Regulation of Insulin Gene Expression in Rat Islets of Langerhans

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ABSTRACT

The rate of glucose stimulated *in vitro* (pro)insulin synthesis in islets of Langerhans isolated from fed male rats was determined. Increases in the glucose concentration of the incubation medium, over the physiological range (2 to 20 mM), stimulated the rates of both (pro)insulin and total protein synthesis during a two hour incubation. This stimulation was preferential for (pro)insulin synthesis and had a sigmoidal dose response curve with a concentration threshold of between 2.5 mM and 5.0 mM and a maximal rate at 20 mM glucose. Inhibition of islet RNA synthesis by actinomycin D depressed the rate of total protein synthesis within 30 minutes of the application of a 20 mM glucose stimulus. A specific inhibition of (pro)insulin synthesis by actinomycin D, that occured 60 minutes after the application of a 20 mM glucose stimulus, was thought to reflect the inhibition of glucose stimulated preproinsulin mRNA synthesis.

The effect of glucose on islet preproinsulin mRNA content during *in vitro* incubations was also determined. To achieve this a dot blot hybridization assay for preproinsulin mRNA was developed which used a ^{32}P-labeled cloned human insulin gene as the hybridization probe. The assay proved to be sensitive enough to detect preproinsulin mRNA in as few as fifty islets. Incubation of islets, for 2 hours, at 2.5 mM glucose had no effect on islet preproinsulin mRNA content but incubation at 20 mM glucose increased islet preproinsulin mRNA content. Actinomycin D had no effect on this pattern of glucose stimulation and it is suggested that the degradation of preproinsulin mRNA is in some way dependent on RNA synthesis and that its inhibition by glucose plays an important role in the regulation of islet preproinsulin mRNA content.

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ABBREVIATIONS

^{3}H-leucine	: L-[4,5-^{3}H]-leucine
AIS-Sepharose	: anti insulin serum Sepharose 4B
NGPS-Sepharose	: normal guinea pig serum Sepharose 4B
bp	: base pairs
Kb	: kilobase pairs
cAMP	: cyclic adenosine-2',3'-monophosphoric acid
dbcAMP	: dibutyl cyclic adenosine-2',3'-monophosphoric acid
cDNA	: copy deoxyribonucleic acid
dATP	: 2'-deoxyadenosine-5'-triphosphate
dCTP	: 2'-deoxycytidine-5'-triphosphate
dGTP	: 2'-deoxyguanosine-5'-triphosphate
dTTP	: thymidine-5'-triphosphate
DEPC	: diethylpyrocarbonate
DNA	: deoxyribonucleic acid
DNAse I	: deoxyribonuclease I
EDTA	: diaminoethanetetra-acetic acid
HEPES	: N-2-hydoxyethylpiperazine-N'-2-ethanesulphonic acid
mRNA	: messenger ribonucleic acid
oligo(dT)cellulose	: oligo(2'-deoxythymidylic acid)cellulose
poly A^{+} RNA	: polyadenylated ribonucleic acid
poly A^{-} RNA	: non-polyadenylated ribonucleic acid
RNA	: ribonucleic acid
RNAse	: ribonuclease
TCA	: trichloroacetic acid
Tris-Cl	: tris(hydroxymethyl)aminomethane buffer
tRNA	: transfer ribonucleic acid

Chapter 1

General Introduction

Historical Survey

The first clinical description of the disease diabetes was made in the first century A.D. by the Roman physician Celsus. However many of its symptoms, such as polyuria (Ebers papyrus, Egypt ~1500 B.C.), had been recorded much earlier. The clinical description of diabetes improved with the recognition of further symptoms. However significant progress towards a treatment was not made until Dobson, in 1775, made the crucial observation that the sweetness of diabetic's urine was due to sugar. This led to the adoption of various diet regimens for the treatment of diabetics, none of which were particularly effective. An early example was the limited carbohydrate, high protein and fat diet prescribed by Rollo (~1800).

In 1869 Paul Langerhans described a previously unknown cell type of the pancreas. They occurred in 'heaps' or 'islands' situated between the acini and were not connected to the exocrine duct system. The embryology of these cells was studied by Laguesse who named them the 'Islets of Langerhans' and speculated that they might be the source of an 'internal secretion' (Laguesse 1894). Von Mering and Minkowski first implicated the pancreas in the control of the diabetic state with their classic experimental pancreatectomies of dogs inducing the rapid onset of diabetic symptoms (Von Mering & Minkowski 1889). This led to the suggestion, by De Meyer (1909), that the islets of Langerhans were the source of a 'hormone' (a term first used by Starling to describe 'internal secretions' (Starling 1905)) responsible for controlling the

diabetic state; he named this hormone 'insuline' after its proposed tissue of origin (De Meyer 1909). Early histological studies of the islets revealed two cell types, the α- and β-cells, named after their content of acidophilic and basophilic granules respectively (Lane 1907). It was suggested that these two cell types were indicative of a dual secretory function for the islets (Bensley 1914).

In the 1890's an extract of thyroid gland effective in the treatment of endemic goitre, cretinism and myxoedema was discovered. This encouraged the search for a similar extract of the pancreas able to control the diabetic state. Two of the most successful extracts, those of Zeulzer (1908) and Paulesco (1920), partially reduced glycosuria in pancreactectomized dogs but caused severe toxic side effects. Banting and Best (1922) were the first to develop a clinically successful pancreatic extract for the treatment of diabetes. This extract, which they called 'insulin', revolutionized the treatment of diabetes and has saved the lives of countless diabetics. This achievement was acknowledged by the award of the 1923 Nobel prize for medicine.

Soon after the discovery of insulin the dual secretory role postulated for the islets (Bensley 1914) was confirmed by the identification of a hyperglycemic factor, glucagon, in an aqueous extract of pancreas (Murlin et al 1923).

Islet Cellular Composition

Immunocytochemical studies have confirmed the β-cells as the site of insulin production (Lacy & Davies 1959) and the α-cells as the site of glucagon production (Baum et al 1962;

Okada et al 1968). There are two other cell types of the islets, the D-cells and the PP-cells, which have similarly been shown to be the sites of synthesis of somatostatin (Luft et al 1974) and pancreatic polypeptide (Larsson et al 1975) respectively.

The cellular composition of islets varies and they are classified by their location in either the duodenal portion (the head) or the splenic portion (the tail) of the pancreas. The β-cells are located in the core of the islet (Carpenter 1966) and are the most abundant cell type of both splenic and duodenal islets (~78% of the total cell mass). They are surrounded by an outer layer of varying amounts of the other three cell types (Orci et al 1976). The outer layer in splenic islets is comprised of α-cells, D-cells and PP-cells (28%, 4% and 2% of the total cells respectively) whilst in duodenal islets these proportions are different (2%, 5% and 20% respectively).

Techniques Used in the Study of Insulin

Initially bioassays were the only techniques available for the measurement of insulin. Many of these utilized the hypoglycemic action of insulin in a second animal connected either by cross-circulation (Zunz & La Barre 1927) or pancreatic transplantation (Houssay & Deulofeu 1939). An early refinement was the development of *in vitro* perfusion of rat pancreas in which insulin release was demonstrated by a bioassay utilizing the hypoglycemic action of insulin in alloxan-diabetic, adrenodemedullated, hypophysectomized rats (Anderson & Long 1948). One of the first *in vitro* assays for

insulin was based on its effect on the uptake of glucose by isolated rat diaphragms (Randle 1956). Once the antigenicity of insulin had been demonstrated (Arquilla & Stavitsky 1956a; Arquilla & Stavitsky 1956b; Moloney & Coval 1955; Berson et al 1956) and a method for labeling insulin with radioactive ^{125}iodine had been developed (Kallee 1952) the use of a radioimmunoassay for insulin became possible (Yalow & Berson 1960).

With the advent of such a reliable *in vitro* insulin assay the scope of insulin research was greatly enhanced and tissue preparations better than perfused rat pancreas (Anderson & Long 1948; Grodsky et al 1963) or rabbit pancreatic slices (Coore & Randle 1964) were necessary. The first major improvement in islet tissue preparation came with the freehand microdissection of the enlarged islets of obese hyperglycemic mice (Hellerström 1964). This produced small quantities of relatively pure islet tissue and the later development of collagenase digestion of chopped guinea pig pancreas dramatically increased the yield of islet tissue (Moskalewski 1965). The collagenase digestion method was applied to rat pancreas and with subsequent improvements formed the basis of all current islet isolation techniques. Two of these improvements were increasing the yield of islets by ductal distension of the pancreas prior to digestion (Lacy & Kostianovsky 1967) and the purification of islets from the digestate by Ficoll density gradient centrifugation (Lindall et al 1969) in place of hand-picking of the islets under microscopic control.

Structure of Insulin

Due to its widespread use in the treatment of diabetes insulin was the first protein to be produced in quantity to a high degree of purity and therefore was the logical choice for Sanger and his co-workers in their pioneering amino acid sequencing techniques. Thus bovine insulin was the first protein to have its amino acid sequence completely elucidated (Ryle et al 1955), an achievement which revolutionized molecular biology and earned Sanger a Nobel prize in 1958.

Insulin is a small protein (5700 daltons) composed of two polypeptide chains, the A- and B-chains, which contain 21 and 30 amino acid residues respectively. These chains are connected by two interchain disulphide bridges and there is a further intrachain disulphide bridge within the A-chain (Figure 1.1). The integrity of these bridges is essential for biological activity (Lens & Neutelings 1950; Wintersteiner 1933). The tertiary and quaternary structures of porcine insulin were determined by X-ray crystallography (Blundell et al 1971; Pullen et al 1976). Insulin is thought to be stored, in the secretory granules, in the form of three dimers of insulin combined with two atoms of zinc to form hexamers (Greider et al 1969).

Many other mammalian, avian and fish insulin sequences have been determined and comparison between species permits the identification of invariant residues possibly involved in the biological activity of insulin (Humbel et al 1972). A number of mutations in human insulin, producing impaired biological activity, have been identified, these mutations have been mapped to amino acid residues responsible for

receptor binding (Tager 1984)(Figure 1.2).

Most species have only one form of insulin, however, rats (Clark & Steiner 1969), mice (Bunzli et al 1972; Markussen 1971), tunafish and toadfish (Humbel et al 1972) have two distinct forms.

Figure 1.1

The Structure of Human Insulin

The amino acid sequence of the A- and B-chains are shown along with the positions of the intrachain and interchain disulphide bonds.

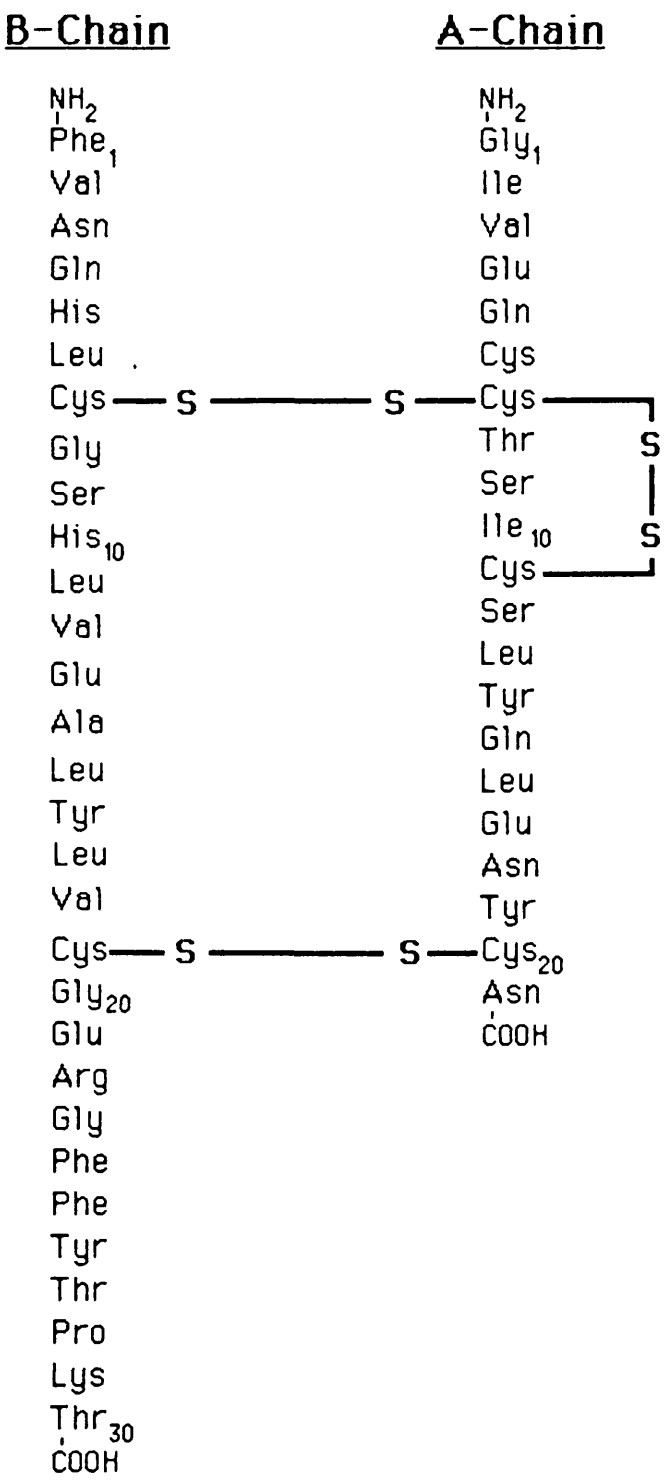

B-Chain
A-Chain
NH2
Phe1
Val
Asn
Gln
His
Leu
Cys
S
S
Cys
Gly
Ser
His10
Leu
Val
Glu
Ala
Leu
Tyr
Leu
Val
Cys
S
S
Cys20
Gly20
Glu
Arg
Gly
Phe
Phe
Tyr
Thr
Pro
Lys
Thr30
COOH
NH2
Gly1
Ile
Val
Glu
Gln
Cys
Cys
Thr
S
Ser
Ile10
S
Cys
Ser
Leu
Tyr
Gln
Leu
Glu
Asn
Tyr
Cys20
Asn
COOH

Figure 1.1

Techniques for the Study of Insulin Synthesis

Initial studies relied on the incorporation of radio-labeled amino acids (e.g. ^{35}S-methionine, ^{3}H-leucine and ^{3}H-phenylalanine) by pancreatic tissue into insulin to assay insulin biosynthetic rate (Pettinga & Rice 1952; Light & Simpson 1956; Vanghαm& Anfinsen 1954). This has remained the basic principal behind such studies ever since. The early use of bovine (Pettinga & Rice 1952; Light & Simpson 1956; Vanghαm & Anfinsen 1954) or foetal canine pancreas (Wagle 1965b) or the 'principal' islets, or Brockmann bodies (Brockmann 1846), of teleost fish (Bauer & Lazarow 1961; Humbel 1963; Humbell & Renold 1963; Humbel et al 1961) has been superseded by the use of rat pancreatic islets (Voelker et al 1962; Wagle 1963; Mallory et al 1964; Taylor et al 1964) and occasionally rat islet β-cell tumors (Steiner & Oyer 1967). Similarly the techniques for the purification of the radiolabeled insulin from such preparations has been improved from a combination of insulin fibril precipitation (Pettinga & Rice 1952), paper (Von Mering & Minkowski 1889) and gel electrophoresis (Clark & Steiner 1969) and gel filtration chromotography (Steiner et al 1967) to the currently favoured immunological precipitation techniques (Taylor et al 1962; Berne 1975a) which permit far quicker purifications in fewer steps.

Synthesis of Insulin

The discovery that insulin consists of two separate peptide chains brought into question its mechanism of

synthesis. The distribution of proline residue specific radioactivity in insulin labeled by *in vitro* radiolabeling experiments with Anglerfish islets supported the hypothesis that the A- and B-chains were synthesised separately *in vivo* (Humbel 1965). Yields of active insulin from recombination experiments between purified A- and B-chains were generally low (Dixon & Wardlow 1960; Du et al 1961). This led to the hypothesis that insulin was synthesised as a single peptide precursor, which enabled the correct peptide folding and disulphide bonding, which was later proteolytically cleaved to yield the mature form of insulin (Givol et al 1965). An early attempt to detect such an insulin precursor in bovine pancreas failed and it was concluded that if such a precursor existed it would be present at less than 10% of the quantity of insulin (Wang & Carpenter 1965). In order to detect such a small amount of precursor a source of tissue much richer in β-cells than bovine pancreas was required. A human β-cell adenoma was found that met this requirement. When used in ^{3}H-leucine and ^{3}H-phenylalanine radiolabeling studies the acid ethanol extracted fraction from this tissue contained two components, only one of which was insulin (Steiner & Oyer 1967). The other component was separated from insulin by gel filtration chromotography and was shown to be of a higher molecular weight (10,800 daltons) than insulin. It was precipitated by insulin antibodies and could be converted to insulin-like material by the action of trypsin. This putative precursor of insulin was named 'proinsulin' and was also found in small quantities in crystalline insulin. Pulse chase radiolabeling studies confirmed that proinsulin was indeed a true precursor of insulin (Steiner et al 1967). The primary structure of

proinsulin was subsequently determined by amino acid sequencing (Steiner et al 1968; Chance et al 1968; Nolan et al 1971; Steiner et al 1969).

Proinsulin is a single polypeptide chain (81 to 86 residues long and ~9000 daltons) consisting of the A and B-chains linked by a connecting peptide, or C-peptide, which is of variable length (31 residues in man, 26 in sheep and 23 in dogs (Humbel et al 1972))(Figure 1.2). Proinsulin has between 2 and 20% of the biological activity of insulin (Rubenstein & Steiner 1971) and the C-peptide is thought to direct the folding of the polypeptide chain to ensure the correct formation of the interchain disulphide bridges (Bromer 1970; Steiner & Clark 1968). Proinsulin is converted to insulin *in vivo* by the proteolytic removal of pairs of basic residues at either end of the C-peptide (Kemmler & Steiner 1970).

In pulse chase radiolabeling studies the incorporation of label into proinsulin was detectable within one to two minutes and the incorporation into insulin within 10 to 15 minutes. Incorporation of label into insulin occurred at a linear rate thereafter but the incorporation of label into proinsulin reached a plateau after several hours. The overall half-time for the conversion of proinsulin into insulin is approximately 30 minutes (Gold et al 1986). When the β-cell is stimulated to secrete insulin newly synthesised insulin does not appear in the medium until one hour later (Steiner 1967; Howell & Taylor 1967). Electron microscopic autoradiography has revealed that the pathway of newly synthesised insulin through the β-cell is from its site of synthesis at the rough endoplasmic reticulum through the Golgi apparatus to the mature secretory granules

(Howell et al 1969) where the insulin is stored awaiting its secretion. The conversion of proinsulin to insulin occurs in the Golgi and secretory granules (Clark 1969; Steiner et al 1970).

Cell free *in vitro* translation of polyadenylated RNA isolated from rat (Chan et al 1976), bovine (Lomedico & Saunders 1976) and fish islets (Permutt et al 1976) produced a protein that was some 2000-3000 daltons larger than proinsulin and yet was precipitated by insulin antibodies. Edman degradation and peptide mapping of this *in vitro* translated product confirmed that it was a precursor to proinsulin which had an additional 23 amino acid residues at its N-terminal end (Chan et al 1976). The discovery of this precursor, named preproinsulin, followed that of similar precursors for immunoglobulins (Milstein et al 1972). Preproinsulin has 23 to 30 additional N-terminal residues (varying between species) forming a hydrophobic 'leader' sequence to proinsulin (Chan et al 1976), a feature in common with many other secretory proteins (Blobel & Dobberstein 1975a; Kemper et al 1974; Chan et al 1976; Milstein et al 1972). These leader, or 'signal', sequences are believed to assist in the formation of the ribosome/membrane junction in rough endoplasmic reticulum, and thus permit the vectorial discharge of the nascent polypeptide into the cisternal space (Milstein et al 1972; Blobel & Dobberstein 1975a). Preproinsulin is difficult to detect in intact tissue (Schmeckpeper et al 1975; Sussman et al 1976) possibly due to rapid removal of the leader by a peptidase associated with rough microsomes, a view which is supported by studies with reconstituted systems (Blobel & Dobberstein 1975b; Boime et al 1977). Although generally thought to be

concurrent with translation there is evidence that this cleavage of the leader sequence may occur post-translationally (Eskridge & Shields 1983).

Figure 1.2

Structure of Human Proinsulin

The amino acid sequence of human proinsulin is shown with the positions of the intrachain and interchain disulphide bonds. Residues thought to be involved in insulin receptor binding are boxed and the pairs of basic residues proteolytically removed to convert proinsulin to insulin are in dotted boxes.

