responses of terminal ileum longitudinal muscle preparations to ACh may have been temporary since, in the six months study, such responses remained depressed and although $E_{max}$ values were slightly higher than in the untreated diabetic group this difference was not significant.

In contrast to the results obtained using the terminal ileum longitudinal muscle, responses of the oesophagus muscularis mucosa preparations to transmural nerve stimulation and to exogenous ACh were not found to be changed by either diabetes or by an ARI. Variations in diabetes induced changes between different parts of the digestive tract have been observed previously (Schmidt et al, 1983; Nowak et al, 1986; Lincoln et al, 1984; Belai et al, 1991) although the reasons for such variations are not clear.

The failure to demonstrate cholinergic neuropathy after the relatively
short 30 day period of diabetes is consistent with several previous neurochemical and histological studies. Lincoln et al (1984) found the activities of the cholinergic nerve marker choline acetyltransferase to be increased rather than decreased in the ileum after eight weeks of streptozotocin-induced diabetes. Schmidt et al (1983) observed abnormalities in mesenteric axons after six or more months of streptozotocin-induced diabetes in the rat but not after three months. Similarly, Maltingly and Fisher (1985) observed axon dwindling in the common peroneal nerve after six or twelve months of such diabetes but not after four. The observation in this study of continued depression of responses to ACh after six months and 1 year of streptozotocin-induced diabetes with no evidence of decreased EC-50 values (in fact mean EC-50 values were increased) suggests that cholinergic denervation may not occur in these two tissues even after this longer period. They do not appear, therefore, to offer appropriate experimental models of diabetic neuropathy.

4.6 OUABAIN-SENSITIVE SODIUM-POTASSIUM ATPase & Ta LEVELS

The polyol/sorbitol pathway hypothesis proposes that increased flux through the pathway leads to reduced motor nerve conduction velocity, reduced levels of myo-inositol and a decrease in Na⁺K⁺ ATPase activity (Greene et al, 1985; Greene & Lattimer, 1983). Reduced concentrations of myo-inositol lead to restricted metabolic synthesis and turnover of phosphoinositides (PI) and reduced catabolites of PI, diacylglycerol and inositol-1,4,5-triphosphate, which stimulate Na⁺K⁺ ATPase and protein kinase C activity (Simmons et al, 1982; Berridge, 1984; Greene & Lattimer, 1986).

Administration of an aldose reductase inhibitor (ARI) or myo-inositol prevents myo-inositol depletion, preserves normal Na⁺K⁺ ATPase activity
and normalizes nerve conduction velocity in peripheral nerve (Greene & Lattimer, 1983; Tomlinson et al, 1982; Finegold et al, 1983; Gillon et al, 1983; Tomlinson et al, 1984; Greene et al, 1975; Mayer & Tomlinson, 1983). Correlation between reduced membrane phospholipids and Na+K+ ATPase activity is however not verified by recent data. Researchers found reduced Na+K+ ATPase activity concurrent with increased membrane phospholipid content and Ca²+ ATPase activity in humans suffering from diabetes mellitus (Mazzati et al, 1990). In addition reduced motor nerve conduction velocity has been shown to coexist with increased Na+K+ ATPase activity in galactose fed mice (Calcutt et al, 1990). Phosphoinositide turnover in postsynaptic ganglion cells is activated by a range of stimulants, electrical, K+ and muscarinic cholinergic or α-adrenergic agonists, and confirms PI's role as a secondary messenger (Nagata et al, 1973; Bone et al, 1984; Bone & Mitchell, 1985; Heilbron E., 1985; Nishizuka Y., 1984; Burt & Lartabee, 1976; Majerus et al, 1985). In diabetes reduced ganglionic XI levels may hinder synaptic transmission by affecting PI-mediated postsynaptic muscarinic signal transduction. Reduced Na+K+ ATPase activity, responsible for maintaining the transmembrane ion gradient, in diabetic ganglia may interfere with neuronal transmission by affecting neuronal repolarization. Therefore, alterations in PI metabolism and Na+K+ ATPase activity in autonomic ganglia in diabetes may directly contribute to autonomic dysfunction. Also the transmembrane ion gradient is intimately linked to transport eg. amino acids, any reduction in the gradient would adversely affect transport of essential components and may lead to the development of neuropathy (Calcutt et al, 1988).

Slowed motor nerve conduction velocity, reduced nerve XI content and reduced Na+K+ ATPase activity are all normalised by treatment with an
ARI or MI (Greene & Lattimer, 1983; Greene et al, 1975; Mayer & Tomlinson, 1983), but restoration of Na⁺ K⁺ ATPase activity by ARI's has not always been found (Lambourne et al, 1988). However sciatic nerves of genetically diabetic mice do not show a deficit in ouabain-sensitive Na⁺ K⁺ ATPase activity, or increased sorbitol and fructose content or reduced NAD content, even though there is reduced motor nerve conduction velocity (Whiteley & Tomlinson, 1985; Calcutt et al, 1988; Bianchi et al, 1987).

These findings have suggested that the polyol pathway is not the central mechanism by which diabetes reduces Na⁺K⁺ ATPase activity and nerve conduction velocity.

An alternative hypothesis suggests that hypothyroidism is responsible for the neuropathy prevalent in diabetes. Diabetic rats are hypothyroid, a condition which has been shown to depress Na⁺ K⁺ ATPase activity (Ganguly et al, 1987; Norgaard et al, 1987). We examined the roles of both hypothyroidism and the polyol pathway in skeletal (diaphragm) and cardiac muscle, superior cervical ganglion and sciatic nerve.

We found significantly reduced T₃ plasma levels in 6 weeks streptozotocin-induced diabetic rats (p<.01). These were corrected by T₃ administration but not by treatment with ponasterat. These reduced T₃ plasma levels concurred with previous studies which showed reduced T₃ levels in 2, 4, and 8 weeks streptozotocin-induced diabetic rats (Akiyama et al, 1989), and neither T₃ or ponasterat treatment was found to affect blood glucose concentrations.

Na⁺K⁺ ATPase activity was significantly reduced in the diaphragm from the untreated diabetic animals (p<.01). It was normalized by treatment with either T₃ or ponasterat. The same pattern was observed in the superior cervical ganglion where reduced Na⁺K⁺ ATPase activity in the
untreated diabetic animals was corrected by treatment with an ARI or $T_3$. This data agreed with the results of a previous study which found that reduced $Na^+ K^+$ ATPase activity and $M_1$ levels, in the superior cervical ganglion from 8 weeks streptozotocin-induced diabetic rats, were normalized by the administration of an ARI, sorbinil (Greene & Mackway, 1986). These results suggest that the $Na^+ K^+$ ATPase depression observed in the diaphragm and in the superior cervical ganglion was, at least in part, due to hypothyroidism.

We found depressed $Na^+ K^+$ ATPase activity in cardiac muscle from untreated diabetic rats ($p<.05$) which was corrected but not normalized by treatment with either ponalrestat or $T_3$. Since $\alpha$-adrenoreceptor density is reduced in the hypothyroid rat (Limas & Limas, 1987), a feature of the diabetic state, this may affect PI turnover which would lead to a decrease in $Na^+ K^+$ ATPase activity; as shown in our data. However neither ponalrestat or $T_3$ administered singly adjusted this dysfunction. It could be that both are partially responsible rather than wholly for the dysfunction occurring in the diabetic condition. Mean plasma $T_3$ levels of the diabetic treated with $T_3$ group were still lower that the control groups. However, plasma $T_3$ levels may not be an appropriate indicator of hypothyroidism in diabetes since sensitivity to $T_3$ has been reported to be depressed in diabetic patients (Bagchi, 1982; Cantanni et al, 1988). This implies that hypothyroidism might, in effect, be more prevalent and more severe in the diabetic population than plasma $T_3$ measurements would suggest.

The effectiveness of ARI treatment has been questioned by a recent study which examined $Na^+ K^+$ ATPase transport activity in lens from 2 weeks streptozotocin-induced diabetic rats and the effect of ARI (sorbinil) treatment. They suggested that restoration of lenticular $Na^+ K^+$ ATPase
activity is not secondary to a normalization of XI levels and may provide evidence that the two parameters are not strictly associated in diabetic tissues (Yeh et al, 1987). A recent study has found further evidence by examining XI levels, motor nerve conduction velocity and Na⁺K⁺ ATPase activity in sciatic nerve from 4 weeks galactose-fed mice (Calcutt et al, 1990). They suggested that exaggerated flux through the polyol pathway can cause a motor nerve conduction velocity deficit that is unrelated to either XI levels or Na⁺K⁺ ATPase activity (Calcutt et al, 1990). Previously they had suggested that motor nerve conduction nerve velocity was not linked to Na⁺K⁺ ATPase activity. Ganglioside treatment was effective in normalizing reduced Na⁺K⁺ ATPase activity whilst failing to restore reduced motor nerve conduction velocity (Calcutt et al, 1988).