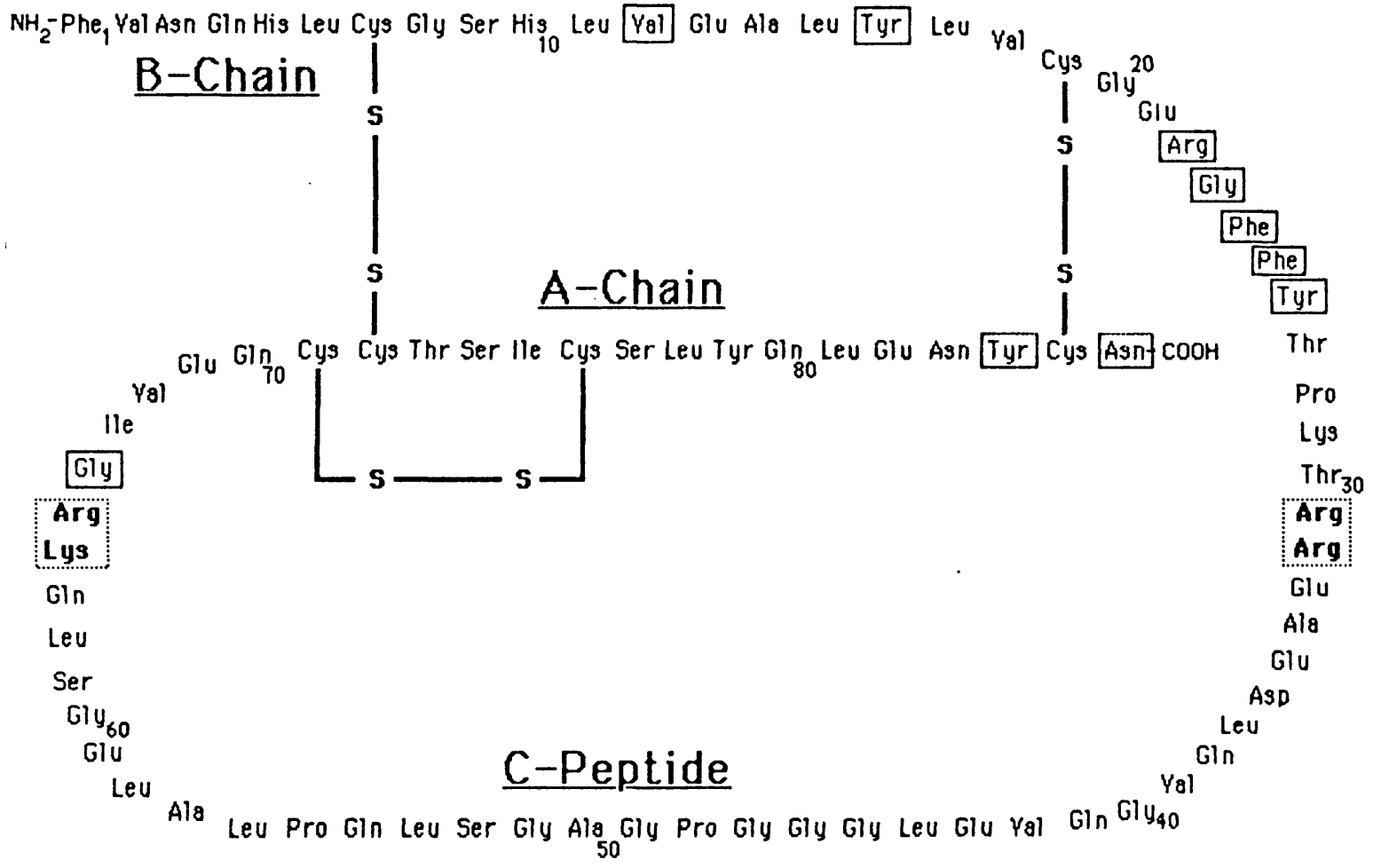

NH2-Phe1 Val Asn Gln His Leu Cys Gly Ser His10 Leu Val Glu Ala Leu Tyr Leu
B-Chain
S
S
S
S
A-Chain
Val Cys Gly20 Glu Arg Gly Phe Phe Tyr
Glu Gln70 Cys Cys Thr Ser Ile Cys Ser Leu Tyr Gln80 Leu Glu Asn Tyr Cys Asn-COOH
Thr
Pro
Lys
Thr30
Arg
Arg
Glu
Ala
Glu
Asp
Leu
Gln
Val
Gly40
Val
Ile
Gly
Arg
Lys
Gln
Leu
Ser
Gly60
Glu
Leu
Ala
C-Peptide
Leu Pro Gln Leu Ser Gly Ala50 Gly Pro Gly Gly Gly Leu Glu Val Gln

Figure 1.2

Structure of Insulin Genes

The preproinsulin gene and/or cDNA sequences are available for mammals [rat (Lomedico et al 1979), human (Bell et al 1980), Old World monkey, *Macaca fascicularis* (Wetekam et al 1982), guinea pig (Chan et al 1984), chinese hamster (Bell & Sanchez-Pescador 1984)], fishes [carp (Hahn et al 1983), salmon (Sorokin et al 1982), anglerfish (Hobart et al 1980), hagfish (Chan et al 1981)] and birds [chicken (Perler et al 1980)]. The gross structure of all these genes is similar. There are generally two introns, one large one interrupting the C-peptide coding sequence (499 bp and 786 bp in rat and human respectively) and a smaller one in the 5'-non-coding region (119 bp and 179 bp in rat and human respectively). The rat insulin I gene is the exception to this as it has only the small intron (Lomedico et al 1979) (Figure 1.3).

Evolutionary constraints are probably different for the various coding regions, thus the A and B-chains are highly conserved between species (except in the case of hystricomorph rodents such as guinea pig where different evolutionary pressures are thought to apply (Chan et al 1984)) whilst that of the C-peptide, due to its less crucial 'spacing' role in proinsulin, is not as highly conserved (Steiner 1978). Also the hydrophobic character, and certain other features, of the N-terminal signal peptide is conserved between species. The usual eukaryotic concensus sequences necessary for the transcription and processing of genes (e.g. the 'Hogness' box, 5'-capping site, 3'-polyadenylation signal) are all present (Bell et al 1980). Recombinant DNA experiments have defined other DNA sequences close to the insulin gene responsible for

its tissue specific expression. Initial studies showed that sequences up to 300 bp upstream of the rat I, human (Walker et al 1983) and rat II (Hanahan 1985) insulin genes were entirely sufficient to mediate tissue specific expression. This corresponded to a region of DNAse sensitivity situated between 250 bp and 300 bp upstream of the rat insulin II gene that was only observed in insulin expressing tissues (Crossley 1974). A 11 Kb DNA fragment containing the human insulin gene has been successfully used to produce transgenic mice which expressed human insulin in a tissue specific manner (Bucchini et al 1986). A similar study produced transgenic mice that not only expressed human insulin in a tissue specific manner but in which the serum level of human insulin responded appropriately to treatment with glucose, amino acids and tolbutamide (Selden et al 1986). Subsequent deletional experiments have defined a region between approximately 103 bp and 333 bp upstream of the rat insulin I gene as a tissue specific 'enhancer' element (Edlund et al 1985). In addition to this positive acting *trans* transcriptional element a *cis* acting negative transcriptional, or 'silencer', element has been reported to lie between 2 Kb and 4 Kb upstream of the gene (Nir et al 1986; Laimins et al 1986). It is thought likely that these two elements, the enhancer and the silencer, are together entirely responsible for the tissue specific and developmentally regulated expression of the insulin gene.

Approximately 500 bp upstream from the human insulin gene there lies a highly polymorphic region, of unknown function, mainly consisting of tandem repeats of the sequence ACAGGGGTGTGGGG (Bell et al 1982). This region is not present in the rat insulin I gene. Two classes of allele occur at this

locus, the U-allele which consists of large insertions (1600-2200 bp) and the L-allele which consists of small insertions (0-600 bp). Positive linkage between the U-allele and type II diabetes has been shown (Owerbach & Nerup 1982; Rotwein et al 1981). There was no linkage observed with either the U- or the L-alleles with type I diabetes. This pattern suggests that the U-allele may in some way alter the expression of the human insulin gene.

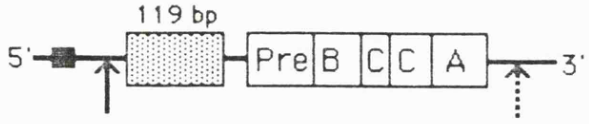
Methylation of cytosine residues plays an important role in eukaryotic gene expression. Many genes have been shown to be undermethylated only in expressing tissues (Cate et al 1983). This was found to be true of the insulin gene. However the correlation between undermethylation and insulin gene expression does not occur in certain tumours and cell lines which express rat insulin genes (Cate et al 1983).

Figure 1.3

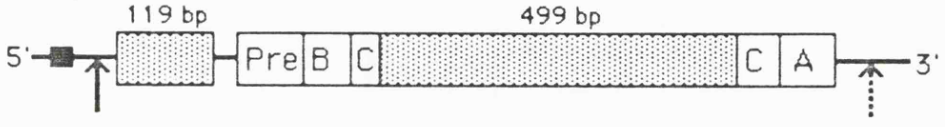
Schematic Diagram of the Rat, Canine and Human Insulin Genes

Protein coding regions are shown as open boxes and introns as shaded boxes. Small black blocks represent the 'Hogness' box and the 5'-capping and the 3'-polyadenylation sites are shown as solid and dashed arrows respectively.

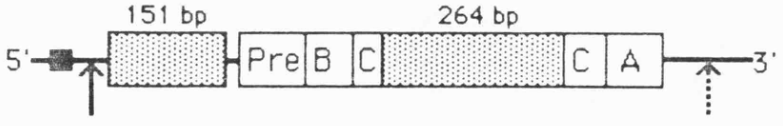
Rat I



Rat II



Canine



Human

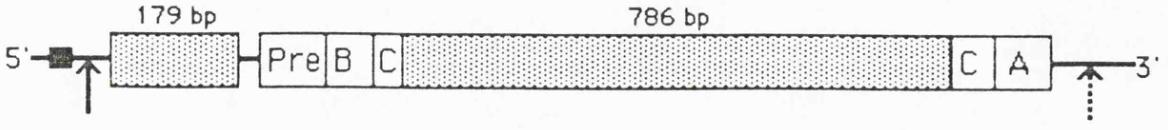


Figure 1.3

Regulation of Insulin Synthesis

A large number of nutrient, hormonal and pharmacological compounds have been tested for their effect on insulin synthesis, many of them have some effect (Table 1.1).

Glucose was first shown to stimulate the incorporation of radiolabeled amino acids into insulin in pancreatic slices (Taylor et al 1964) and later in isolated islets (Howell & Taylor 1966) and it is now accepted that the concentration of glucose reaching the β-cell is the most critical factor in the regulation of insulin synthesis (Taylor 1964; Lin & Haist 1973). Since glucose is also the primary stimulus for insulin secretion the concomitant stimulation of synthesis ensures that the β-cell insulin pool is maintained during long periods of glucose stimulation (Logothetopoulos et al 1964). Insulin biosynthetic rate responds to increasing glucose concentration in a sigmoidal fashion with a threshold of 2 to 4 mM and a maximal rate at 15 to 20 mM (Pipeleers et al 1973; Bone & Howell 1977; Ashcroft et al 1978). Insulin secretion responds to glucose in a similar fashion reaching a maximal rate at a similar concentration but its threshold is higher at 4 to 6 mM (Taylor 1972; Ashcroft et al 1972a).

Although they share the same primary stimulus insulin synthesis and secretion are not obligatorily linked. Their threshold glucose concentrations differ as do their ionic requirements; omission of Ca^{2+} from the medium abolishes secretion but not synthesis (Lin & Haist 1973; Pipeleers et al 1973b) and conversely omission of Mg^{2+} depresses synthesis (Lin & Haist 1973) but not secretion (Grodsky & Bennett 1966;

Milner & Hales 1967). There are also a number of inhibitors capable of dissociating the two processes. Glucose induced synthesis, but not secretion, is inhibited by cycloheximide (Morris & Korner 1970a) and puromycin (Lin et al 1972) whereas trifluoperazine (Gagliardino et al 1980), diazoxide (Lin & Haist 1973) or activation of β-cell α-receptors by catecholamines (Lin & Haist 1975) potently inhibit secretion but have no effect on synthesis.

The physiological status of the animal can affect the regulation of insulin synthesis; in pregnant rats the response of biosynthetic rate to glucose, over the range of 2 to 20mM is elevated whilst that of 48 hour starved rats is diminished (Bone & Howell 1977).

Table 1.1

Effect of Various Agents on (Pro)insulin Synthesis

Agents are classified according to their effect on (pro)insulin synthesis. The references quoted are as follows; (Howell & Taylor 1966)$_1$, (Lin & Haist 1969)$_2$, (Morris & Korner 1970a)$_3$, (Permutt & Kipnis 1970)$_4$, (Asplund 1973)$_5$, (Schatz et al 1973)$_6$, (Baird & Bauer 1976)$_7$, (Berne 1975a)$_8$, (Lin & Haist 1975)$_9$, (Tanese et al 1970)$_{10}$, (Jain & Logothetopoulos 1977)$_{11}$, (Ashcroft et al 1978)$_{12}$, (Niki et al 1976)$_{13}$, (Lin et al 1976)$_{14}$, (Maldonato et al 1976)$_{15}$, (Taylor & Parry 1967)$_{16}$, (Steiner 1969)$_{17}$

Effect of Various Agents on Proinsulin Biosynthesis

Stimulates	Inhibits	No Effect
Glucose[1,2,3,4,5]	Methysergide[9]	Glucagon, cAMP,
Glucagon[5,7], cAMP,	Alloxan[13]	dbcAMP (in absence of
(in presence of	Fluoride[14]	Glucose)[6]
Glucose)[6]	Streptozotocin[15]	Secretin,
Octanoate[8]	Mannoheptulose[12,2]	Pancreozmincholecytoskinin,
Glucosamine[9]		Gastrin[5]
Caffeine[12], reduced pyridine		Palmitate, Oleate[8]
nucleotides[7]		L-Glucose, Fructose (in the
Inosine, Guanosine,		absence of glucose),
Galactose,		3-O-methylglucose, Sorbitol[9]
Adenosine[11]		Serotonin, Dopamine,
Dihydroxyacetone[12]		Epinephrine[9]
Fructose (in presence of		Xylitol, Ribose, Pyruvate[2]
Glucose)[12]		Tolbutamide[16,17]
Mannose[2]		
Theophylline[10]		

Insulin Degradation and the Homeostasis of β-Cell Insulin Content

A balance between insulin synthesis, secretion and intracellular degradation is responsible for maintaining β-cell insulin pool size (Halban et al 1980). Intracellular degradation involves lysosomal (Richardson et al 1975) or microsomal (Kotulas et al 1965; Zühlke et al 1977) enzymes acting via a crinophagic mechanism (Halban & Wollheim 1980). Under certain conditions degradation may be responsible for a fraction of the insulin turnover that can match, or even exceed, that due to secretion (Halban & Wollheim 1980). Insulin degradation is inhibited at high glucose concentrations either by glucose directly or its metabolism (Schäfer et al 1983). The relative level of β-cell insulin stores also plays a part in the regulation of insulin degradation rate (Halban & Renold 1983). A simple two secretory granule pool model (one available for secretion the other for degradation) has been proposed to explain these effects on the rate of insulin degradation (Halban & Renold 1983).

Diabetes Mellitus and Insulin Synthesis

The pathology of diabetes mellitus has been associated either with the loss of β-cells, as in type I diabetes, or with some derangement of the insulin secretory mechanism, as in type II diabetes, and not directly with any abnormality in insulin synthesis. There are, however, a number of animal

models which may implicate alterations in the insulin biosynthetic process in the pathogenesis of some particular forms of diabetes.

Infection of whole mice (Hellqvist & Taylor 1980) or cultured isolated mouse islets (Hellqvist et al 1981) with encephalomyocarditis virus (a diabetogenic virus) causes an inhibition of insulin biosynthetic rate. Spiny mice (*Acomys cahirinus*) have a defective glucose induced insulin secretory profile resembling that seen in latent or type II diabetes in man, they also have a different response pattern of glucose induced insulin synthesis from normal mice and rats (Trueheart et al 1976). These models must be viewed in the light of preliminary measurements of insulin biosynthetic rate in human islets obtained from human patients with type II diabetes which did not show any significant difference from normal human islets (Lohmann et al 1980).

Mechanism of Glucose Stimulation of Insulin Synthesis

It has been postulated that glucose exerts its stimulatory effect on insulin synthesis, and secretion, via a 'glucoreceptor'. This may be of the 'regulator-site' type, where the glucoreceptor is a membrane bound protein, or of the 'substrate-site' type, where the glucoreceptor is one or more of the enzymes responsible for the metabolism of glucose (Randle et al 1968). The vast majority of available evidence favours the substrate-site model.

The ability of certain sugars to stimulate insulin synthesis and secretion is closely linked to their metabolism by the β-cell (Ashcroft 1980). Compounds such as

glyceraldehyde, dihydroxyacetone and inosine that can, directly or indirectly, enter glycolysis at the triose phosphate level all stimulate insulin synthesis and secretion (Ashcroft et al 1978; Ashcroft et al 1973; Kapito & Hedeskov 1976; Hellman et al 1974b). Mannoheptulose, an inhibitor of glucose phosphorylation, inhibits the metabolism of glucose and mannose and their stimulation of insulin synthesis and secretion. Mannoheptulose, however, does not inhiit the metabolism or stimulation of insulin synthesis and secretion by any other metabolisable sugar (Ashcroft et al 1978). Phloretin, on the other hand, preferentially inhibits N-acetylglucosamine metabolism and therefore only inhibits the stimulation of insulin synthesis and secretion by N-acetylglucosamine (Ashcroft et al 1978). These and other data, such as the stimulation of insulin secretion by increased glycogenolysis in glycogen loaded islets (Malaisse et al 1977) and the anomeric specificity of glucose induced insulin secretion (α-D-glucose over β-D-glucose)(Niki et al 1974), constitute the main body of evidence supporting the hypothesis that the substrate-site glucoreceptor is a rate limiting step of glucose utilization. Glucose phosphorylation is proposed as the rate limiting step in the case of stimulation by glucose, and N-acetylglucosamine transport in the case of stimulation by N-acetylglucosamine since it is not inhibited by mannoheptulose (Ashcroft 1976; Ashcroft 1980). A D-glucose-ATP-phosphotransferase activity (with a K_m of 16 mM for glucose), distinct from hexokinase, has been identified in mouse and rat islet extracts but the association of this activity with the glucoreceptor remains tentative (Ashcroft & Randle 1970; Matschinsky & Ellermann 1968;

Malaisse et al 1976).

Glucose metabolism does not appear to stimulate insulin synthesis merely by making more ATP available for protein synthesis since inhibition of insulin synthesis by low concentrations of streptozotocin can be achieved without alteration of cellular ATP content (Maldonato et al 1976).

Cyclic AMP has been implicated in the mechanism of stimulation of synthesis since agents which elevate islet cAMP content such as methylxanthines (Tanese et al 1970), dbcAMP and glucagon (Schatz et al 1973) potentiate glucose induced insulin synthesis. Glucose (Charles et al 1973), mannose (Grill & Cerasi 1976), inosine, α-oxoisocaproic acid and N-acetylglucosamine (Campbell & Taylor 1981), all of which stimulate insulin synthesis, also raise islet cAMP levels. It is unlikely that cAMP acts as a true second messenger in this situation since it seems to sensitize the insulin biosynthetic mechanism to glucose since the threshold concentrations for increases in islet cAMP are higher than those for stimulation of insulin synthesis (Ashcroft 1980). This sensitizing role for cAMP is supported by the observation that the altered glucose stimulatory profiles for insulin synthesis in starved rats can be partly restored to normal by incubation with 3-isobutyl-methyxanthine, a phosphodiesterase inhibitor known to increase islet cAMP content (Bone & Howell 1977).

Level of Insulin Gene Expression at Which Glucose Exerts Control

Generally there are three types of control possible, transcriptional, translational and post-translational. The

major post-translational modification is the conversion of proinsulin to insulin and the half-life of this process is unaltered by varying glucose concentration (Baird 1971) although it is decreased by tolbutamide treatment (Gold et al 1986). There is no data implicating post-translational controls in insulin gene expression by glucose. Therefore transcriptional and translational mechanisms must account for the observed glucose control. Evidence will be discussed for glucose modulation of both the synthesis of new preproinsulin mRNA as well as the translation of existing preproinsulin mRNA.

Early investigations were hindered by the lack of a specific assay for insulin gene transcription and it was not until the advent of specific preproinsulin mRNA assays, utilizing recombinant DNA techniques, that the relative contributions of transcriptional and translational controls could be assessed. A point to be borne in mind is that although the effect of glucose is tissue specific, in that it stimulates protein synthesis in islet tissue and not in other tissues such as liver (Morris & Korner 1970a), the action of glucose on protein synthesis isn't completely specific to insulin. The synthetic rate of non-insulin proteins is stimulated but that of insulin is stimulated to a higher degree (Morris & Korner 1970a; Permutt & Kipnis 1972a). Another consideration, at least in the rat which has two forms of insulin, is that there may be differential expression of rat insulin I and II with a preference for the synthesis of rat insulin I, over that of Insulin II, under glucose stimulation (Kakita et al 1982).

(a) Translational Controls

Preliminary evidence for this form of control was circumstantial, relying on the use of actinomycin D, an inhibitor of RNA synthesis. Glucose stimulated proinsulin synthesis in islets of normal rats (Steiner et al 1972; Permutt & Kipnis 1970; Morris & Korner 1970b; Permutt & Kipnis 1972a) or 48 hour starved rats (Jahr et al 1980b) was unaffected, in the short term, by the inhibition of RNA synthesis by actinomycin D. Also the rate at which insulin biosynthetic rate returned to pre-stimulatory levels, after the removal of a 45 minute 5.5 mM glucose stimulus, was unaffected by actinomycin D (Kaelin et al 1978).

Direct support for translational controls came with the use of specific DNA/RNA hybridization studies proving that glucose stimulated proinsulin synthesis, over a period of one hour, was possible without a concomitant change in preproinsulin mRNA level (Rosenzweig & Yip 1979; Itoh et al 1978). A similar observation was made with 15 day foetal rat islets (Kakita et al 1983).

Isolation of polyribosomes from islets subjected to various concentrations of glucose for 45 minutes showed that increased glucose concentration promoted a shift of translation of preproinsulin mRNA on mono-ribosomes to trisomes (Permutt & Kipnis 1972). The effect of glucose was found to be on the rate of polypeptide chain initiation rather than chain elongation (Permutt 1974). Cell-free translation of preproinsulin mRNA in competition with other poly A^+ containing RNA, and the inhibition of chain initiation by aurintricarboxylic acid, was used to produce

data in support of the Lodish model (Lodish 1971) for regulation of preproinsulin mRNA translation by glucose (Lomedico & Saunders 1977). This model proposes that all mRNAs have an intrinsic rate constant for initiation of translation and that those mRNAs with lower rate constants, such as preproinsulin mRNA (Lomedico & Saunders 1977), are translated slower than those mRNAs with higher rate constants when the concentration of translation initiation factor(s) is limiting. It is proposed that the metabolism of glucose increases the pool size of initiation factor(s) and allows those mRNAs with lower rate constants (e.g. preproinsulin mRNA) to compete more efficiently for translation initiation factor(s) (Lomedico & Saunders 1977). This model is attractive since it accounts for the effect of glucose on general islet protein synthesis as well as that on proinsulin synthesis.

(b) Transcriptional Controls

As in the case of translational controls the early evidence for transcriptional controls was circumstantial as only total RNA synthesis could be measured until the advent of a specific preproinsulin mRNA assay.

Glucose stimulated the incorporation of ^{14}C-uridine into RNA in islet tissue (Jarrett et al 1967; Puchinger & Wacker 1972; Gold et al 1982) independent of the effects on transport of uridine (Gold et al 1982), and was actinomycin D sensitive (Jarrett et al 1967). This effect was not seen in other tissues (Jarrett et al 1967) and the actinomycin D sensitivity was observed 45 minutes after the

start of glucose stimulation (Permutt & Kipnis 1972a). The rate of turn off of glucose stimulated insulin synthesis in islets, after removal of a 45 minute glucose stimulus, was found to decline faster in the presence of actinomycin D for a glucose stimulus of 20 mM but not for ones of 2.5 mM or 5.5 mM (Kaelin et al 1978).

Specific DNA probe hybridization assays permitted the correlation of changes in preproinsulin mRNA with proinsulin biosynthetic rate. After a general fall in mRNA levels in the pancreas of four day starved rats preproinsulin mRNA activity increased more rapidly than that of other species of mRNA on refeeding. This was true of measurements of preproinsulin mRNA made by both cell-free translation and cDNA hybridization (Giddings et al 1981). A similar study on refeeding, and glucose injection, of three day fasted rats showed a detectable increase in preproinsulin mRNA within two hours, and restoration to control levels within twelve hours, which was blocked by actinomycin D (Giddings et al 1982).

There is no direct evidence for a glucose stimulus eliciting an increase in preproinsulin mRNA in animals under normal physiological conditions. Isolated mouse islets cultured for seven days in medium containing 20 mM glucose showed no change in preproinsulin mRNA level. In islets incubated at 3.3 mM glucose the level dropped by 90% after 24 hours. Subsequent culture in medium containing 20 mM glucose restored the preproinsulin mRNA level to that of the controls after three days. The insulin biosynthetic rates correlated well with the observed levels of preproinsulin mRNA (Brunstedt & Chan 1982). However, there

is evidence that glucose may modulate the levels of preproinsulin mRNA in islets under normal physiological conditions since pulse-chase ^{14}C-uridine labeling studies, in the presence of actinomycin D or α-amanitin to inhibit RNA synthesis, have shown an increase in preproinsulin mRNA half-life at high glucose concentrations (Welsh et al 1985).