Our data of mean increased, but not significantly so, Na⁺K⁺ ATPase activity in diabetic rat sciatic nerve seemed to support this suggestion, but clashed with previous work which found reduced Na⁺K⁺ ATPase activity in the diabetic rat sciatic nerve, however, it should be noted this was not a valid comparison since there were insufficient number of animals in our group for proper statistical analysis (Greene et al, 1985; Greene & Lattimer, 1983). Treatment with ponalrestat and T₃ reduced this excess Na⁺K⁺ ATPase activity.

The existence of XI depletion in the nerves of humans with diabetes mellitus is contentious (Hale et al, 1987; Dyck et al, 1988) and its significance in experimental animal diabetic neuropathy may not be reflected in human neuropathy.

T₃ treatment is effective in normalizing Na⁺K⁺ ATPase activity in skeletal muscle (diaphragm) and nervous tissue (superior cervical ganglion) and may be linked. Iodide ions are actively taken up to
synthesize T₃ and T₄, it may be that an initial diabetes-induced derangement of Na⁺-K⁺ ATPase activity reduces the Na⁺ gradient and thus inhibits the uptake of iodide ions. This would lead to reduced T₃ and T₄ levels and the resulting hypothyroid state would reduce α-adrenoceptor density (Limas & Limas, 1987), and may affect phosphatidylinositol turnover which would lead to a decrease in Na⁺-K⁺ ATPase activity. Thus a self-reinforcing cycle, of reduced Na⁺-K⁺ ATPase activity leading to hypothyroidism which further reduced Na⁺-K⁺ ATPase activity, would be established. In which case joint treatment with ponasterat and T₃ would be worth investigating, especially in the heart which responded partially to each treatment. This, however, begs the question as to what initially causes Na⁺-K⁺ ATPase activity depression in the diabetic state. Only when this is established will it be possible to develop effective treatment.

It may be deficiencies in the anterograde axonal transport, with a reduction in the transport of critical molecules to distal axons, which leads to axon shrinkage; this supports the above proposal since Na⁺-K⁺ ATPase depression has been suggested to be linked to decreased axonal transport (Calcutt et al, 1988; Tomlinson & Mayer, 1984; Hoffman et al, 1987). Studies have shown reduced substance P axonal transport in 3 weeks streptozotocin-induced diabetic rats (Tomlinson et al, 1988). More selective effects on conduction could arise from deficient delivery of voltage-sensitive Na⁺ channels; because the latter are carried by fast anterograde axon transport (Tomlinson et al., 1988). Anything that impairs Na⁺ channel delivery may have a significant effect on nerve conduction velocity.

Impaired axonal transport could contribute to eventual neuropathy by many mechanisms i.e. if one or more of any number of vital cellular
components were not supplied to the axon and nerve endings at a rate sufficient to prevent depletion then neuropathy could follow. This may be particularly important in neurones with longer axons and could explain why neuropathy is often detected in the feet (Mackury et al, 1994). A requirement for long axons for vulnerability to diabetic neuropathy could, of course, mean that small laboratory animals, such as the rat, may be relatively resistant to such damage.

It is apparent from the data that the central mechanism responsible for axon dysfunction remains to be elucidated and that M1 levels may be less significant whilst conversely T3 levels maybe more significant than previously realised.

4.7 CONCLUSION

In all the tissues that we examined, in our attempt to demonstrate the streptozotocin-induced diabetic rat as a model for human diabetic neuropathy, we were unable to show sustained and conclusive evidence that neuropathy had occurred or was occurring. In tissues innervated by the cholinergic and tissues innervated by the adrenergic system neuropathy was not evident. The tissues used, the anococcygeus muscle, the oesophagus, the terminal ileum longitudinal muscle, sciatic nerve, vas deferens, superior cervical ganglion, the heart and the diaphragm all failed to show distinct evidence of neuropathy. The different periods of diabetes used also failed to provoke the neuropathy we were searching for.