Thus the effect of glucose on the translation and transcription of preproinsulin mRNA under conditions of long term starvation and refeeding are well characterized. Due to practical limitations, however, the effect of glucose on the rate of *in vitro* insulin gene transcription in normal islets over short term stimulations has not been well characterized. This thesis describes the development of a novel assay for preproinsulin mRNA and its application to the study of the effect of glucose on insulin gene transcription in islets of Langerhans isolated from normally fed rats over incubation periods of 2 and 24 hours. Parallel measurements of the rate of (pro)insulin synthesis were also carried out. The results are discussed in the context of the control of β-cell insulin gene expression.

Chapter 2

Experimental

Introduction

This chapter describes in detail the methods used to obtain the data presented in this thesis. It is divided into two main parts, the first dealing with the isolation of islet tissue and the measurement of insulin synthesis, and the second with the measurement of preproinsulin mRNA.

Reagents

Collagenase (C-9263), 8-hydroxyquinolone, deoxyribonuclease I (D-0876), Soybean trypsin inhibitor (T-9003), actinomycin D (A-4262), tetracycline (T-3383), dextran blue, ribonuclease A (R-5500), diethyl pyrocarbonate, spermidine, dATP (D-6500), dGTP (D-4010), dTTP (D-0251), dithiothreitol (D-0632), phenol red, dextran sulphate (D-6001), polyvinylpyrrolidone (PVP-360), guanidine hydrochloride (G-4630), salmon sperm DNA (D-1626), glycerol, and bovine insulin (I-5500) were purchased from Sigma Chemical Co. Ltd., Poole, Dorset.

Plasmid phins214 was kindly donated by Dr. G.I. Bell (Chiron Corporation, Emeryville CA, USA). The plasmid contains a 1605 bp HincIII/BglI fragment which includes the entire human preproinsulin gene plus 58 5' and 117 3' flanking nucleotides. BamHI linkers were added to this fragment and it was cloned into the BamHI site of pBR322. The host *E. coli* strain is HB101 and the bacteria are Amp^S and Tet^S. The bacteria were stored as a suspension in 50% (v/v) glycerol at -20°C until use.

Plasmid pD1462 was kindly donated by Dr. D.F. Steiner (University of Chicago, Chicago IL, USA). The plasmid contains a 1290 bp SstI fragment containing the entire canine preproinsulin gene (Kwok et al 1983). BamHI linkers were added to this fragment and it was cloned into the BamHI site of pBR322. The host *E. coli* strain is RR1 (Amp^S and Tet^S). The bacteria were stored as a suspension in 50% (v/v) glycerol at -20°C until use.

Plasmid pKA30 and sequencing primer were kindly donated by Dr. W. Kastern (Hagedorn Research Laboratory, Gentofte, Denmark). This plasmid contains a 400-450 bp fragment of a rat insulin cDNA cloned into the PstI site of pBR322 by G-C tailing. The host *E. coli* strain is MC1061.

Single stranded M13 phage clones containing either a 180 bp fragment of a rat insulin cDNA cloned into the HpaII site or a $170^{+}_{-}10$ bp fragment of the α-1-microglobulin gene cloned into the EcoRI site were kindly donated by Dr. W. Kastern. The clones were supplied as purified DNA and were stored at -20°C until use.

Lambda DNA-HindIII digest (27-4048-01), oligo(dT)cellulose, type 7 (27-5543-0), BamHI restriction enzyme, PstI restriction enzyme, HinfI restriction enzyme, hexanucleotides (27-2166-01), Sephadex G-50 fine and Sepharose 4B were purchased from Pharmacia Ltd., Milton Keynes.

Hams F12 tissue culture medium was purchased from Flow Laboratories, Irvine, Scotland.

Foetal calf serum (011-6290) was purchased from GIBCO Europe Ltd., Uxbridge.

Bovine serum albumin for insulin assays was purchased from Armour Pharmaceutical Co. Ltd., Eastborne.

Formamide (puriss p.a.), guanidinium thiocyanate (purum) and sodium N-lauroylsarcosine (pract.) were purchased from Fluka AG, CH-9470 Buchs, Switzerland.

Agarose (electrophoresis grade), low melting point agarose (electrophoresis grade), nuclease free bovine serum albumin (5561) and DNA polymerase I (*E. coli*) were purchased from Bethesda Research Laboratories, Inc., Maryland, USA.

Bacto agar, Bacto yeast extract and Bacto tryptone were purchased from Difco Laboratories, Detroit, Michigan, USA.

FisoFluor 2 scintillation fluid, phenol (AR grade), caesium chloride, 38% (w/v) formaldehyde solution, 2-methoxyethanol, iso-amyl alcohol, diethyl ether and isopropanol were purchased from Fisons PLC, Loughborough.

Insulin binding reagent and human insulin standards for insulin radio-immunoassay were purchased from Welcome Reagents Ltd., Beckenham.

Guinea pig anti-porcine insulin serum was purchased from Miles-Yeda Ltd., Rehovot, Israel.

Bromophenol blue, xylene cyanole FF and β-mercaptoethanol were purchased from Bio-Rad Laboratories, Richmond, California, USA.

L-[4,5-^{3}H]Leucine (120-190 Ci/mmol in aqueous solution containing 2% ethanol) and deoxycytidine 5'-[α-^{32}P]triphosphate (~3000 Ci/mmol in ethanol/water 1:1) were purchased from Amersham International PLC, Amersham.

Ethidium bromide (research grade) was purchased from Serva Feinbiochemica GmbH & Co., Heidelberg, FDR.

Collagenase was purchased from Boehringer Corporation (London) Ltd., Lewes, East Sussex.

All other reagents used were Fisons (AR grade) or similiar.

^{125}I-labelled insulin was prepared by a modification of the method of Greenwood et al (1963). This employs chloramine T as an oxidant to activate ^{125}I and facilitate covalent linkage to the phenolic ring of certain tyrosyl residues in the insulin molecule.

All sterile water used in solutions for RNA work was treated with 0.1% diethylpyrocarbonate for several hours. Excess diethylpyrocarbonate was removed by autoclaving for 20 minutes at 15 psi.

SSC solution was prepared as a 20x stock solution (1xSSC is 0.15 M NaCl, 15 mM sodium citrate pH 7.0) and autoclaved for 20 minutes at 15 psi.

Denatured salmon sperm DNA was prepared by passing a 10 mg/ml solution (in water) repeatedly through a 18 G needle and boiling for 10 minutes. Aliquots were stored at -20°C until use.

Phenol was redistilled at 160°C, to remove contaminants that cause breakdown or crosslinking of RNA and DNA, and stored under nitrogen at -20°C in small aliquots. Before use aliquots were melted at 68°C and 8-hydroxyquinoline added to a final concentration of 0.1% (w/v). The melted phenol was extracted several times with an equal volumes of 1.0 M Tris-Cl pH 8.0 followed by 0.1 M Tris-Cl pH 8.0, 0.2% β-mercaptoethanol until the pH of the aqueous phase was greater than pH 7.6. The phenol solution was stored under equilibriation buffer for up to one month.

4 M Guanidinium thiocyanate stock solution was prepared as follows. 100 ml of water, 10 ml of 1 M Tris-Cl pH 7.6 and 10.6 ml of 0.2 M EDTA are added to a 100 g bottle of guanidinium thiocyanate and stirred overnight. To this was added 21.2 ml of 20% (w/v) sodium N-lauroylsarcosine, 2.1 ml of β-mercaptoethanol and the volume was adjusted to 212 ml by the addition of water. The solution was then filtered and stored at 4°C in a tightly sealed brown glass bottle for a maximum of one month before use.

Agarose beads were prepared by dissolving 100 mg of agarose in 50 ml of water by immersion in a boiling water bath.The agarose was allowed to set and then syringed through progressively smaller needles until it ran freely through a 23 G needle. The beads were stored at 4°C until use.

Formaldehyde agarose beads were prepared by dissolving 100 mg of agarose in 8.7 ml of 38% (w/v) formaldehyde, 1 ml of 1 M $NaHPO_4$ pH 7.6, 5 ml of glycerol, 35.3 ml of water with 5 mg of bromophenol blue by immersion in a boiling water bath (in a fume cupboard). The agarose was allowed to set and then syringed through progressively smaller needles until it ran freely through a 23 G needle. The beads were stored at 4°C until use.

Materials

Capped test tubes (1.5 ml and 0.5 ml) and disposable 4 ml test tubes were purchased from Sarstedt, Leicester.

Petri dishes (90 mm) were purchased from Sterilin Ltd., Feltham.

Nitrocellulose paper was purchased from Sartorius, Surrey.

Disposable polypropylene Econo-column chromotography columns were purchased from Bio-Rad Laboratories, Richmond, California, USA.

X-ray film (RX) was from Fuji Photo Film Co., Ltd.

Glass fibre filter disks (GF/C disks) were purchased from Whatman.

Anti-insulin serum coupled to Sepharose 4B (AIS-Sepharose) was prepared according to the outlines set down by the manufacturer (Pharmacia) modified by Berne (1975a). 1 ml of guinea pig anti-porcine insulin serum was coupled to 5 g of Sepharose 4B and as a control 0.4 ml of normal guinea pig serum was coupled to 2 g of Sepharose 4B (NGPS-Sepharose). The prepared gels were stored at 4°C at a concentration of 100 mg/ml in binding buffer (0.2 M glycine, 2.5 mg/ml BSA, pH 8.8). No gel preparations more than one month old were used.

Glassware and plasticware were siliconized by rinsing with dimethyldichlorosilane solution and allowed to air dry. They were then rinsed with hot water several times and finally with distilled water and dried in a drying oven.

(1) Isolation of Pancreatic Islets and Measurement of Insulin Synthesis

Isolation of Pancreatic Islets

The islets used in this study were all isolated from male or female rats of an albino Wistar strain (body weight 250 to 300g) allowed free access to food and water.

(i) Isolation Principle

Pancreatic islets are liberated from the pancreatic stroma by the action of the enzymatic complex collagenase, whose action is assisted by the mechanical disruption of the pancreas by ductal distension and chopping of the pancreas prior to digestion (Howell & Taylor 1966).

This isolation procedure does not appear to affect the functional integrity of the islets since detailed secretory and ultrastructural studies have failed to demonstrate any significant differences from islets isolated by the much less traumatic procedure of microdissection (Lacy & Kostianovsky 1967; Lacy et al 1968).

(ii) Islet Isolation Medium

Throughout the isolation procedure the islets were bathed in Gey & Gey medium (Gey & Gey 1936) of the following composition;

NaCl	120.0 mM
$NaHCO_3$	27.0 mM
Na_2HPO_4	1.0 mM
KCl	5.0 mM
KH_2PO_4	0.2 mM
$MgCl_2$	1.0 mM
$MgSO_4$	0.3 mM
$CaCl_2$	2.0 mM
Glucose	2.5 mM

Phenol red (2.5 µg/ml) was added as a pH indicator and the medium was equilibrated to pH 7.4 by gassing with O_2 + CO_2 (95:5) immediately prior to use.

(iii) Isolation Method

Rats were killed by cervical dislocation and the spleen and pancreas exposed by a ventral incission. Both organs were removed and immediately placed in 200 ml of ice-cold medium. Alternatively the pancreas was ductally distended *in situ* as follows. Firstly the pancreatic duct was securely ligated as close as possible to its point of drainage into the duodenum. The common bile duct was then dissected out, tensioned using a pair of curved forceps and a small transverse nick made superior to the pancreas. Then a fine cannula was introduced into the common bile duct and firmly tied in place. The pancreas was then gently distended with 20 ml of medium administered by syringe through the cannula.

The pancreas and spleen were then removed as described above.

The spleen and any excess fat or connective tissue were dissected away and, in the case of the non-distended pancreas, distended by subperitoneal injection of medium with a syringe fitted with a 23 gauge hypodermic needle. The pancreas was chopped with scissors into pieces of a uniform size of approximately 1-2 mm and washed with 15 ml of medium and pelleted by brief centrifugation (500g for 5 seconds). The tissue pellet was transferred to a stoppered 25 ml conical flask along with collagenase (12 mg Boehringer, 2 mg Sigma), 2 mg DNAse and sufficient medium to give a 2:1 ratio of medium to tissue as judged by eye.

Digestion was carried out by vigorous shaking (Stuart flask shaker) in a water bath (37°C) for 10 to 12 minutes. The end point of the digestion was determined by periodic examination of aliquots of the digestate under a binocular microscope (magnification X15). Further digestion was prevented by dilution of the digestate to 15 ml with medium and the diluted tissue was pelleted by brief centrifugation (500g for 5 seconds), this was repeated once more and the tissue pellet made up to 15 ml with medium.

Hand picking of the islets was carried out on black bottomed Petri dishes using a finely drawn siliconized Pasteur pipette under a binocular microscope (magnification X15). Small aliquots of the digestate were placed in the Petri dish and further diluted with medium and agitated by repeated aspiration through a Pasteur pipette to break up any aggregates of tissue. The islets appeared as white oval bodies amongst a background of fine acinar debris. As far as

possible only evenly sized islets free of attached acinar material were picked and they were placed in freshly gassed medium.

Two rats were used per digestion with up to four rats used per isolation. All isolations were started between 9 and 11 am and the time from animal death to the end of the isolation procedure was 90 minutes or less. The yield from two rat pancreata was typically 300-400 islets which was normally increased by up to 50% by *in situ* distension of the pancreata.

Islet Incubation Medium

Incubations were carried out in Gey and Gey medium supplemented with 3 mg/ml BSA containing various concentrations of glucose. Where appropriate actinomycin D (10 µg/ml) was added from a stock solution (1 mg/ml) in ethanol with equivalent amounts of ethanol added to the control incubations.

Islet Incubation Conditions

Islets were transferred to siliconized 1.5 ml capped test tubes with a finely drawn Pasteur pipette and gently pelleted using a MSE Micro Centaur microfuge (2 seconds, low speed). Medium was removed from the pelleted islets and replaced with 75 µl of incubation medium containing 2.5 mM glucose and the tubes were gassed briefly with O_2 + CO_2 (95:5), tightly capped and placed in a gently shaking water bath (37°C) to preincubate for 45 minutes.

At the beginning of the incubation period the glucose concentration was adjusted to the desired level by the addition of 25 µl of fresh incubation medium containing the appropriate concentration of glucose. The radiolabelling period was started by the addition of 20 µl of ^{3}H-Leucine and 5 µl of incubation medium with an appropriate concentration of glucose to compensate for dilution by the addition of ^{3}H-Leucine. After all additions to the incubation tubes they were regassed. During both preincubation and incubation the islets were agitated occasionally by gentle flicking of the tubes.

Incubations were terminated by washing the islets with 1 ml of ice-cold preincubation medium, pelleting of the islets in a MSE Micro Centaur microfuge (10 seconds, high speed) and aspiration of the medium with a finely drawn Pasteur pipette. This wash was repeated once more and the islets resuspended in 500 µl of ice-cold sonication buffer and sonicated (see below).

Sonication of Islets

Islets were suspended in 1 ml of sonication buffer (0.2 M glycine containing 2.5 mg/ml BSA and 25 µg/ml Soybean trypsin inhibitor) and sonicated on ice by two 15 second bursts at medium power with a 3 mm probe (MSE 150 watt Ultrasonic Disintegrator). Particulate material was removed from the sonicate by pelleting (MSE Micro Centaur microfuge, 10 minutes, high speed) and the supernatant transferred to a fresh tube. Islet sonicates were stored at -20°C until used.

Gel Fractionation of Islet Sonicates

Samples in running buffer (1 M acetic acid containing 0.2 mg/ml BSA) supplemented with 10% (w/v) glucose were layered on the top of a Sephadex G-50 fine column equilibrated in running buffer (bed volume 44 ml; 56 cm x 1 cm diameter). Elution was carried out at a rate of 1.7 ml/hour with running buffer and 0.64 ml fractions were collected using a LKB 7000 Ultrorac fraction collector. The elution rate was kept constant by the use of a Mariotte reservoir and the column was pre-run with a 1 mg sample of bovine insulin to prevent sample adsorbtion. The void volume of the column was calculated using dextran blue. The radioactivity (cpm) in each fraction was determined by liquid scintillation counting of a 200 µl aliquot of each fraction in 4 ml of FisoFluor 2 scintillation fluid.

Assay of (Pro)Insulin Synthesis

(i) Principle

Insulin synthesis was determined by following the incorporation of ^{3}H-Leucine into (pro)insulin which was purified from other labelled islet proteins by an immuno-binding technique using anti-insulin serum coupled to a Sepharose gel (AIS-Sepharose) (Berne 1975a). The assay was performed in a batch manner using normal serum coupled to Sepharose (NGPS-Sepharose) as a control for non-specific binding of labeled islet proteins to the AIS-Sepharose.

(ii) Method

Four 1.5 ml capped test tubes per sample were used (two each for AIS- and NGPS-Sepharose). Each tube contained 100 µl of islet sonicate and 20 mg of AIS- or NGPS-Sepharose in a total of 1 ml of binding buffer. The capped tubes were end over end mixed for 90 minutes and the gel then washed four times by end over end mixing with 1 ml of fresh binding buffer each time. Buffer was aspirated between washes after pelleting of the gel (MSE Micro Centaur microfuge, 1 minute, high speed). Bound material was eluted from the gel by end over end mixing for 120 minutes with 1 ml of elution buffer and pelleting the gel as before. The radioactivity in duplicate 200 µl aliquots of supernatant was determined by liquid scintillation counting after the addition of 4 ml of FisoFluor 2 scintillation fluid.

Assay of Total Protein Synthesis

Total protein synthesis was estimated as the amount of ^{3}H-leucine incorporated into TCA insoluble material in islets incubated in the presence of ^{3}H-leucine. Duplicate 50 µl aliquots of islet sonicate and 200 µg of carrier BSA (50 µl of 4 mg/ml stock) were added to 4 ml of 5% (w/v) TCA and stood on ice for two hours. The precipitate was then pelleted (MSE Mistral 3000 bench centrifuge, 2,000 rpm at 4°C for 15 minutes), drained, washed with a further 4 ml of ice-cold 5% TCA and repelleted as above. This final pellet was drained and dissolved in 0.5 ml of 0.1 M NaOH over one hour and the radioactivity in duplicate 100 µl aliquots was determined by liquid

scintillation counting after the addition of 4 ml of FisoFluor 2 scintillation fluid.

Comparison of the counting efficiency of ^{3}H-leucine spiked samples revealed no significant differences in quenching between insulin synthesis samples (see above) and total protein synthesis samples.

Calculation of Fractional (Pro)Insulin Synthesis Rate

The fractional (pro)insulin synthesis rate was calculated as the ratio of the rate of (pro)insulin synthesis to that of total protein synthesis.

Assay of Insulin Content

Determination of islet insulin content was by radio-immunoassay (RIA). The assay relies on the competition of unlabelled and ^{125}I-labelled insulin for a fixed number of immune-binding sites and the determination of the amount of bound ^{125}I-insulin by gamma counting.

The Welcome insulin RIA kit was used with Welcome human insulin standards. Samples were diluted appropriately with assay buffer (between 5 and 1000 fold) to bring them onto the standard curve. Samples were assayed in duplicate and counted for one minute on a Beckman Gamma 5500 counter.

(2) Measurement of Preproinsulin mRNA

General Procedures

Since ribonuclease is present on the human skin and is an extremely stable and active enzyme, it is necessary to adopt working practices in all operations involving RNA to reduce the risk of contamination of samples with ribonuclease. Thus gloves were worn during all of the following procedures and contamination of glassware and reagent solutions by ribonuclease was reduced by sterilization. Glassware was sterilized by baking for 2 hours at 200°C, plasticware was sterilized by autoclaving for 20 minutes at 15 psi and all reagent solutions were prepared using DEPC treated water (DEPC is a strong inhibitor of ribonuclease (Fedorcsak & Ehrenberg 1966)). Wherever possible solutions were sterilized by autoclaving for 20 minutes at 15 psi before use. Since very small quantities of nucleic acids were used all glassware, such as Corex tubes, and plasticware were siliconized before use to prevent losses due to adsorbtion on vessel walls.

Isolation of Total Cellular RNA

Total cellular RNA was isolated from rat pancreas and liver by the guanidinium/hot phenol method (Maniatis et al 1982). In the case of liver the rat was fasted overnight before use to reduce liver glycogen which otherwise coprecipitates with RNA (Giddings & Rotwein 1986). The animals were killed by cervical dislocation and a ventral incision

made. The pancreas was then removed, trimmed of fat and connective tissue and blotted dry. It was then weighed and coarsely chopped. The tissue was then homogenized, in guanidinium thiocyanate stock solution (5 ml per gram of tissue) in a screw top bottle with a Polytron homogenizer (full speed for 20 seconds). The homogenate was heated to 60°C in a water bath and an equal volume of preheated phenol (60°C) and chloroform/isoamyl alcohol (24:1 (v/v)) together with half a volume of preheated aqueous buffer (0.1 M sodium acetate pH 5.2 containing 10 mM Tris-Cl pH 7.4 and 1 mM EDTA at 60°C) were added. The bottle was tightly capped and vigorously shaken at 60°C for 15 minutes. This mixture was cooled on ice and the two phases separated by centrifugation in 15 ml Corex tubes (MSE Mistral 3000 bench centrifuge, 2000 g at 4°C for 20 minutes). The aqueous (upper) phase was carefully aspirated into a fresh screw top bottle. The aqueous phase was re-extracted with phenol/chloroform and then twice with an equal volume of chloroform/isoamyl alcohol at room temperature. The RNA in the extracted aqueous phase was precipitated by the addition of two volumes of ethanol followed by storage at -20°C for several hours. Recovery of the RNA precipitate was by centrifugation (Sorvall HB4 rotor, 6,000 rpm at -10°C for 20 minutes) and the pellet was dissolved in 10 mM Tris-Cl pH 7.5. The RNA was reprecipitated by addition of a one tenth volume of 3 M sodium acetate pH 5.2 and 2 volumes of ethanol followed by storage at -20°C for one hour. Recovery of the precipitate was as above and the final pellet was dried under vacuum and dissolved in DEPC treated water (1 ml per gram of original tissue) on ice. The purified RNA was stored in small aliquots at -70°C until use.

Preparation of Poly A^+ RNA

Since virtually all mammalian mRNAs carry at their 3' ends a polyadenylate tract, affinity chromotography on oligo(dT)cellulose can be used to selectively enrich total cellular RNA preparations for polyadenylate containing RNA (Poly A^+ RNA) (Edmonds et al 1971; Aviv & Leder 1972).

Total cellular RNA solution was heated to 68°C for 5 minutes and an equal volume of double strength loading buffer added (x1 buffer was 40 mM Tris-Cl ph 7.6 containing 0.5 M NaCl and 1 mM EDTA). Once cooled to room temperature the RNA was loaded onto a disposable polypropylene column containing 1 ml of oligo(dT)cellulose, equilibrated in loading buffer. The effluent was collected, reheated to 68°C, cooled to room temperature and reloaded onto the column. The column was then washed with 5 column volumes of loading buffer followed by 4 column volumes of loading buffer containing 0.1 M NaCl. All column effluents thus far were collected and precipitated in the same manner as poly A^+ RNA (see below) to give the non-polyadenylated RNA (poly A^- RNA) fraction. The bound poly A^+ RNA was eluted from the column with 3 column volumes of elution buffer (10 mM Tris-Cl pH 7.5 containing 1 mM EDTA). The eluent was adjusted to 0.5 M NaCl by the addition of solid NaCl and taken through another round of oligo(dT)cellulose chromotography. Between each use of the column it was regenerated by washing with three column volumes of water, three column volumes of 0.1 M NaOH containing 5 mM EDTA and finally with water until the effluent was less than pH 8.0. The poly A^+ RNA was precipitated from the final eluent by addition of a one tenth volume of 3 M sodium acetate pH 5.2

and 2.2 volumes of ethanol followed by storage at -20°C overnight. The precipitate was recovered by centrifugation (Sorvall HB4 rotor, 10,000 rpm at -10°C for 30 minutes), and rinsed in cold 70% ethanol, repelleted as above and finally dried under vacuum. The final pellet was dissolved in DEPC treated water to a concentration of ~1 mg/ml and stored in 20 µl aliquots at -70°C until use. Up to 5 mg of total cellular RNA could be processed per ml of oligo(dT)cellulose.

Spectrophotometric Determination of RNA and DNA Concentration

The absorbance of total cellular RNA, poly A^+ RNA and DNA preparations were measured in quartz cuvettes with a Pye Unicam SP800 dual beam spectrophotometer. The concentration of RNA and DNA was calculated from the absorbance at 260 nm; an absorbance of 1.0 corresponding to a concentration of 40 µg/ml for single stranded RNA and 50 µg/ml for double stranded DNA. The relative purity of the RNA or DNA was estimated from the ratio of the absorbance at 260 nm to that at 280 nm; a ratio of 2.0 is obtained with pure RNA or 1.8 for pure DNA, contamination with other substances such as phenol lowers these values (Maniatis et al 1982).

Isolation of Plasmid DNA

In this study three plasmids phins214, pDI462 and pKA30 were used to amplify specific preproinsulin gene sequences for use as hybridization probes. Since all three plasmids are derived from pBR322 the same plasmid isolation protocol was applicable to all of them.

(i) Storage of bacteria

Bacteria were stored as a 50% (v/v) glycerol suspension in L-broth (see below) at -20°C. Periodically the viability of the bacteria was checked by overnight growth on agar plates (1.5% (w/v) agar in L-broth) at 37°C. The addition of ampicillin (50 µg/ml) or tetracyclin (15 µg/ml) to the agar was used to check the presence of the expected plasmid borne antibiotic resistance.

(ii) Growth of Bacteria and Amplification of the Plasmid

Growth of bacteria was carried out in L-broth which was prepared with the appropriate antibiotic. This was 50 µg/ml ampicillin for all three plasmids which are all Amp^R Tet^S. L-broth was prepared by dissolving 10 g each of Bactotryptone and NaCl, 5 g of Bactoyeast extract and 1 g of glucose in water, adjusting the pH to 7.0, making the volume up to 1000 ml and then autoclaving for 20 minutes at 15 psi. Antibiotic supplement was added after the L-broth had cooled. Standard asceptic technique was used throughout this and all other procedures where necessary.

Bacteria were streaked onto L-broth agar plates (1.5% (w/v) agar in L-broth) and grown overnight at 37°C. A 5 ml L-broth starter culture was innoculated with a single colony and grown at 37°C for eight hours in a shaking incubator. This starter culture was in turn used to innoculate 250 ml of L-broth in a 2000 ml baffle flask which was incubated overnight at 37°C in a shaking incubator.

(iii) Plasmid Isolation

The cells from a 250 ml culture were pelleted (MSE 6x250 ml rotor, 5,000 rpm at 4°C for 5 minutes), the supernatant was aspirated and the cells placed on ice. The cells were then gently resuspended in 8 ml of lysis buffer A (25 mM Tris-Cl pH 8.0 containing 10 mM EDTA) precooled on ice. 2 ml of lysis buffer B (lysis buffer A containing 10 mg/ml lysozyme) was then gently mixed in and left for 5 minutes. 20 ml of 0.2 M NaOH containing 1% (w/v) SDS was then added and the mixture left for a further 5 minutes. The chromosomal DNA from the lysed cells was precipitated by the addition of 15 ml of 3 M potassium acetate pH 5.0, precooled on ice and the mixture was then poured into a 100 ml polycarbonate screw-top tube and centrifuged (MSE 6x100 ml rotor, 8,000 rpm at 4°C for 10 minutes with the brake off). The entire contents of the tube were poured through a funnel plugged with glass wool into another screw-top tube and 50 ml of isopropanol added. This mixture was centrifuged (as above but this time with the brake on) and the pellet drained carefully. The pellet was then dehydrated by covering for 5 minutes with 3 ml of ethanol and then with 3 ml of ether and finally dried under a stream of nitrogen. The dried pellet was slowly resuspended in 7.5 ml of TE buffer (10 mM Tris-Cl pH 8.0 containing 1 mM EDTA) and solid caesium chloride was added (1.12 times the weight of the solution). The salt was dissolved thoroughly by repeated inversion of the tube. The solution was then mixed with 50 µl of 10 mg/ml ethidium bromide solution and then loaded

into a 10 ml polycarbonate screw-top centrifuge tube. The tube was centrifuged (MSE 10x10 ml titanium rotor, 40,000 rpm) at 20°C for 48 hours.

Illumination of the centrifuge tube with short wave ultraviolet light normally revealed only one fluorescent band, this was the plasmid band. Occasionally a band higher in the tube was seen, this was chromosomal DNA. The plasmid band was carefully removed using a syringe fitted with a long needle and the ethidium bromide removed by several extractions with caesium chloride saturated isobutanol. The caesium chloride was in turn removed from the plasmid DNA by dialysis against four changes of 2000 ml of TE buffer. The purified plasmid was stored at a concentration of ~1 µg/ml at -20°C until use.

Restriction Digestion of DNA

All digests were carried out at 37°C in capped test tubes using the same medium ionic strength digestion buffer (50 mM NaCl containing 10 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$ and 1 mM dithiothreitol), added as a 10x stock solution. The concentration of DNA in the digest was between 0.1 µg/ml and 1 µg/ml. One unit of restriction enzyme per µg of DNA was used. Digestions were set up ensuring that the restriction enzyme never contributed more than one tenth of the total digestion volume to avoid inhibition of the enzyme by the glycerol in the restriction enzyme storage buffer.

^{32}P-Labeling of DNA for Use as Hybridization Probes

Two different methods were used for ^{32}P-labeling of double stranded DNA, nick translation and oligonucleotide primer extension. For the ^{32}P-labeling of single stranded M13 DNA a second strand synthesis method was used. In the case of double stranded DNA derived from plasmids the plasmid insert was isolated before labeling. A different insert isolation procedure was adopted for DNA labeled by nick translation and for oligonucleotide primer extension. Since the insert sequences of both pDI462 and phins214 were cloned into the BamHI site of pBR322 it was possible to adopt the same preparation protocol for both inserts. The plasmid insert of pKA30 was excissed by digestion with HinfI restriction enzyme.

(i) Nick Translation

(a) Isolation of Plasmid Inserts

Plasmid DNA was digested for 3 hours with BamHI and the digestion was stopped by the addition of a 1/10 volume of DNA loading buffer (0.42% (w/v) bromophenol blue, 0.42% (w/v) xylene cyanol, 40% (w/v) sucrose and 0.2% agarose beads in 10xTAE buffer (x1 TAE buffer is 40 mM Tris-Acetate pH 8.6 containing 2 mM EDTA)). 2.5 µg of digested plasmid DNA was loaded into each 1 cm sample well on a 0.7% (w/v) agarose gel. The gel was electrophoresed at 25 mA in TAE buffer until the bromophenol blue dye front had migrated about 8 cm from the sample wells. The gel was stained in TAE buffer containing 0.2 µg/ml

ethidium bromide for one hour. The stained gel was then illuminated with short wave ultraviolet light. The DNA band corresponding to the plasmid insert was cut out of the gel with as little extraneous agarose as possible and placed in a sterile 10 ml beaker. Sufficient 0.3 M sodium acetate pH 7.0 containing 1 mM EDTA to just cover the gel slice was added and the beaker was placed in the dark for 30 to 40 minutes and the contents occasionally swirled. Each gel slice was then placed in a 0.5 ml capped test tube which had been pierced through the bottom with a 23 G needle and plugged with siliconized glass wool. The tube was capped and immersed in liquid nitrogen for ten minutes. The frozen tube was then immediatley centrifuged, within a decapped 1.5 ml capped test tube, at high speed for ten minutes in a MSE Micro Centaur microfuge. The supernatant in the larger, outer, tube was removed from the agarose pellet and recentrifuged, as above, in a fresh 1.5 ml capped test tube. The plasmid insert DNA was precipitated by the addition of a 1/100 volume of 10% (w/v) acetic acid containing 1 M $MgCl_2$ and 2.5 volumes of ethanol and then placing the tube in a dry ice/ethanol bath for 45 minutes. The precipitated DNA was recovered by centrifugation (MSE Micro Centaur microfuge, high speed for 10 minutes) and the pellet dried under vacuum. The dried pellet was resuspended in 30 µl of sterile water and stored at -20°C until use.

The yield of DNA, from this procedure, was estimated by running a sample of the purified insert (equivalent to 0.1 mg assuming 100% recovery) with a range of samples of plasmid digest equivalent to between 0.0125 µg and 0.1 µg

of insert on a 0.7% (w/v) agarose gel as described above. The relative fluoresence of the purified insert sample, as judged by eye, in comparison to those of the standard digested plasmid samples was used to estimate the percentage yield of the isolation procedure.

(b) Labeling Method

0.1 µg of purified plasmid insert DNA (denatured at 100°C for 3 minutes and then quenched on ice) was added to a 0.5 ml capped test tube containing the following; 2.5 µl of 10xnick mix (x10 is 0.5 M Tris-Cl pH 7.8 containing 50 mM $MgCl_2$ and 0.1 M β-mercaptoethanol), 2 µl each of dATP, dGTP and dTTP (50 µM in sterile water), 1 µl of spermidine (0.1 M in sterile water) and sterile water to a final volume of 18 µl. Then 20 µCi of α-^{32}P-dCTP and 4 µl of DNAse I (0.01 µg/ml from a stock solution of 0.1 mg/ml in 50% (v/v) in glycerol) was added and the tube incubated at room temperature for 5 minutes. 1 µl of DNA polymerase I was then added, the tube contents mixed gently and incubated at 15°C for 3 hours.

(ii) Oligonucleotide Primer Extension

(a) Isolation of Plasmid Inserts

Plasmid DNA was digested with either BamHI (in the case of phins214 and pDI462) or HinfI (in the case of pKA30) for three hours and the digestion stopped by the addition of a tenth volume of DNA loading buffer. 4 to

8 µg of digested plasmid DNA was loaded per 1 cm sample well on a 1% (w/v) low melting point agarose gel. The gel was run at 100 volts in TAE buffer until the bromophenol blue dye front had migrated about 8 cm from the origin. The gel tank was placed in an ice bath to prevent the gel melting. The gel was stained in TAE buffer containing 0.2 µg/ml ethidium bromide for one hour and illuminated with short wave ultraviolet light. The DNA band corresponding to the plasmid insert was cut out of the gel with as little extraneous gel as possible. The gel slice was placed in a pre-weighed 1.5 ml capped test tube and 1.5 ml of sterile water per gram of gel added. The DNA was denatured at 100°C for 7 minutes and stored at -20°C until use. The concentration of DNA was calculated from the weight of plasmid loaded per slot and the size of the plasmid insert (in base pairs) assuming 100% recovery from the gel. Before labeling the plasmid insert was denatured at 100°C for 3 minutes and kept at 37°C for 10 to 30 minutes to ensure that the agarose was liquid.

(b) Labeling Method

Stock solutions were as follows; TE buffer (3 mM Tris-Cl pH 7.0 containing 0.2 mM EDTA), solution O (1.25 M Tris-Cl pH 8.0 containing 0.125 M $MgCl_2$ stored at 4°C), solution B (2 M HEPES titrated with 4 M NaOH to pH 6.6 and stored at 4°C), dATP, dGTP and dTTP (each 0.1 M in TE buffer stored at -20°C), solution A (1000 µl solution O, 18 µl of β-mercaptoethanol and 5 µl each of dATP, dGTP and dTTP, prepared fresh for each labeling procedure) and

solution C (50 OD units of hexadeoxynucleotides suspended in 550 µl of TE buffer stored at -20°C).

The labeling reaction was set up in a 0.5 ml capped test tube by the addition of the following in order; water to a final volume of 25 µl, 5 µl of OLB buffer (10 µl of solution A, 25 µl of solution B and 15 µl of solution C), 1 µl nuclease free BSA, 25 ng of purified plasmid insert DNA, 2.5 µl of α-^{32}P-dCTP and 0.5 µl of Klenow (large fragment) polymerase I (2 units). This was then incubated at room temperature overnight.

(iii) Second Strand Synthesis of M13 DNA

Radioactively labeled single stranded DNA was prepared by second strand synthesis of M13 vectors using sequencing primer and α-^{32}P-dCTP. The M13 vectors used contained the coding strand of the cloned DNA so the anti-sense strand was radioactively labeled by this procedure.

M13 DNA was annealed to sequencing primer by heating 1 µl of M13 DNA, 1 µl of 10x sequencing buffer (x10 is 0.1 M Tris-Cl pH 7.5 containing 0.5 M NaCl and 0.1 M $MgCl_2$), 1 µl of sequencing primer (2.5 µg/ml in sterile water) and 7 µl of sterile water in a 0.5 ml capped test tube to 55°C for 15 minutes. The tube was then allowed to cool very slowly to room temperature over a period of one hour. Then 5 µl of nucleotide buffer (0.1 mM each of dATP, dGTP and dTTP in 5x sequencing buffer), 5 µl of α-^{32}P-dCTP , 7 µl of sterile water and 6 units of Klenow (large fragment) polymerase I were added to the tube. The tube was then incubated at room temperature for 15 minutes.

Purification of ^{32}P-Labeled DNA

At the end of all the DNA labeling reactions the unincorporated α-^{32}P-dCTP was separated from the ^{32}P-labeled DNA by gel filtration on Sephadex G-100. The whole of the labeling reaction was loaded onto a Sephadex G-100 fine column (equilibriated in 10 mM Tris-Cl pH 8.0 containing 1 mM EDTA) packed in a glass wool plugged Pasteur pipette to within 5 mm from the top of the pipette. The column was washed with equilibriation buffer and 100 µl aliquots were collected. The radioactivity in each fraction was monitored using a hand β-radiation monitor and the relevent fractions were pooled for use.

Measurement of α-^{32}P-dCTP Incorporation

The specific radioactivity of the α-^{32}P-dCTP labeled DNA was estimated from the incorporation of α-^{32}P-dCTP into TCA insoluble material.

Before gel filtration triplicate 1 µl aliquots of the labeling reaction were removed and each were added to 0.5 ml of 5 µg/ml denatured salmon sperm DNA in 2 mM EDTA pH 8.0 in a disposable 4 ml test tube. 150 µl of ice-cold 50% (w/v) TCA was added and the tubes stood on ice for 15 minutes. The tube contents were then filtered through a 2.5 cm GF/C filter disc and then washed four times with 20 ml of ice-cold 5% (w/v) TCA and finally with 10 ml of ice-cold ethanol. After being air dried the radioactivity on each filter disc was measured by liquid scintillation counting in 4 ml of FisoFluor 2

scintillation fluid.

Measurement of Total ^{32}P-Radioactivity

Sample aliquots were spotted onto GF/C disks, air dried for 60 minutes and placed in a scintillation vial. The radioactivity was determined by liquid scintillation counting in 4 ml of FisoFluor 2 scintillation fluid.

Tissue Culture of Islets

Aseptic techniques were used throughout all tissue culture operations. Freshly isolated islets were tissue cultured in 20 ml of Hams F12 medium supplemented with 10% (v/v) foetal calf serum, 1 mM pyruvate, 1 mM glutamine and 2 mM $CaCl_2$. Up to ~800 islets were cultured in one 90 mm Petri dish. Culture of the islets was carried out at 37°C in a LEEC incubator with a 4% CO_2 atmosphere. Tissue culture medium was equilibriated in Petri dishes in the incubator for one hour before use. Islets were removed from the tissue culture medium by hand-picking under a binocular microscope (magnification x15) with a finely drawn, siliconized, Pasteur pipette.

Dot Blot Assay of Preproinsulin mRNA

(i) Principle

The assay method is based on one previously reported for the measurement of specific RNA sequences in small samples of cultured cells (Cheley & Anderson 1984).

Guanidine hydrochloride solubilization of the islets followed by an ethanol precipitation was used to selectively enrich the samples for RNA. Serial dilutions of these samples were applied to a nitrocellulose filter using a specially designed filtration manifold which ensures consistent and reproducible application. The samples were immobilised onto the nitrocellulose filter by baking and then hybridized with ^{32}P-labeled DNA complementary in sequence to preproinsulin mRNA. After hybridization any unbound DNA was washed off and the nitrocellulose filter autoradiographed. This resulted in exposed 'dots' appearing on the X-ray film whose density was proportional to the amount of probe. The amount of preproinsulin mRNA present in the original islet samples was estimated by comparison of the optical densities of the sample dots with those of known RNA standards. Thus in any experiments that required quantitation RNA standards (5 µg each of poly A^+ RNA isolated from rat pancreas, as a positive control, and liver, as a negative control) were assayed along with the islet samples.

(ii) Method

Islets were solubilized by addition of 1 ml of 7.6 M guanidine hydrochloride in 0.1 M potassium acetate pH 5.0 followed by 5 to 10 rapid aspirations through a sterile 25 G needle mounted in a 1 ml sterile disposable syringe. The solubilized islets were transferred to a 15 ml Corex tube and the RNA precipitated overnight at -20°C after addition of 0.6 ml of 95% (v/v) ethanol. The precipitate was

recovered by centrifugation (Sorvall HB4 rotor, 6,000 rpm at -10°C for 20 minutes) and the pellet drained and the walls of the tube carefully dried with tissues.

From this point onwards any RNA standards were carried through the same protocol as that for islets. The pellet (or RNA standard) was dissolved in 250 µl of 15% (w/v) formaldehyde by vigorous shaking for 10 minutes (Stuart flask shaker) and then 250 µl of 20xSSC added. The samples were heated to 50°C for 15 minutes and then quenched on ice. Serial dilutions of the samples (typically 2 fold for islet samples and 2/3 fold for RNA standards) were made in 10xSSC in 4 ml disposable plastic test tubes on ice.

Nitrocellulose paper and two sheets of Whatman 3MM paper were cut to fit the filtration manifold. These were thoroughly wetted by floating on 10xSSC. Any nitrocellulose filters that did not wet evenly were discarded. The filtration manifold was assembled with the nitrocellulose on top of the two layers of 3MM paper and clamped firmly in place (Figure 2.1). Vacuum was applied to the manifold and 50 µl aliquots of the diluted samples were loaded into each sample well. After application of the samples each well was washed three times with 50 µl of 10xSSC. The nitrocellulose sheet was then removed from the manifold and air-dried and baked under vacuum at 80°C for one hour to immobilize the RNA. The nitrocellulose paper was then hybridized, washed and autoradiographed as described below.

Figure 2.1

Assembly of Dot Blot Filtration Manifold

Prewetted nitrocellulose, cushioned on two layers of prewetted 3MM paper, is placed in position over the sample wells of the support plate. The top plate and rubber gasket are then firmly clamped on top of the support plate and into the bottom plate by the clamping screws. These screws run through holes in the top and support plates and locate into threaded holes in the bottom plate. A vacuum is applied to the pipe in the bottom plate and the samples are loaded into the wells on the top plate. The vacuum draws the samples through the manifold and filters them onto the nitrocellulose. Cross-contamination between sample wells is prevented by the rubber gasket and an adequate vacuum seal is maintained by the rubber seal on the bottom plate.

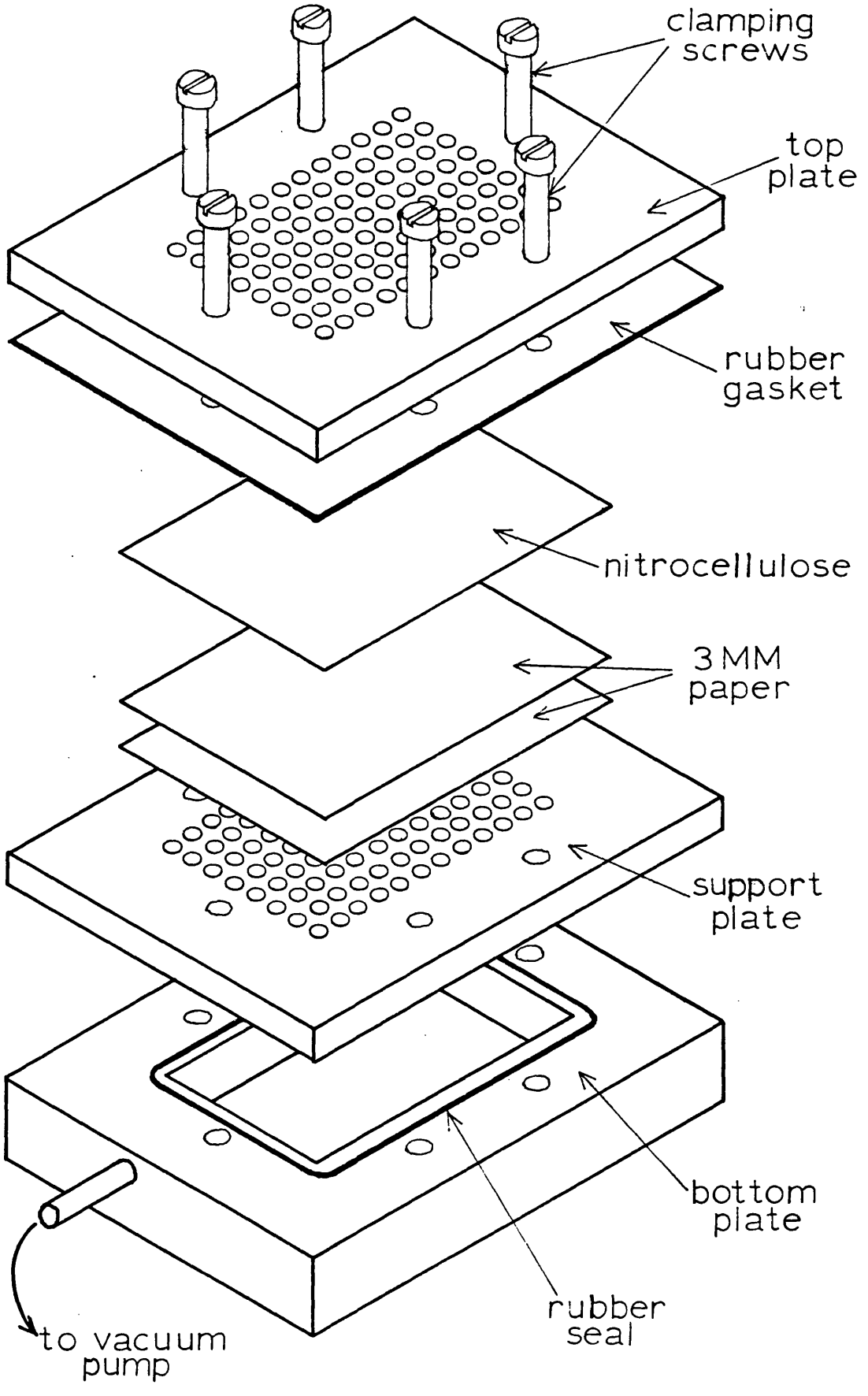

clamping screws
top plate
rubber gasket
nitrocellulose
3MM paper
support plate
bottom plate
rubber seal
to vacuum pump

Figure 2.1

Northern Blotting

RNA samples were vacuum dried, redissolved in 10 µl of denaturation mix (50% (v/v) formamide and 2.2 M formaldehyde in 20 mM $NaHPO_4$ pH 7.6), incubated at 60°C for 10 minutes and then quenched on ice. Ten microlitres of formaldehyde agarose beads were added and the samples loaded onto a 1.0% (w/v) agarose gel in 2.2 M formaldehyde, 25 mM $NaHPO_4$ pH 7.6. Lambda DNA digested with HindIII denatured with 1/10 volume of 1.5 M NaOH, 0.1 M EDTA at room temperature for ten minutes was used as molecular weight markers (~4 ng per marker lane). The gel was run at 100 volts until the bromophenol blue dye front had migrated about 8 cm. All operations involving formaldehyde were carried out in a fume cupboard. The gel was removed from the electophoresis tank and soaked in water at 60°C for 10 minutes, this was replaced with cold water for a further 10 minutes and finally 20xSSC for twenty minutes. All soaking steps were accompanied by gently agitation.