The results from the 1 year diabetic rats were particularly disappointing, since this length of time represented a considerable proportion (about 40%) of the life-expectancy of the rat, and so could
be considered, in this respect, equivalent to long-term human diabetes. The mortality rate among this group was high and it may be that there were rats in this group that were vulnerable to nervous/vascular damage by diabetes but that these were among those rats that died before testing.

Several changes were observed in the diabetic rats, the reduction in the uptake of \(^{3}H\)-NAD and the changes in smooth muscle, though these were not proof of neuropathy. Indeed certain changes between the six week and 1 year diabetic rats, as is discussed elsewhere, may have been due to ageing. Differences between age-matched control and diabetic groups were not due to ageing, but defects caused by diabetes which were not indicative of neuropathy. The relevance of these changes to human diabetic neuropathy may be marginal, and it could be that these changes are peculiar to the diabetic rat.

Our failure to demonstrate neuropathy meant that the role of the polyol pathway in diabetes could not be properly evaluated. It may be that the polyol pathway is a required element in the development of human diabetic neuropathy, though whether it is THE element remains doubtful. The normalization of Na\(^{+}\)K\(^{-}\) ATPase activity in diabetic rats treated with T\(_{3}\) is strong evidence that hypothyroidism may contribute to it's depression by diabetes. Further work on this aspect of diabetes could prove fruitful. However, the link between reduced Na\(^{+}\)K\(^{-}\) ATPase activity and neuropathy, itself, remains speculative.

Testing of the hypothyroidism hypothesis is now essential and data generating from this may provide a crucial insight into human diabetic neuropathy. Also, it may be, that no one mechanism is responsible for the varied dysfunctions found in human diabetes and it will only be through the correction of several that the cure will be found.
The development of an effective model of neuropathy still remains a high priority. It will be only through this that the biochemical and physiological basis for neuropathy will be understood. An appropriate model also allows the evaluation of the different treatments suggested for diabetic neuropathy. As described, it is very difficult to assess the etiology of neuropathy or the efficacy of treatments/preventative measures in human diabetes. It is ethically questionable to treat one set of diabetics whilst allowing another to develop the symptoms of diabetic neuropathy, or, alternatively, to subject patients to side effects of treatments for conditions which many would not, in any case, develop.

There still remains much further work to be carried out in this field before a proper understanding of diabetic neuropathy is achieved. It is possible that the period of diabetes which we used in the streptozotocin-induced rat was insufficient to properly mimic the defects occurring in human diabetes. A longer period of diabetes in the rat may show the development of the defects that we were searching for. However, as previously noted, it may be that the rats that do survive the ravages of diabetes are peculiarly resistant to neuropathy, as is the case in some long-term human diabetics.

In human diabetes various tissues are differently affected due to diabetes and it could be that the same situation is prevalent in the diabetic rat. It could be that the tissues we examined for signs of neuropathy were not susceptible to diabetic dysfunction. We used tissues that we thought were relevant to human diabetes, such as the aorta, oesophagus, heart etc., and it may be that neuropathy is occurring in other tissues. Further research could be conducted into a new set of tissue to establish whether diabetic neuropathy is occurring. However,
if tissues are used from the diabetic rat which do not suffer dysfunction in human diabetics then the problem of interpretation arises. It is questionable as to how relevant would it be to establish neuropathy in tissues from diabetic rats which have no direct correlation with the dysfunctions occurring in human diabetes? It may well be that the diabetic rat is only susceptible to early changes which it then corrects and that it is unusually resistant to diabetic neuropathy. In this light it would seem that this species offers little opportunity to evaluate diabetic neuropathy. The rat is relatively resistant to atherosclerosis and if this underlies diabetic neuropathy, as has been suggested (Low et al, 198), then this might explain the rats resistance to diabetic neuropathy.

It may be possible that the defects that are absent in the diabetic rat are present in other species. Since different patients vary considerably in their vulnerability to diabetic neuropathy it would not be surprising if species also differed in this respect. Whilst other species have been used, such as rabbit, mice and genetically-diabetic Chinese hamsters, the predominant work has been carried out on the rat. Following our efforts to show neuropathy in the rat, it could be that another species would provide more fruitful data. Further work should be carried out on different species to evaluate their effectiveness as models of diabetic neuropathy. Some researchers have already moved on from the diabetic rat to diabetic mice to evaluate neuropathy and this trend should be developed; together with other species. A recent study in alloxan-diabetic rabbits reported sympathetic denervation in the carotid artery (Tesfamaria et al, 1990). This species is also more vulnerable to atherosclerosis, induced by a high cholesterol diet, than are rats (Mauriello et al, 1991). This might therefore provide a more appropriate
model for future study despite posing some practical disadvantages, such as greater size regarding housing and higher mortality rate after induction of diabetes.