The apparatus used for blotting of the gel was the same as used for Southern blotting (Maniatis et al 1982). A plastic tray is partly filled with 20xSSC and a stack of glass plates wrapped in 3MM chromotography paper, presoaked in 20xSSC, placed in it. The agarose gel was positioned, with its original underside uppermost, on the 3MM paper avoiding trapping air bubbles beneath it. Nitrocellulose filter and two pieces of 3MM paper were cut to the size of the gel and wet by floating on 20xSSC. The nitrocellulose filter was placed on top of the gel and then the two sheets of 3MM paper on top of that. Any trapped air bubbles were removed by smoothing with a

glass rod. Finally a stack of paper towels (4-5 cm high) was placed on top of these. All the layers were weighted down with a glass plate topped with a 500 g weight and the apparatus was left at 4°C overnight to blot. As the paper towels became wet with blotting buffer they were replaced. After transfer the towels were removed and the positions of the gel slots marked on the filter with a soft pencil. The filter was removed from the gel, blotted dry and baked at 80°C under a vacuum for 2 hours.

Hybridization of Nitrocellulose

The same protocol was used for both the preproinsulin mRNA assay and the Northern blot experiments. All prehybridization and hybridization steps were carried out in Perspex hybridization chambers with screw fitted water tight lids. Prehybridization buffer was prepared by adding stock 125x Denhardts solution (1x Denhardts solution is 0.02% (w/v) each of BSA, Ficoll 400 and polyvinylpyrrolidone) and denatured salmon sperm DNA (10 mg/ml treated at 100°C for 5 minutes before use) to hybridization stock solution (50% (v/v) formamide, 5xSSC, 25 mM $NaHPO_4$ pH 6.5, 0.2% (w/v) SDS) to give final concentrations of 10x Denhardts solution and 250 µg/ml denatured DNA. This buffer was degassed under vacuum and preheated to 42°C.

The baked nitrocellulose filter was placed in the hybridization chamber with 15 ml of prehybridization buffer and the lid of the chamber was firmly screwed shut. Prehybridization was carried out overnight by immersion of the chamber in a water bath at 42°C. At the end of

prehybridization all of the buffer was drained from the filter and replaced with an equal volume of hybridization buffer [4:1 (v/v) prehybridization buffer:50% (w/v) dextran sulphate in DEPC treated water] preheated to 42°C. ^{32}P-Labeled DNA (1-8 x 10^7 cpm of 1-8 x 10^8 cpm/µg) was added to the hybridization buffer taking care to thoroughly mix the DNA with the buffer before it came into direct contact with the filter. The lid of the chamber was screwed firmly shut and hybridization was carried out for 24 hours by immersion in a water bath at 42°C.

At the end of the hybridization period the buffer was drained from the nitrocellulose filter and it was washed in several changes (250 ml each) of wash buffer and finally air dried for autoradiography. The wash buffers contained 0.1% (w/v) SDS and various concentrations of SSC according to the individual experimental protocol. Throughout the washing the nitrocellulose filter was agitated and regularly turned over.

Autoradiography

Nitrocellulose filters were fixed to stiff card and wrapped in cling film before exposure to X-ray film in a film cassette (Harmer, London), using one intensifying screen (Philips Ultra). The exposure was carried out at -70°C for between 2 days and 2 weeks. Exposed X-ray film was developed in an Agfa-Gevaert, Gevaset 437N automatic film processor.

Quantitization of Dot Blot Optical Densities

Optical densities of the autoradiograph spots were determined by scanning the autoradiographs with an LKB Ultroscan 2202 Laser Densitometer using the LKB Gelscan program running on an Apple IIe computer to collect and analyse the data from the densitometer. To ensure accurate measurement each line of spots was scanned three times at slightly different positions across the center and the resulting data merged to give an averaged result. A program written in UCSD-Pascal, called 'Gelmerge', was specifically written to achieve this (see appendix). All spot densities quoted in this study are in the units calculated by the Gelscan program. The estimation of preproinsulin mRNA content of islet samples was achieved as follows. The spot densities for the serially diluted pancreatic poly A^+ RNA standard were plotted against the amount of RNA. This standard curve was used to estimate the amount of preproinsulin mRNA in a sample by reading off the equivalent amount of pancreatic poly A^+ RNA. The sample spot used for this estimation was the lowest dilution possible on the most linear portion of the standard curve.

Chapter 3

The Effect of Glucose on (Pro)insulin Synthesis

Introduction

Glucose was first shown to stimulate the incorporation of radiolabeled amino acids into (pro)insulin in slices of bovine pancreas (Taylor et al 1964; Parry & Taylor 1966). Subsequent studies have attempted to characterize and elucidate the mechanism of glucose induced (pro)insulin synthesis using pancreatic islet tissue derived from a variety of mammalian species.

The rate of (pro)insulin synthesis is usually measured by following the incorporation of radioactively labeled amino acids, such as ^{3}H-Leucine or ^{3}H-phenylalanine, into newly synthesised (pro)insulin. Various types of extraction procedure are commonly used to separate (pro)insulin from other islet proteins, these include gel filtration chromotography (Morris & Korner 1970a), polyacrylamide gel electrophoresis (PAGE)(Baird & Bauer 1976) and immune-binding techniques. Immune-binding procedures use either double antibody precipitation (Zucker & Logothetopoulos 1975) or anti-insulin serum coupled to a cellulose gel matrix (Howell & Taylor 1966; Bone & Howell 1977). It has been demonstrated that glucose stimulates the synthesis of (pro)insulin in rat islets (pregnant (Bone & Howell 1977), foetal, newborn (Asplund 1973) and normal (Morris & Korner 1970a)), human islets (Jahr et al 1980a), a rat islet adenoma (Johnson et al 1984), mouse islets (Schatz et al 1973), rabbit islets (Howell & Taylor 1966) and bovine pancreatic slices (Taylor et al 1964; Parry & Taylor 1966). Glucose also stimulates the synthesis of other islet cell proteins (Morris & Korner 1970a; Permutt & Kipnis 1970). Depriving many types of mammalian

cells of glucose reduces their rate of cellular protein synthesis (Means et al 1968; Hollinger & Hwang 1972; van Venrooji et al 1972; Freudenberg & Mager 1971). However, the stimulation of protein synthesis by changes in the concentration of glucose over the physiological range (2 to 20 mM (Steiner & Williams 1959)) appears to be unique to islet tissue. No such effect has been observed in Landschutz ascites cells, rat liver slices (Morris & Korner 1970a), exocrine pancreatic tissue (Schatz et al 1973) and rat muscle or diaphragm (Manchester & Young 1958). This stimulation is preferential for (pro)insulin synthesis since the fraction of total protein synthesis due to (pro)insulin synthesis increases with glucose concentration (Morris & Korner 1970a).

There are two forms of rat insulin, insulins I and II, and these are present in approximately equal amounts in crystalline insulin extracted and purified from rat pancreas (Clark & Steiner 1969). When the glucose concentration is increased, however, there is marked preferential synthesis of insulin I relative to II. After 4 hours incubation the ratio of synthesis of insulin I relative to II in rat islet tissue was found to be ~1.0 at 5.5 mM and ~10.0 at 16.0 mM glucose (Kakita et al 1982).

The dose response for the effect of glucose on the rate of (pro)insulin synthesis is sigmoidal with a threshold of between 2 and 4 mM glucose and a maximal rate at 15 to 20 mM (Pipeleers et al 1973; Bone & Howell 1977; Ashcroft et al 1978). On raising the glucose concentration from 4 to 20 mM an increase of ~30-fold in synthesis rate of (pro)insulin and of ~4-fold for total protein was observed (Ashcroft et al 1978). At 2 mM glucose (pro)insulin constituted ~5% of the total

protein synthesised and at 20 mM glucose ~21% (Ashcroft et al 1978). Since insulin is synthesised as a prohormone the rate at which proinsulin is converted to insulin is a crucial factor in determining the over all rate of proinsulin synthesis. The half-time of conversion has been reported as 36 minutes (Gold et al 1986) which was reduced to 24 minutes in islets isolated from rats made hyperglycemic by glucose infusion (Logothetopoulos & Jain 1980b) and 20 minutes by pretreatment of rat islets with tolbutamide (Gold et al 1986), a compound which stimulates (pro)insulin secretion (Coore & Randle 1964; Montague & Taylor 1968). Time course studies have shown that incorporation of ^{3}H-leucine into (pro)insulin is linear over 2 hours in islets incubated at 2.8 mM glucose. However, the time course of ^{3}H-leucine incorporation into (pro)insulin in islets incubated at 15.3 mM glucose is biphasic. The first phase is at a higher rate than at 2.8 mM glucose and lasts 45 minutes. This is followed by a second phase at a higher rate (Permutt & Kipnis 1972a). Pretreatment of the islets with actinomycin D, an inhibitor of RNA synthesis (Goldberg et al 1962), reduced the second phase of incorporation and the residual rate was equal to that of the first phase. The incorporation rate at 2.8 mM glucose was unaffected by actinomycin D pretreatment (Permutt & Kipnis 1972a). These results imply that RNA synthesis plays a role in the glucose stimulation of (pro)insulin synthesis. This is supported by the observation that glucose stimulated islet RNA synthesis (Permutt & Kipnis 1972b).

In this chapter the effect of glucose on (pro)insulin and total protein synthesis was determined during short term (up to 2 hours) *in vitro* incubations of rat pancreatic islets. The

possible role of RNA synthesis in any glucose effect was also investigated by using actinomycin D to inhibit RNA synthesis. Studies similar to the work presented in this chapter have been carried out by other workers. It was necessary, however, to repeat these studies in order to correlate the observed rates of *in vitro* (pro)insulin synthesis, in the system used here, with the data on preproinsulin mRNA presented in the next chapter.

Methods

Characterization of AIS-Sepharose

(i) Measurement of AIS-Sepharose Insulin Binding Capacity

This was achieved by measuring the competition for a fixed number of immune-binding sites on a small quantity of AIS-Sepharose between increasing amounts of unlabeled insulin and a trace quantity of ^{125}I-insulin.

A number of capped 1.5 ml test tubes were set up each containing 20 mg of AIS-Sepharose (or NGPS-Sepharose as a control), approximately 20,000 cpm of ^{125}I-insulin and a range of concentrations of unlabeled insulin (0 to 10 µg/ml) all in a total volume of 1 ml of binding buffer. The tubes were then end over end mixed on a rotating platform (30 rpm) for 90 minutes and the gel pelleted (MSE Micro Centaur microfuge, 1 minute, high speed). Duplicate 100 µl aliquots of supernatant were gamma counted for five minutes in a Beckman Gamma 5500 counter.

Binding of ^{125}I-insulin to AIS- and NGPS-Sepharose in the presence of islet sonicate was also examined. Fifty islets were sonicated in 1.8 ml of binding buffer and approximately 10,000 cpm of ^{125}I-insulin added to the sonicate before pelleting of particulate matter (MSE Micro Centaur microfuge, 5 minutes, high speed). 0.5 ml aliquots of the supernatant were loaded onto disposable polypropylene chromotography columns packed with 1 ml of either AIS-Sepharose or NGPS-Sepharose equilibrated in binding buffer. The supernatant was allowed to run into the gel and

left for 120 minutes at room temperature. The columns were then washed with 20 ml of binding buffer followed by 10 ml of elution buffer (1 M acetic acid containing 2.5 mg/ml BSA). 1 ml fractions of both buffers were collected and their radioactivity determined, as described above, as was the radioactivity in duplicate 0.1 ml aliquots of the supernatant sample.

(ii) Measurement of Islet Sonicate Binding by AIS-Sepharose

The capacity of AIS-Sepharose and NGPS-Sepharose to selectively bind *in vitro* labeled (pro)insulin was examined as follows. Sixty islets were incubated in 500 µl of medium containing 16.7 mM glucose and 40 µCi ^{3}H-leucine for 120 minutes. At the end of the incubation the medium was removed and the islets sonicated in 1 ml of sonication buffer. Three different volumes of islet sonicate (100, 50 and 25 µl) were treated with both AIS-Sepharose and NGPS-Sepharose as follows. Each aliquot was mixed (end over end) with 20 mg of gel in a total volume of 1 ml of binding buffer for 120 minutes at room temperature. The gel was then pelleted (MSE Micro Centaur microfuge, 1 minute, high speed) and the supernatant removed. The gel was then washed by mixing (end over end) for 5 minutes with 1 ml of fresh binding buffer which was removed from the gel by centrifugation. The gel was washed a further three times in this manner. Bound material was eluted from the gel by mixing (end over end) it with 1 ml of elution buffer for 180 minutes. The gel was then pelleted as above, the supernatant removed, lyophilized and reconstituted in 0.2 ml of running buffer containing 10% (w/v) glucose (to increase

the density of the sample in order to facilitate sample loading). The treated supernatant was then subjected to G-50 Sephadex gel fractionation along with a sample of the untreated sonicate.

Dose Response of Glucose on (Pro)insulin Synthesis

Groups of 10 islets were incubated for 120 minutes in medium containing 2.5 mM, 5.0 mM, 10.0 mM, 15.0 mM or 20.0 mM glucose and 10 µCi of L-[4,5-^{3}H]-leucine. At the end of the incubation period the islets were pelleted, the supernatant aspirated and the islet pellet was sonicated in 1 ml of sonication buffer. Duplicate aliquots of sonicate were removed for the determination of (pro)insulin synthesis, total protein synthesis and islet insulin content.

Time Course of (Pro)Insulin Synthesis

Groups of 20 islets were incubated for 30, 60, 90, 120 or 180 minutes in medium containing either 2.5 mM or 20.0 mM glucose and 10 µCi of L-[4,5-^{3}H]-leucine. At the end of the incubation period the islets were pelleted, the supernatant aspirated and the islet pellet was sonicated in 1 ml of sonication buffer. Duplicate aliquots of sonicate were removed for the determination of (pro)insulin synthesis, total protein synthesis and islet insulin content.

Time Profile of (Pro)Insulin Synthesis

Groups of 20 islets were incubated for 30, 60, 90 or 120 minutes in medium containing 2.5 mM or 20.0 mM glucose. Thirty minutes before the end of each incubation period 20 µCi of L-[4,5-^{3}H]-leucine was added. Both preincubations and incubations were carried out in either the presence or absence of 10 µg/ml actinomycin D. At the end of the incubation period the islets were pelleted, the supernatant aspirated and the islet pellet was sonicated in 500 µl of sonication buffer. Duplicate aliquots of sonicate were removed for the determination of (pro)insulin synthesis, total protein synthesis and islet insulin content.

Effect of Actinomycin D on (Pro)Insulin Synthesis at Different Glucose Concentrations

Groups of 20 islets were incubated for 120 minutes in medium containing 2.5 mM, 5.0 mM, 10.0 mM, 15.0 mM or 20.0 mM glucose. Sixty minutes before the end of each incubation period 20 µCi of L-[4,5-^{3}H]-leucine was added. Both the preincubations and the incubations were carried out in either the presence or absence of 10 µg/ml actinomycin D. At the end of the incubation period the islets were pelleted, the supernatant aspirated and the islet pellet was sonicated in 500 µl of sonication buffer. Duplicate aliquots of sonicate were removed for the determination of (pro)insulin synthesis, total protein synthesis and islet insulin content.

Results

All results in this chapter are given as mean values with their standard errors. Data from separate experimental islet preparations were standardized by the use of index values. The index value for a particular parameter was calculated as the ratio of the value to that of the standard sample for each experiment. The standard sample was chosen as the sample which had been incubated in 20.0 mM glucose or, in the case of the (pro)insulin synthesis profiles, as the sample which had been incubated for 90 to 120 minutes at 20.0 mM glucose in the absence of actinomycin D. Statistical significance values were calculated using a two-tailed Students T-test.

Characterization of AIS-Sepharose

(i) Measurement of AIS-Sepharose Insulin Binding Capacity

Figure 3.1 shows the percentage of the total ^{125}I-insulin bound plotted against the log of the concentration of unlabeled insulin present. The insulin binding capacity of the AIS-Sepharose was estimated from the point at which the concentration of unlabeled insulin first inhibited the AIS-Sepharose binding of ^{125}I-insulin. Typically AIS-Sepharose preparations had an insulin binding capacity in excess of 16 µg/g of gel. NGPS-Sepharose bound less than 2.5% of the total ^{125}I-insulin at all concentrations of unlabeled insulin indicating a low level of non-specific insulin binding.

Figure 3.1

Insulin Binding of AIS- and NGPS-Sepharose

Trace quantities of ^{125}I-insulin with increasing concentrations of unlabeled insulin were bound to either AIS-Sepharose or NGPS-Sepharose. The bound material was eluted and the radioactivity (cpm) determined and the percentage of the total ^{125}I-insulin bound was plotted against the log of the concentration of the unlabeled insulin for both AIS-Sepharose treated samples (closed triangles) and NGPS-Sepharose treated samples (open triangles).

Each data point represents the mean value of duplicate aliquots from the same sample. The point at which increasing concentrations of unlabeled insulin started to inhibit the binding of ^{125}I-insulin by AIS-Sepharose is indicated by an arrow.

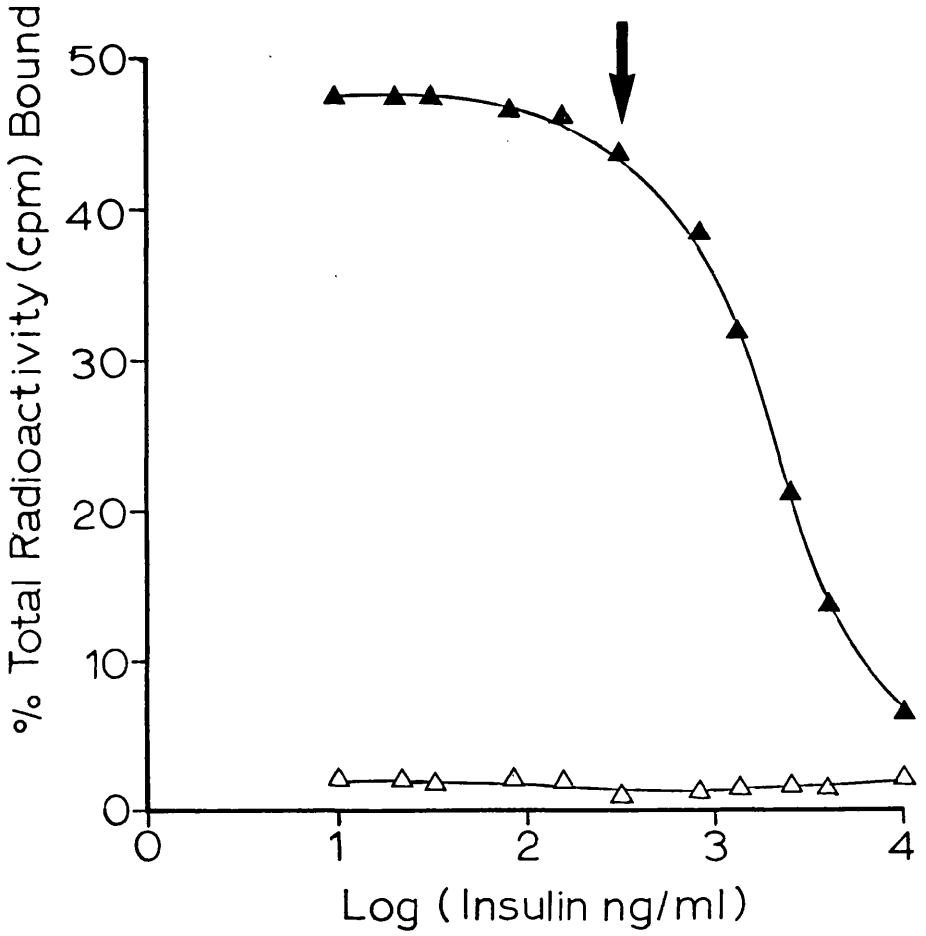

% Total Radioactivity (cpm) Bound
50
40
30
20
10
0
0
1
2
3
4
Log (Insulin ng/ml)

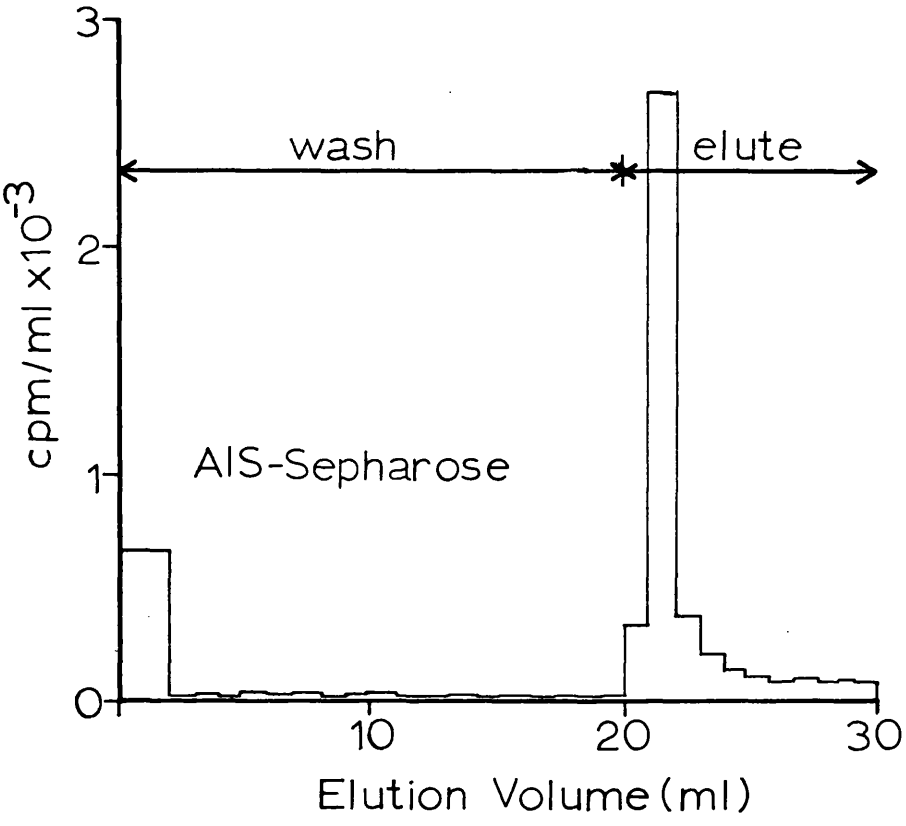
Figure 3.1

Figure 3.2

Binding of ^{125}I-Insulin by AIS-Sepharose and NGPS-Sepharose in the Presence of Islet Sonicate

Samples of islet sonicate spiked with ^{125}I-insulin were bound to columns of either AIS-Sepharose or NGPS-Sepharose and then washed with binding buffer followed by elution buffer. The radioactivity (cpm) in the eluted fractions were determined for both the AIS-Sepharose column and the NGPS-Sepharose column and plotted against the elution volume.