Diabetes, whilst causing no direct excessive discomfort to the patient, has an insidious effect on other components. The interplay of different factors is well known, however, researchers only examine diabetes in isolation. It could be that additional risk factors need to be considered so that diabetic neuropathy is effectively evaluated. It is known that hypertension together with diabetes leads to vasculopathy, and it may well be that vasculopathy is the pivotal step that leads to neuropathy, though a direct correlation between hypertension and neuropathy has not been reported. The interplay of different risk factors needs to be investigated thoroughly and it could be that this finally leads to an effective model of diabetic neuropathy and greater understanding of its etiology and means of prevention.
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CHAPTER SIX
6.1 STATISTICAL ANALYSIS

The significance of difference between groups for the results analysis were assessed initially by one-way analysis of variance and, where p values less than 0.05 were obtained, individual groups were compared by Duncans multiple comparisons (Bruning & Kintz, 1968).

6.1a Duncans Multiple Comparisons Tests

Step 1: The mean score for each group is obtained

\[
\text{Mean of group 1} = \frac{\text{Sum of group 1}}{n}
\]

when \( n \) is the number in the group

Step 2: The standard error of the means is derived by the following formula

\[
\text{s.e.} = \sqrt{\frac{\text{MS within}}{n}}
\]

when \( \text{MS within} \) is the means within a group.

If however the numbers of cases in some groups are unequal then the harmonic mean, \( \bar{n} \), of the numbers is obtained, but only if the numbers between groups are not too disparate. If there are not more than twice as many in the largest group as in the smallest group then it is permissible to use the harmonic mean, \( \bar{n} \), where

\[
\bar{n} = \frac{\text{numbers of groups}}{1/n_1 + 1/n_2 + \ldots + 1/n_l}
\]

when \( n_1, n_2, n_3 \) etc. are the numbers per group in the different groups.

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Step 3: The significant studentized ranges are obtained from the literature. The literature tables have values for different degrees of freedom, df, for the mean square within groups; whilst the exact df may not be listed the next closest df suffices for our purpose. Once the appropriate df column has been assigned then the different ranges of means (k) to be compared are obtained.

Step 4: Each significant range obtained from the literature table is then multiplied by the s.e. obtained in step 2. This results in the minimum mean differences (R) for the given ranges of comparisons.

Step 5: The means obtained are initially ranked from the smallest to the largest so that the differences between the various means can be tested for significance. If the difference between the means, obtained in step 1, is larger than the minimum mean difference, obtained in step 4, for that range then this is considered to be significant.

6.2 BASIS FOR TRIODOTHYRONINE ASSAY

There are several commercially available test kits, employing a variety of principles. The kit used in this study utilised the "competition" principle.

Tubes were supplied which had a specific antibody coated on to the tube wall. Initially an immunological reaction was carried out by placing the serum in the tube together with enzyme labelled antigen. Both antigens, the enzyme labelled and the serum antigens, reacted with the antibody on the tube wall. After a short time the mixture was emptied out and the tube washed several times with buffer solutions (see figure below).
In the next step an indicator reaction took place which produced the colour change which was measured in a spectrometer. To the tube, which had been previously eluted, substrate chromogen was added leading to a colour change. The greater the binding by the enzyme labelled antigen the lower the concentration of serum antigen.

Indicator Reaction

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6.3 Basal Overflow of $^3$H-NA from Anococcygeus Muscle

Basal $^3$H Overflow
($\equiv$ pmol $^3$H-NA /g/min)

Duration of superfusion (h)

Basal overflow of $^3$H expressed as equivalent pmol $^3$H-NA /g from 6 weeks diabetic anococcygeus muscle. The results approximated to first order kinetics and are plotted logarithmically. The regression coefficients were calculated for each tissue ($n = 6$ for each group). Mean values ± s.d. which were used for the above plots were: controls $\bullet$, $-0.564 \pm 0.243$ h$^{-1}$, diabetic $\star$, $-0.467 \pm 0.203$ h$^{-1}$ and diabetic treated with ponalrestat $\star\star$, $-0.454 \pm 0.298$ h$^{-1}$. Difference between these means were not significant.