Arrows indicate the volume of column washing with binding buffer and elution buffer.


3
2
1
0
cpm/ml x10-3
wash
elute
AIS-Sepharose
10
20
30
Elution Volume (ml)

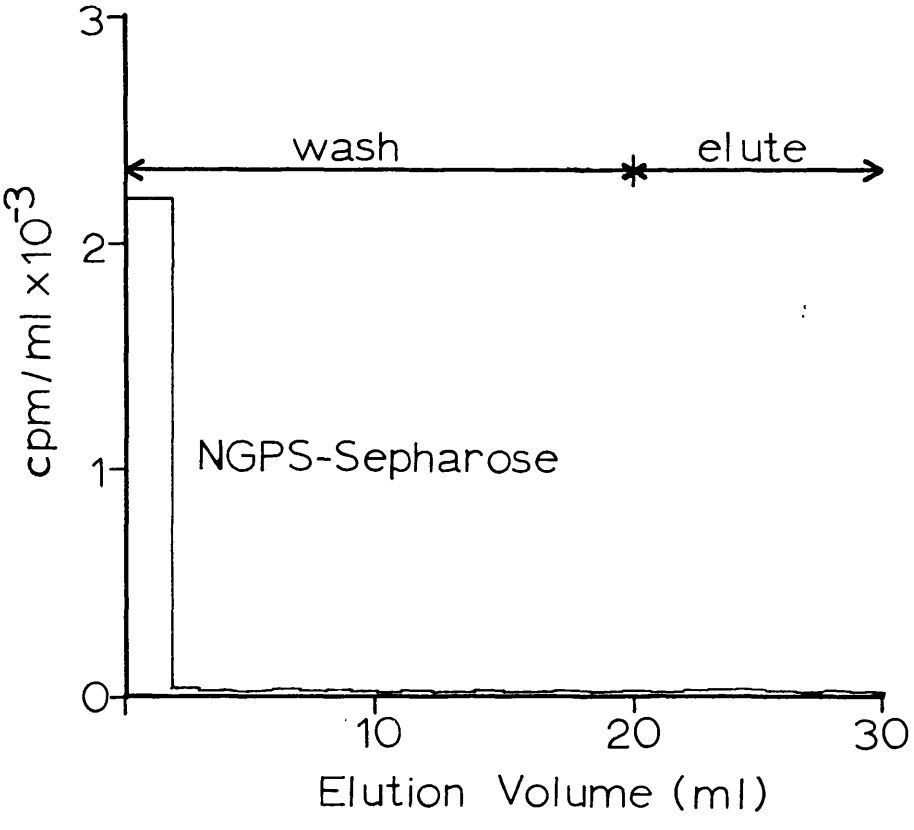

3
2
1
0
cpm/ml x10-3
wash
elute
NGPS-Sepharose
10
20
30
Elution Volume (ml)

Figure 3.2

(ii) Measurement of Islet Sonicate Binding by AIS-Sepharose

Figure 3.3 shows the Sephadex G-50 fractionation profile of untreated islet sonicate. The radioactivity was eluted from the column in four peaks. One at the void volume of the column (V_o) (Peak 1), one at the total volume of the column (V_t) (Peak 4) and two peaks of intermediate mobility (Peaks 2 & 3). Peak 1 consisted of labeled material larger than or at the upper end of the gel size fractionation range (1500-30000 daltons for peptides) that were excluded from the gel matrix and had a small shoulder associated with it. The material in peak 4 was of low molecular weight material not fractionated by the gel and as such probably represents unincorporated ^{3}H-Leucine. Peaks 3 and 4 lay within the size fractionation range of the gel matrix and the position of the slower peak (K_{av}=0.50) corresponded to that of the major peak of insulin immunoreactivity. The position of the faster of the two intermediate peaks (K_{av}=0.30) corresponded to that of a small shoulder in the peak of insulin immunoreactivity.

Figures 3.4, 3.5 and 3.6 show the Sephadex G-50 fractionation profiles of 100 µl, 50 µl and 25 µl aliquots of islet sonicate, which had been treated with AIS- or NGPS-Sepharose, respectively. The fractionation profiles of all three treated aliquot volumes were similar. The AIS-Sepharose treated sonicate profiles contained all the peaks present in the untreated sonicate with the exception of peak 4. The NGPS-Sepharose treated sonicate profiles only contained peak 1.

Figure 3.3

Sephadex G-50 Fractionation Profile of Untreated Islet Sonicate

Islets were incubated in medium containing 16.7 mM glucose and ^{3}H-Leucine for 120 minutes, sonicated and then fractionated on a Sephadex G-50 column. The radioactivity (cpm) (solid line) and the immunoreactive insulin content (dashed line) of each fraction were determined.

Numbered arrows indicate the positions of the peaks in eluted radioactivity (Peaks 1 to 4). The void volume (V_o) and the total volume (V_t) of the column are marked by arrows.

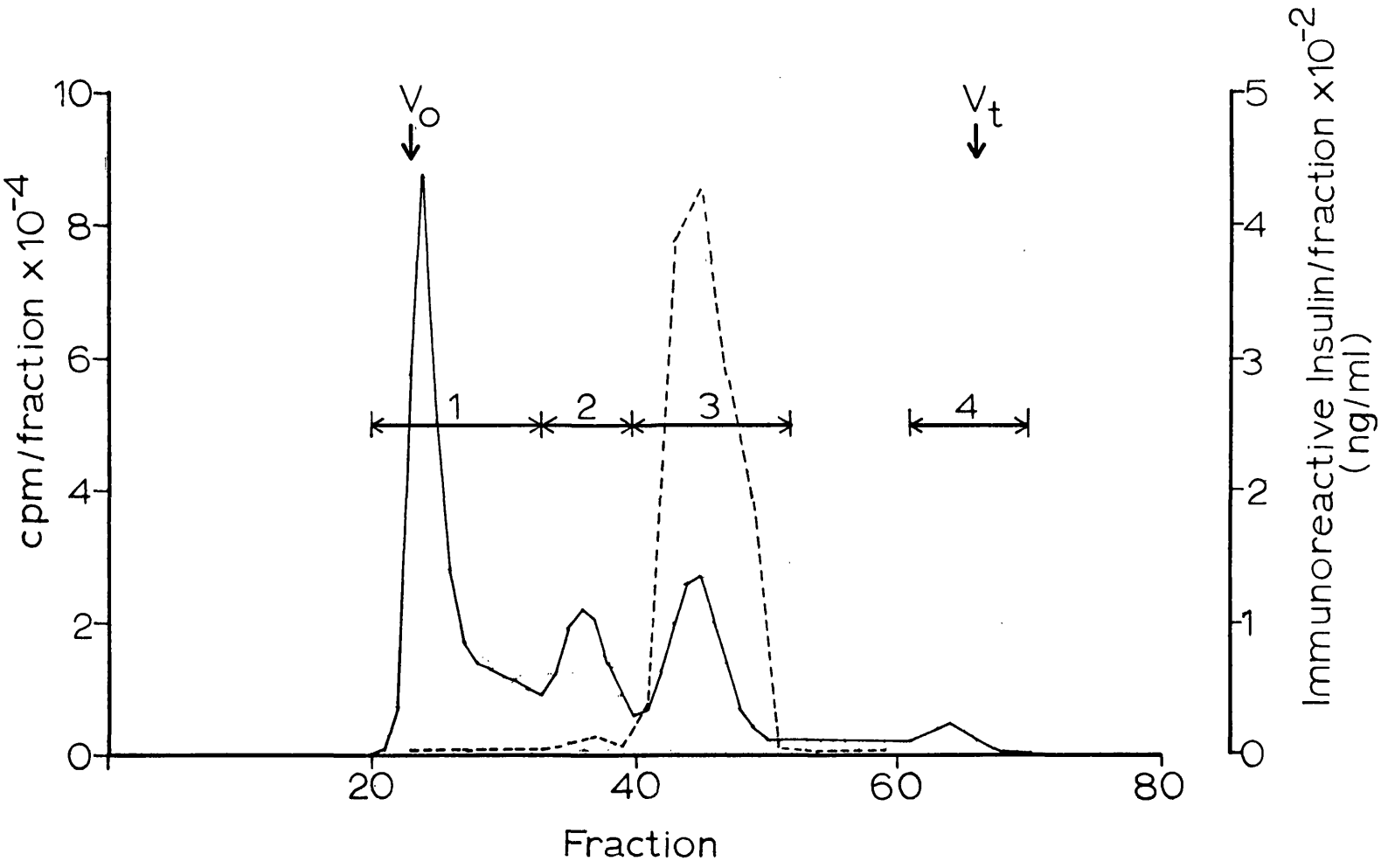

cpm/fraction x10⁻⁴
Immunoreactive Insulin/fraction x10⁻²
(ng/ml)
Vo
Vt
1
2
3
4
Fraction
0
2
4
6
8
10
20
40
60
80
1
2
3
4
5

Figure 3.3

Figure 3.4

Sephadex G-50 Fractionation Profile of AIS-Sepharose and NGPS-Sepharose Treated 100 µl Aliquots of Islet Sonicate

Sixty islets were incubated in medium containing 16.7 mM glucose and ^{3}H-leucine for 120 minutes and then sonicated. 100 µl aliquots of sonicate were bound to either AIS-Sepharose or NGPS-Sepharose and the bound material was fractionated on a Sephadex G-50 column. The radioactivity of each fraction was determined for the AIS-Sepharose treated sample (solid line) and the NGPS-Sepharose treated sample (dashed line).

The results were adjusted to the original volume of the sonicated islet sample. The positions of the two intermediate mobility peaks are given as their K_{av} values. The void volume (V_o) and the total volume (V_t) of the column are marked by arrows.

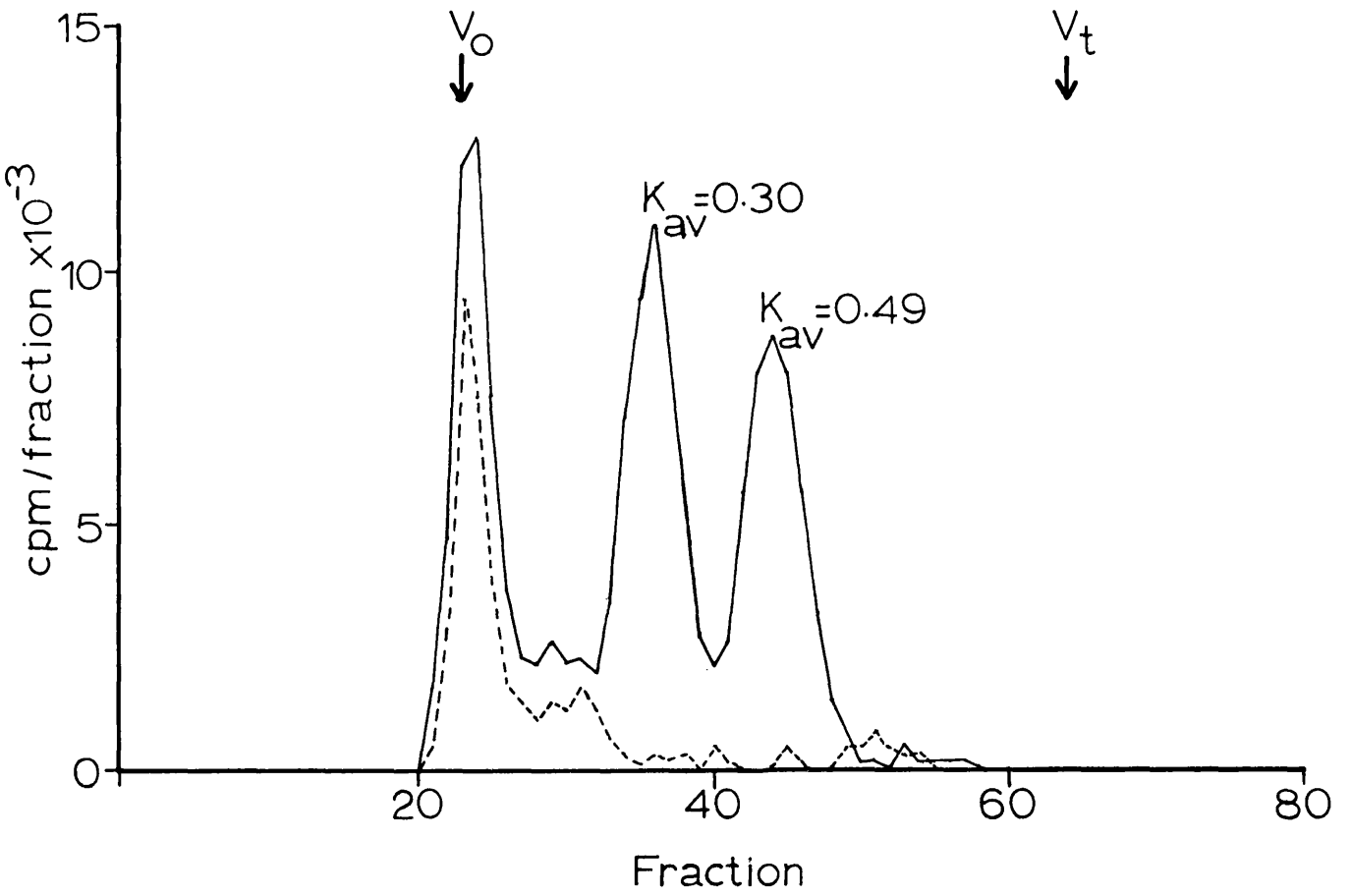

15
10
5
0
cpm / fraction x10^-3
V_o
V_t
K_av=0.30
K_av=0.49
20
40
60
80
Fraction

Figure 3.4

Figure 3.5

Sephadex G-50 Fractionation Profile of AIS-Sepharose and NGPS-Sepharose Treated 50 µl Aliquots of Islet Sonicate

Sixty islets were incubated in medium containing 16.7 mM glucose and ^{3}H-Leucine for 120 minutes and then sonicated. 50 µl aliquots of sonicate were bound to either AIS-Sepharose or NGPS-Sepharose and the bound material was fractionated on a Sephadex G-50 column. The radioactivity of each fraction was determined for the AIS-Sepharose treated sample (solid line) and the NGPS-Sepharose treated sample (dashed line).

The results were adjusted to the original volume of the sonicated islet sample. The positions of the two intermediate mobility peaks are given as their K_{av} values. The void volume (V_o) and the total volume (V_t) of the column are marked by arrows.

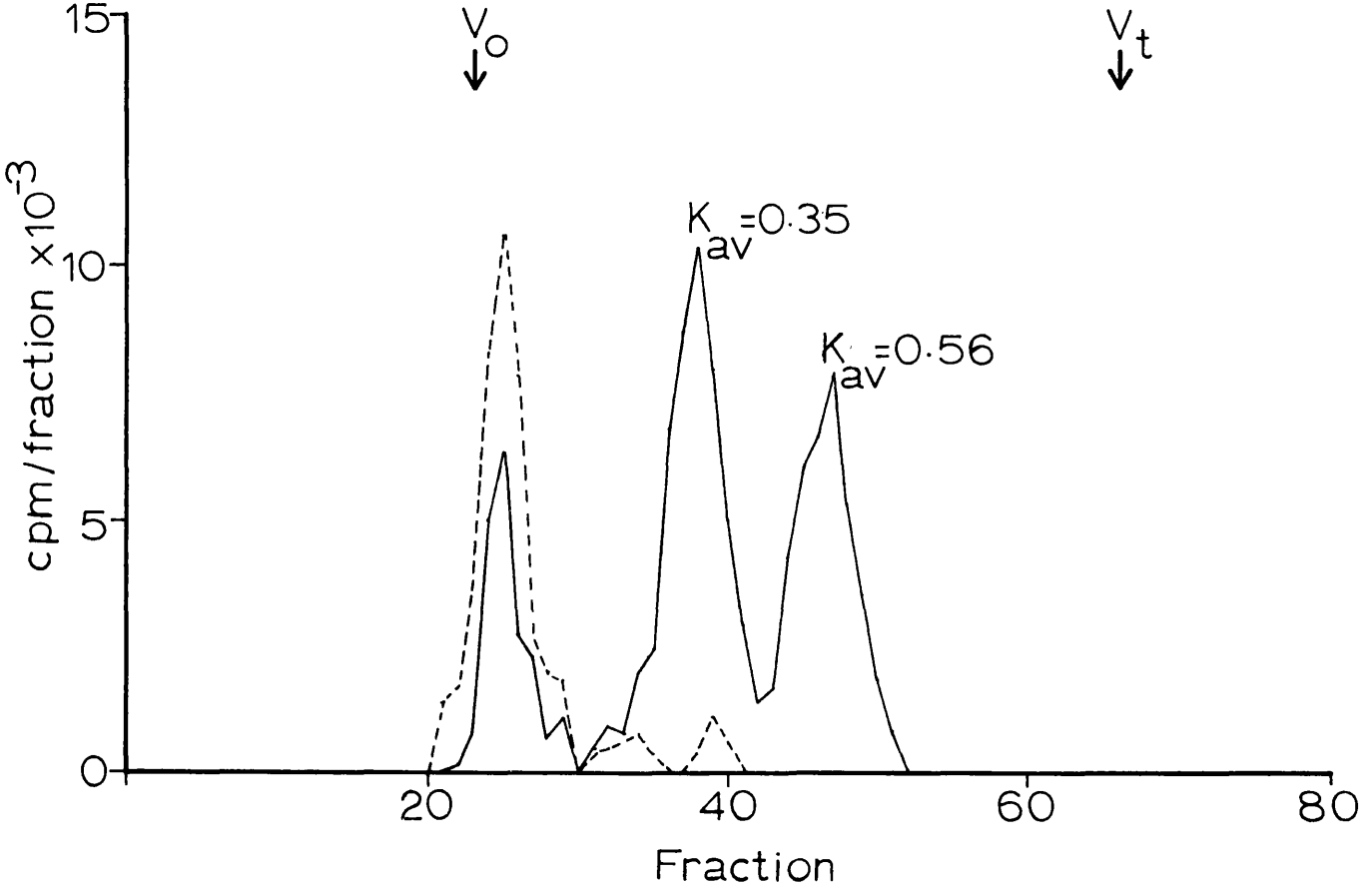

15
10
5
0
cpm/fraction x10-3
Vo
Vt
Kav=0·35
Kav=0·56
20
40
60
80
Fraction

Figure 3.5

Figure 3.6

Sephadex G-50 Fractionation Profile of AIS-Sepharose and NGPS-Sepharose Treated 25 µl Aliquots of Islet Sonicate

Sixty islets were incubated in medium containing 16.7 mM glucose and ^{3}H-Leucine for 120 minutes and then sonicated. 25 µl aliquots of sonicate were bound to either AIS-Sepharose or NGPS-Sepharose and the bound material was fractionated on a Sephadex G-50 column. The radioactivity of each fraction was determined for the AIS-Sepharose treated sample (solid line) and the NGPS-Sepharose treated sample (dashed line).

The results were adjusted to the original volume of the sonicated islet sample. The positions of the two intermediate mobility peaks are given as their K_{av} values. The void volume (V_o) and the total volume (V_t) of the column are marked by arrows.

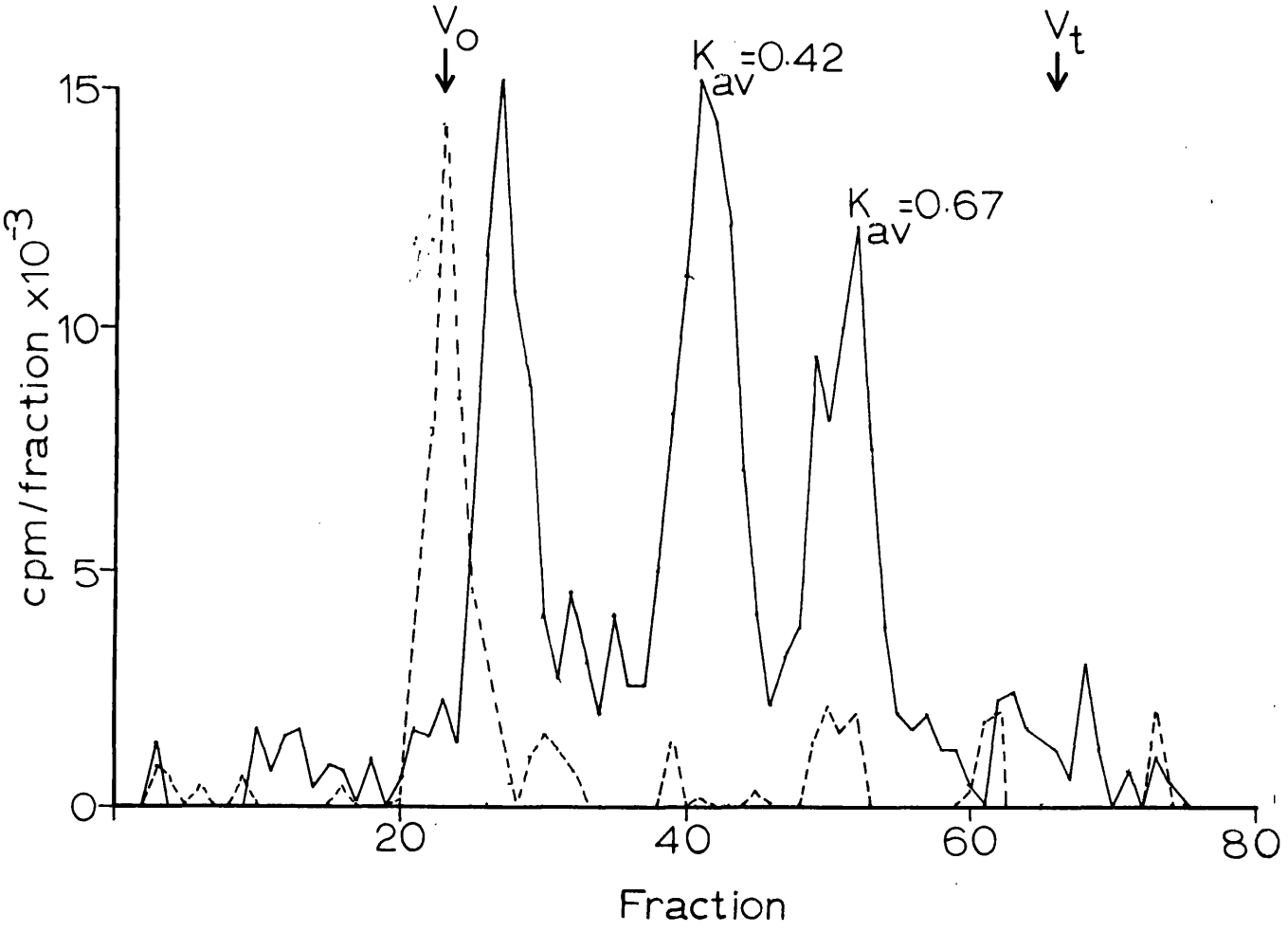

V_o
K_{av}=0.42
V_t
K_{av}=0.67
15
10
5
0
cpm/fraction x10^{-3}
20
40
60
80
Fraction

Figure 3.6

Table 3.1 shows the recoveries of ^{3}H-leucine labeled material in peaks 1,2 and 3 (see Figure 3.3) of the Sephadex G-50 fractionated AIS-Sepharose and NGPS-Sepharose treated aliquots of islet sonicate (100 µl, 50 µl and 25 µl) as a percentage of the peaks in the untreated islet sonicate.

The recoveries of peaks 2 and 3 in the 25 µl sonicate sample were ~50% greater than in the 100 µl and 50 µl sonicate samples. The recoveries for the NGPS-Sepharose treated samples were similar for all three sonicate volumes.

Percentage Recovery of Fractionated Peaks

Sample	Peak 1	Peak 2	Peak 3
Whole sonicate	100.0%	100.0%	100.0%
Treated sonicate (100 µl aliquot)	17.2% (10.3%)	48.3% (0%)	31.7% (0%)
Treated sonicate (50 µl aliquot)	5.0% (12.2%)	47.5% (0%)	27.2% (0%)
Treated sonicate (25 µl aliquot)	27.8% (12.9%)	77.4% (0%)	48.8% (0%)

Table 3.1 - Recoveries of G-50 Fractionated ^{3}H-Leucine Labeled Material

Different volumes of islet sonicate, from islets incubated at 16.7 mM glucose for 180 minutes in the presence of ^{3}H-leucine, were bound to AIS-Sepharose or NGPS-Sepharose and the bound material was subjected to Sephadex G-50 gel fractionation (see Figures 3.3, 3.4, 3.5 and 3.6). The radioactivity (cpm) eluted in peaks 1,2 and 3 (see Figure 3.3) was determined and the percentage recovery of each peak, relative to that of a sample of untreated sonicate, was calculated for both AIS-Sepharose bound material and NGPS-Sepharose bound material (in brackets).

NGPS-Sepharose Controls

In all experiments NGPS-Sepharose control samples bound ~10%±2% of the TCA precipitable radioactivity (cpm). There was no statistically significant difference in this value between experiments or between samples in the same experiment.

Islet Insulin Contents

The mean islet insulin content was 69.3±28.7 ng (n=88) for all the experiments. There was no statistically significant difference between islet insulin contents for different tissue preparations.

Dose Response of Glucose on (Pro)insulin Biosynthesis

Figure 3.7 shows the dose-response for glucose stimulated (pro)insulin synthesis. (Pro)insulin synthesis increased with glucose concentration in a sigmoidal fashion. Only the values at 15.0 mM and 20.0 mM glucose were statistically significantly different from that at 2.5 mM glucose ($P<0.05$ and $P<0.005$ respectively). The threshold for glucose stimulation was between 2.5 mM and 5 mM. The half-maximal rate occurred at ~12 mM glucose and the maximal rate, at 20.0 mM glucose, was 5-fold higher than that at 2.5 mM glucose. The dose-response was steepest between 5.0 mM and 15.0 mM glucose.

Figure 3.8 shows the same data as in Figure 3.7 expressed as fractional (pro)insulin synthesis. Overall the dose-response curve had the same shape as that seen in Figure 3.7

but with a different half-maximal value (~8 mM glucose) and the maximal rate at 20.0 mM glucose was only ~2.5-fold higher than that at 2.5 mM glucose. Once again only the values at 15.0 mM and 20.0 mM glucose were statistically significantly different from that at 2.5 mM glucose ($P<0.05$ and $P<0.0005$ respectively).

Figure 3.7

Glucose Dose Response for (Pro)Insulin Synthesis

Islets were incubated in medium containing ^{3}H-Leucine and various concentrations of glucose for 120 minutes. At the end of the incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin synthesis rate. The radioactivity (cpm) in the AIS-Sepharose bound material was plotted against the glucose concentration of the incubation medium.

The data points are the means of a number of observations, which are noted beside each data point, with standard error bars.

* $P<0.05$ relative to 2.5 mM glucose incubation

** $P<0.005$ relative to 2.5 mM glucose incubation

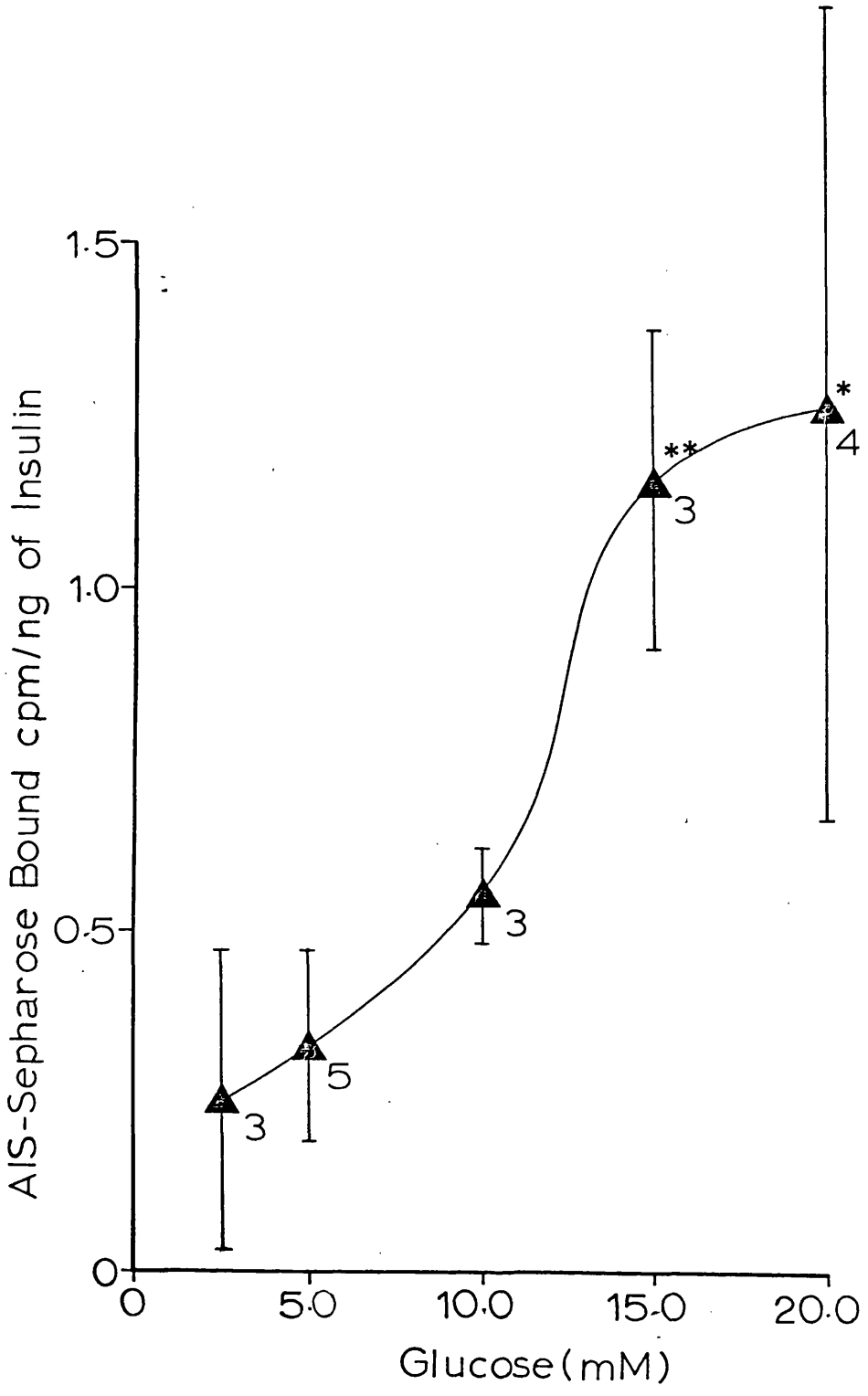

1·5
1·0
0·5
0
AIS-Sepharose Bound cpm/ng of Insulin
0
5·0
10·0
15·0
20·0
Glucose (mM)
3
5
3
**
3
*
4

Figure 3.7

Figure 3.8

Glucose Dose Response for Fractional (Pro)Insulin Synthesis

Islets were incubated in medium containing ^{3}H-leucine and various concentrations of glucose for 120 minutes. At the end of the incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin and total protein synthesis rates. The index values of the fractional (pro)insulin synthesis rate were calculated and plotted against the glucose concentration of the incubation medium.

The data points are the means of a number of observations, which are noted beside each data point, with standard error bars.

* $P<0.05$ relative to 2.5 mM glucose incubation

*** $P<0.0005$ relative to 2.5 mM glucose incubation

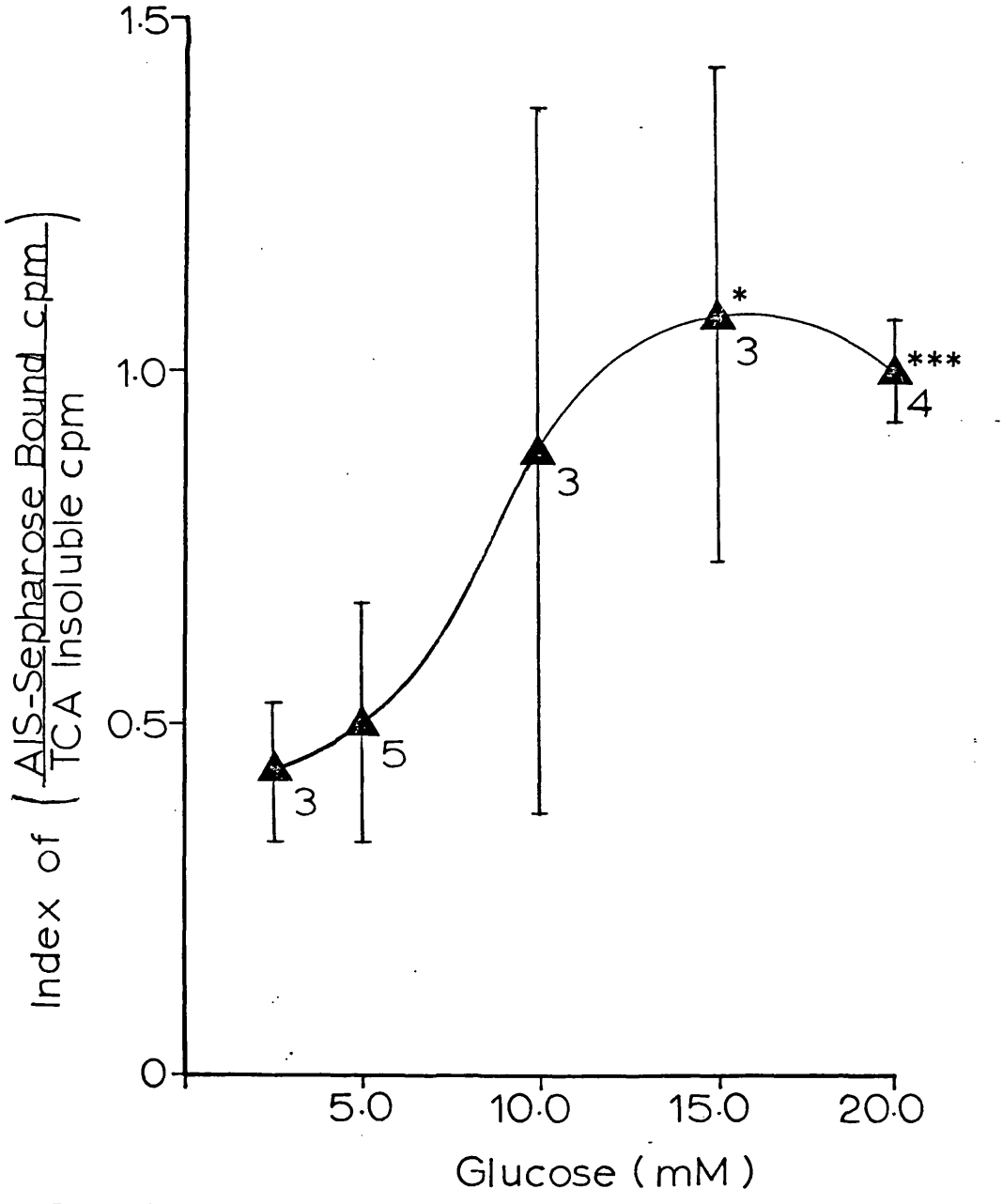

1·5
1·0
0·5
0
Index of (AIS-Sepharose Bound cpm / TCA Insoluble cpm)
5·0
10·0
15·0
20·0
Glucose (mM)
3
5
3
*
3
***
4

Figure 3.8

Time Course of (Pro)Insulin Biosynthesis

Figure 3.9 shows the time course of (pro)insulin synthesis in islets incubated at either 2.5 mM or 20.0 mM glucose. (Pro)insulin synthesis at 2.5 mM glucose was linear over the 180 minutes of the experiment. Synthesis at 20.0 mM glucose was the same as that at 2.5 mM glucose after 30 minutes but had increased rapidly to a value ~3-fold higher after 60 minutes. From 60 minutes onwards the synthesis rate at 20.0 mM glucose was linear and roughly paralleled that at 2.5 mM glucose but at a higher rate. The only statistically significant difference between the synthesis rates at 2.5 mM and 20.0 mM glucose was at the 180 minutes time point ($P<0.05$).

Figure 3.10 shows the time course of total protein synthesis in islets incubated at either 2.5 mM or 20.0 mM glucose. The rate of synthesis at 2.5 mM glucose was linear over the whole 180 minutes of incubation and was closely paralleled by that at 20.0 mM glucose for the first 120 minutes. After 120 minutes the synthetic rate at 20.0 mM glucose appeared to plateau slightly. There were no statistically significant differences between the 2.5 mM and 20.0 mM glucose values at any of the time points.

Figure 3.11 shows the time course of fractional (pro)insulin synthesis. The synthetic rate at 2.5 mM glucose remained constant at ~1.5% of total islet protein synthesis over the whole of the 180 minute incubation period. For the 20.0 mM glucose incubations there was a sharp increase in the synthetic rate from ~3% to ~7% between 30 and 60 minutes which then levelled out to 5% after 90 minutes. Only the 60 and 180

minute time points showed a statistically significant difference between the 2.5 mM and 20.0 mM glucose values ($P<0.005$ for both).

Figure 3.9

Time Course of (Pro)insulin Synthesis

Islets were incubated in medium containing ^{3}H-leucine and either 2.5 mM or 20 mM glucose for various times. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin synthesis and insulin content. The ratio of (pro)insulin synthesis to the islet insulin content was plotted against time for incubations in medium containing 2.5 mM (open triangles and dashed line) and 20 mM (closed triangles and solid line) glucose.

The data points are the mean values of duplicate aliquots from pairs of samples with standard error bars.

* $P<0.05$ relative to same incubation time in 2.5 mM glucose.

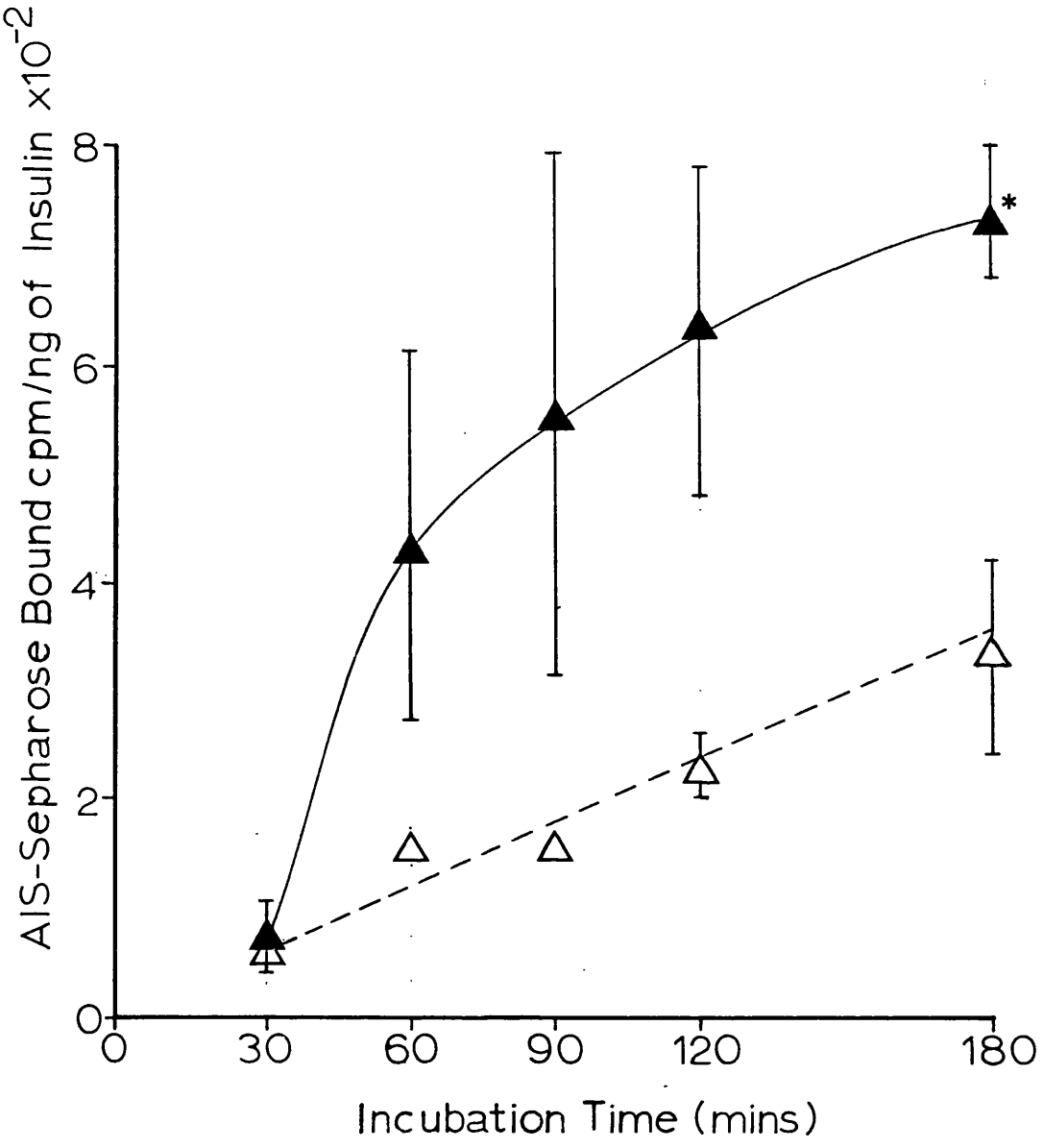

AIS-Sepharose Bound cpm/ng of Insulin x10⁻²
0
2
4
6
8
0
30
60
90
120
180
Incubation Time (mins)
*

Figure 3.9

Figure 3.10

Time Course of Total Protein Synthesis

Islets were incubated in medium containing ^{3}H-leucine and either 2.5 mM or 20 mM glucose for various times. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for total protein synthesis synthesis and insulin content. The ratio of total protein synthesis to the islet insulin content was plotted against time for incubations in medium containing 2.5 mM (open triangles and dashed line) and 20 mM (closed triangles and solid line) glucose.

The data points are the mean values of duplicate aliquots from pairs of samples with standard error bars.

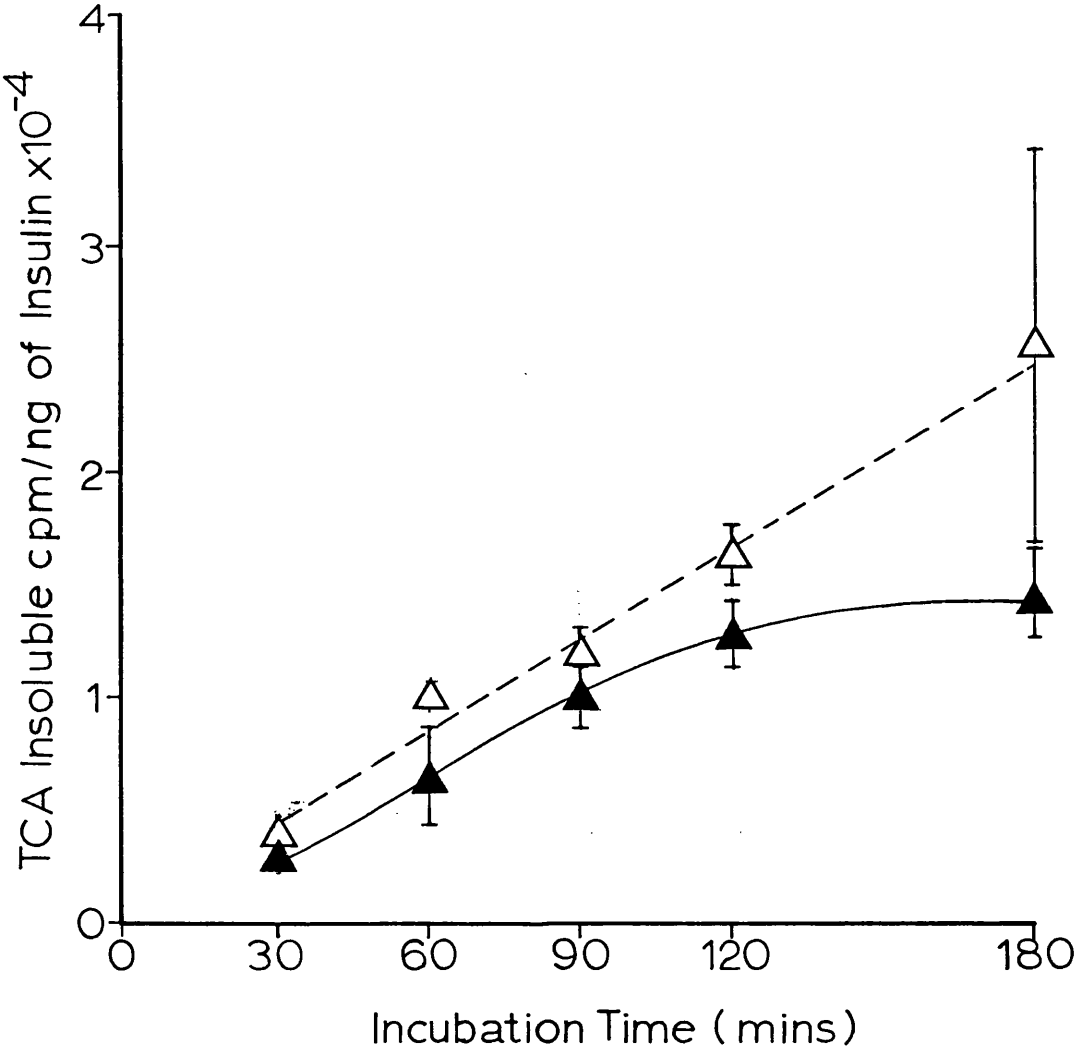

TCA Insoluble cpm/ng of Insulin x10-4
4
3
2
1
0
0
30
60
90
120
180
Incubation Time (mins)

Figure 3.10

Figure 3.11

Time Course of Fractional (Pro)insulin Synthesis

Islets were incubated in medium containing ^{3}H-leucine and either 2.5 mM or 20 mM glucose for various times. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin and total protein synthesis. The fractional (pro)insulin synthesis rate was calculated and plotted against time for incubations in medium containing 2.5 mM (open triangles and dashed line) and 20 mM (closed triangles and solid line) glucose.

The data points are the mean values of duplicate aliquots from pairs of samples with standard error bars.

** $P<0.005$ relative to same incubation time in 2.5 mM glucose.

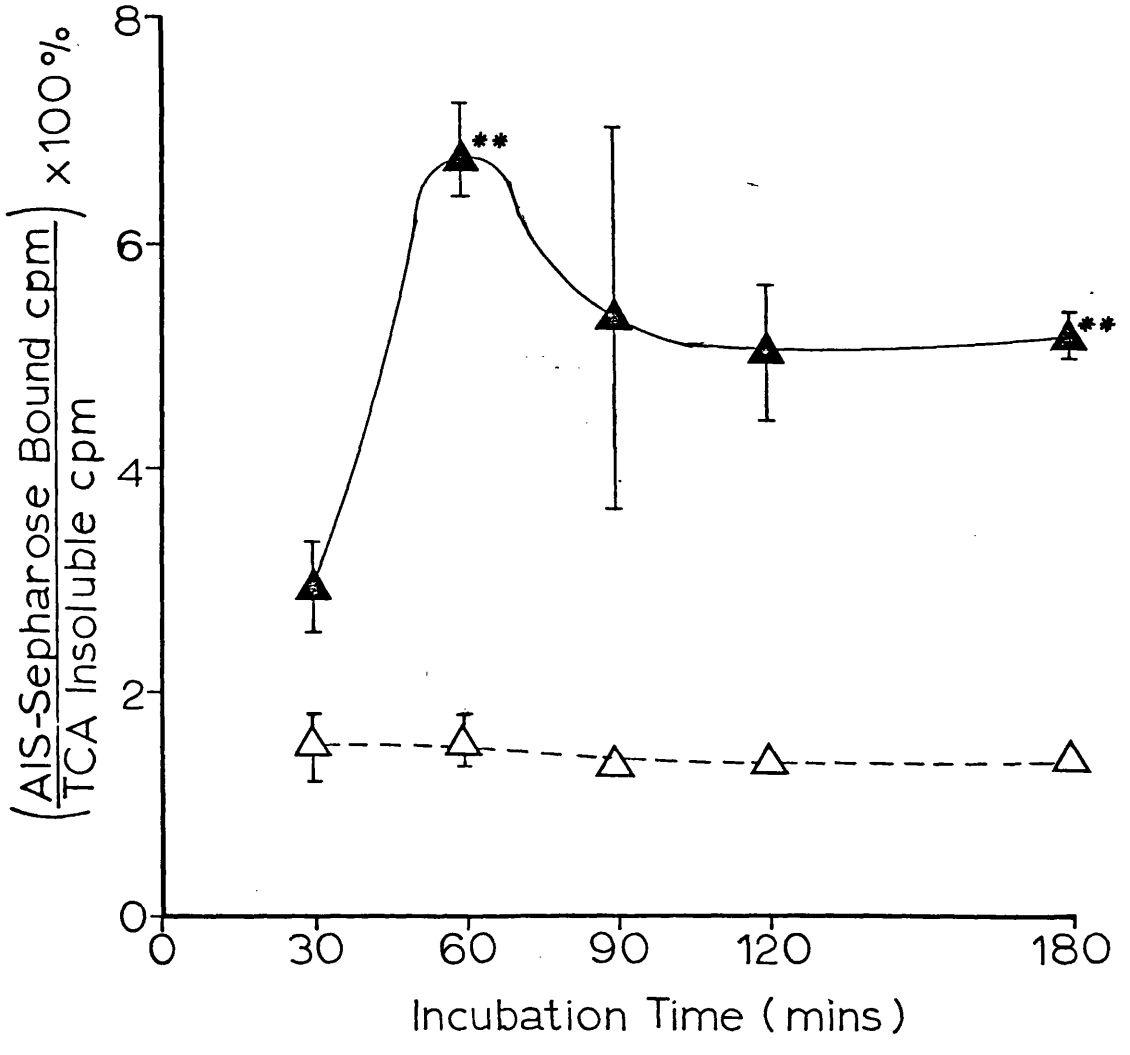

(AIS-Sepharose Bound cpm / TCA Insoluble cpm) x100%
8
6
4
2
0
**
**
0
30
60
90
120
180
Incubation Time (mins)

Figure 3.11

Time Profile of (Pro)Insulin Biosynthesis

Figures 3.12, 3.13 and 3.14 show the time-dependent profiles for various synthesis parameters for a 120 minute incubation at 2.5 mM or 20.0 mM glucose in the presence or absence of actinomycin D. Figure 3.12 shows the profile for (pro)insulin synthesis. At both 2.5 mM and 20.0 mM glucose, in the absence of actinomycin D, synthesis appeared to increase slightly to a peak value and then decrease over the rest of the 120 minute incubation. In the case of 2.5 mM glucose synthesis reached a peak after 30-60 minutes whereas at 20.0 mM glucose it reached a peak value after 60-90 minutes. The addition of actinomycin D did not have any statistically significant effect on synthesis at 2.5 mM glucose at any of the time points. At 20.0 mM glucose, however, the addition of actinomycin D reduced synthesis by a statistically significant amount at the 30-60 minute, 60-90 minute and 90-120 minute time points($P<0.0005$, $P<0.0005$ and $P<0.005$ respectively). Actinomycin D had no statistically significant effect on synthesis at 20.0 mM glucose at the 0-30 minute time point.

Figure 3.13 shows the profile for total protein synthesis. In the absence of actinomycin D the pattern was similar to the results in Figure 3.12 for (pro)insulin synthesis. The pattern of synthesis in the presence of actinomycin D was also similar to that for (pro)insulin synthesis. At 2.5 mM glucose actinomycin D had no statistically significant effect on synthesis at any of the time points but at 20.0 mM glucose there was a statistically significant decrease in synthesis at the 0-30 minute, 30-60 minute, 60-90 minute and 90-120 minute time points ($P<0.05$,

$P<0.0005$, $P<0.005$ and $P<0.005$ respectively).

Figure 3.14 shows the profile for fractional (pro)insulin synthesis. Synthesis at 2.5 mM glucose remained constant and was unaffected by actinomycin D at all time points. Synthesis at 20.0 mM glucose, in the absence of actinomycin D, increased to a peak at 60-90 minutes and then decreased slightly. Actinomycin D reduced synthesis slightly at all the 20.0 mM time points but was only statistically significant at the 60-90 minute time point ($P<0.0005$).

Figure 3.12

Time Profile of (Pro)Insulin Synthesis

Islets were incubated in medium containing either 2.5 mM or 20 mM glucose in the presence or absence of actinomycin D for various times. 30 minutes before the end of each incubation ^{3}H-Leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin synthesis. The index values of the rate of (pro)insulin synthesis were plotted against the ^{3}H-Leucine labeling period for incubations both in the presence of actinomycin D (cross hatched bars) and the absence of actinomycin D (open bars).

Data points are the mean values of a number of samples, which are given beside each bar, with standard error bars.

** $P<0.005$ relative to the rate in the absence of actinomycin D.

*** $P<0.0005$ relative to the rate in the absence of actinomycin D.

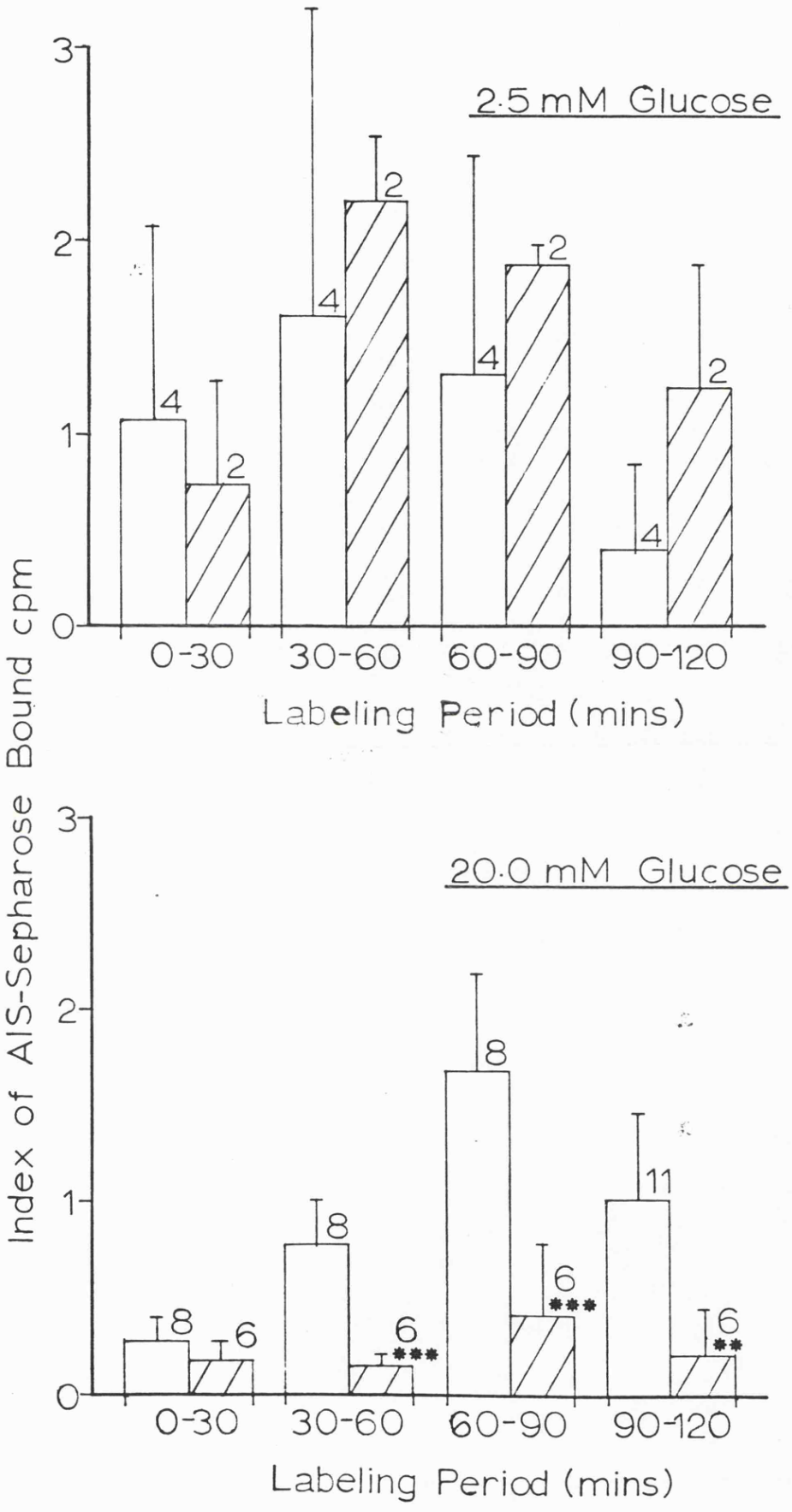

2·5 mM Glucose
20·0 mM Glucose
Index of AIS-Sepharose Bound cpm
Labeling Period (mins)
0-30
30-60
60-90
90-120
0
1
2
3
4
2
8
6
11
***
**

Figure 3.12

Figure 3.13

Time Profile of Total Protein Synthesis

Islets were incubated in medium containing either 2.5 mM or 20 mM glucose in the presence or absence of actinomycin D for various times. 30 minutes before the end of each incubation ^{3}H-leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for total protein synthesis. The index values of the rate of total protein synthesis were plotted against the ^{3}H-leucine labeling period for incubations both in the presence of actinomycin D (cross hatched bars) and the absence of actinomycin D (open bars).

Data points are the mean values of a number of samples, which are given beside each bar, with standard error bars.

* $P<0.05$ relative to the rate in the absence of actinomycin D.

** $P<0.005$ relative to the rate in the absence of actinomycin D.

*** $P<0.0005$ relative to the rate in the absence of actinomycin D.

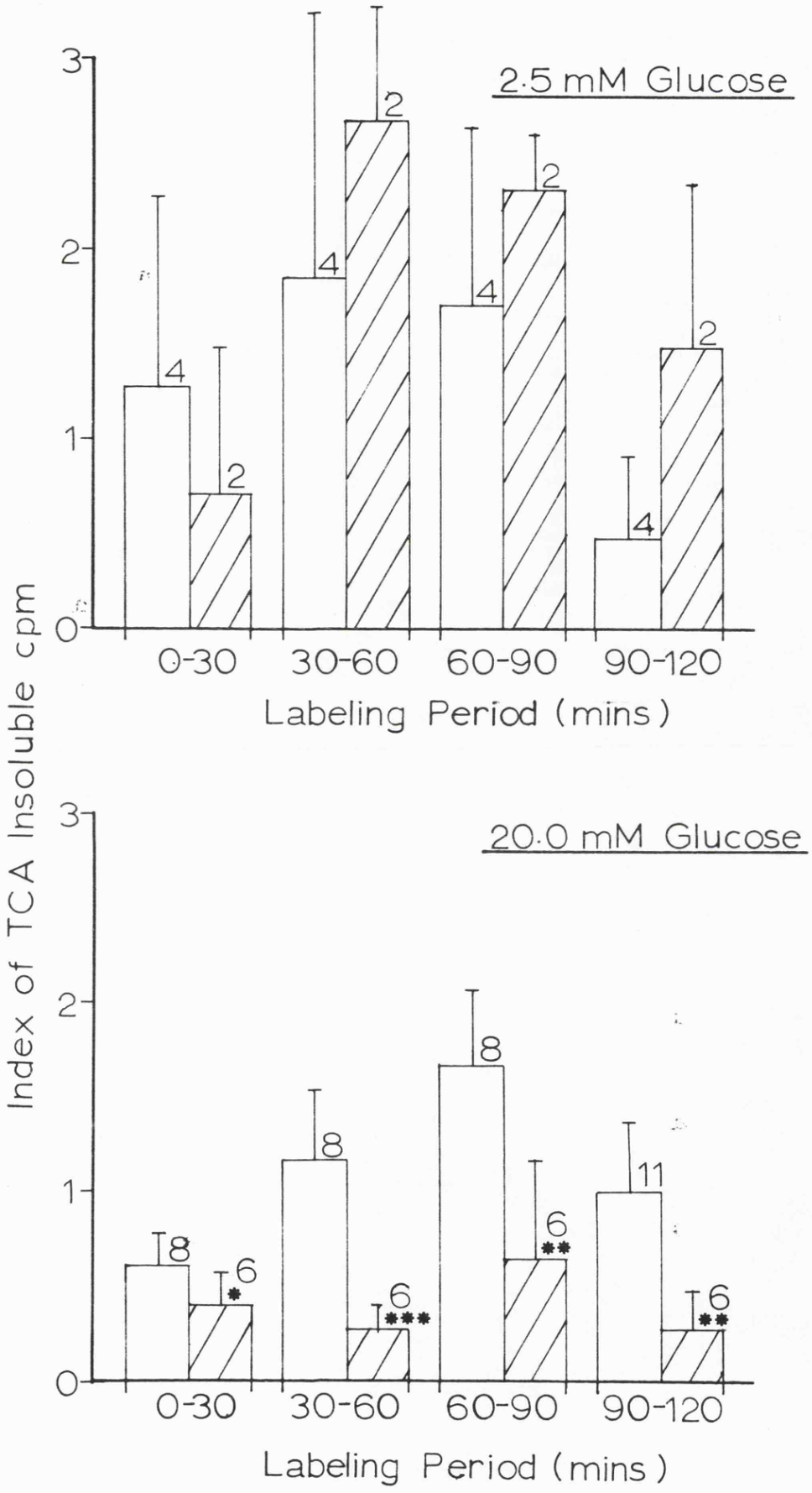

2.5 mM Glucose
20.0 mM Glucose
Index of TCA Insoluble cpm
0
1
2
3
0-30
30-60
60-90
90-120
Labeling Period (mins)
4
2
4
2
4
2
4
2
8
6
8
6
8
6
11
6
*
***
**
**

Figure 3.13

Figure 3.14

Time Profile of Fractional (Pro)Insulin Synthesis

Islets were incubated in medium containing either 2.5 mM or 20 mM glucose in the presence or absence of actinomycin D for various times. 30 minutes before the end of each incubation ^{3}H-Leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin and total protein synthesis. The index values of the fractional rate of (pro)insulin synthesis were plotted against the ^{3}H-Leucine labeling period for incubations both in the presence of actinomycin D (cross hatched bars) and the absence of actinomycin D (open bars).

Data points are the mean values of a number of samples, which are given beside each bar, with standard error bars.

*** $P<0.0005$ relative to the rate in the absence of actinomycin D.

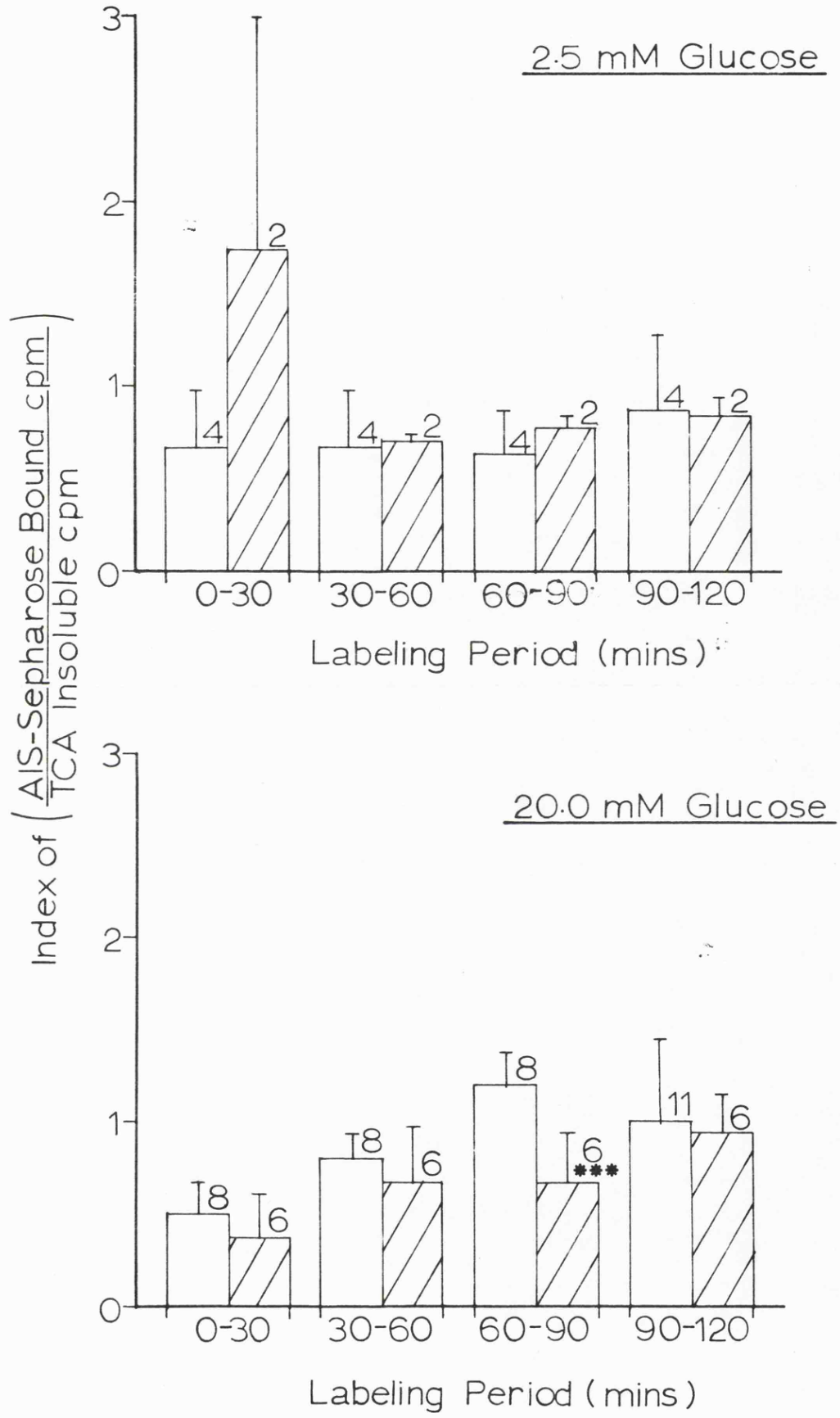

2.5 mM Glucose
20.0 mM Glucose
Index of (AIS-Sepharose Bound cpm / TCA Insoluble cpm)
0
1
2
3
0-30
30-60
60-90
90-120
Labeling Period (mins)
4
2
8
6
11
***

Figure 3.14

Effect of Actinomycin D on (Pro)Insulin Synthesis at Different Glucose Concentrations

Figures 3.15, 3.16 and 3.17 show different synthesis parameters for the second hour of incubation, in the presence or absence of actinomycin D, at different glucose concentrations.

Figure 3.15 shows the rates of (pro)insulin synthesis. In the absence of actinomycin D (pro)insulin synthesis increased with glucose concentration to a maximum at 15.0 mM glucose and then was slightly lower at 20.0 mM glucose. In the presence of actinomycin D synthesis was inhibited to a constant low level at all glucose concentrations. The addition of actinomycin D caused a statistically significantly reduction in the synthesis rate at 10.0 mM, 15.0 mM and 20.0 mM glucose ($P<0.025$, $P<0.005$ and $P<0.005$ respectively).

Figure 3.16 shows the rates of total protein synthesis. In the absence of actinomycin D total protein synthesis was highest at 5.0 mM glucose and decreased either side of this value. The addition of actinomycin D caused a reduction in synthesis rate to a constant low level at all glucose concentrations but there was only a statistically significantly reduction at 10.0 mM, 15.0 mM and 20.0 mM glucose ($P<0.005$ for all three).

Figure 3.17 shows the rates of fractional (pro)insulin synthesis. Fractional (pro)insulin synthesis steadily increased from 2.5 mM to 20.0 mM glucose in the absence of actinomycin D. The addition of actinomycin D had no statistically significant effect on synthesis at any of the glucose concentrations.

Figure 3.15

Effect of Actinomycin D on (Pro)Insulin Synthesis at Different Glucose Concentrations

Islets were incubated in medium containing various concentrations of glucose, in the presence or absence of actinomycin D, for 120 minutes. 60 minutes before the end of each incubation ^{3}H-leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin synthesis and insulin content. The ratio of the rate of (pro)insulin synthesis to the islet insulin content was plotted against the glucose concentration of the medium for incubations carried out in the presence (cross hatched bars) and the absence (open bars) of actinomycin D.

The data points are the mean values from duplicate aliquots of eight samples with standard error bars.

* $P<0.05$ relative to the rate in the presence of actinomycin D.

** $P<0.005$ relative to the rate in the presence of actinomycin D.

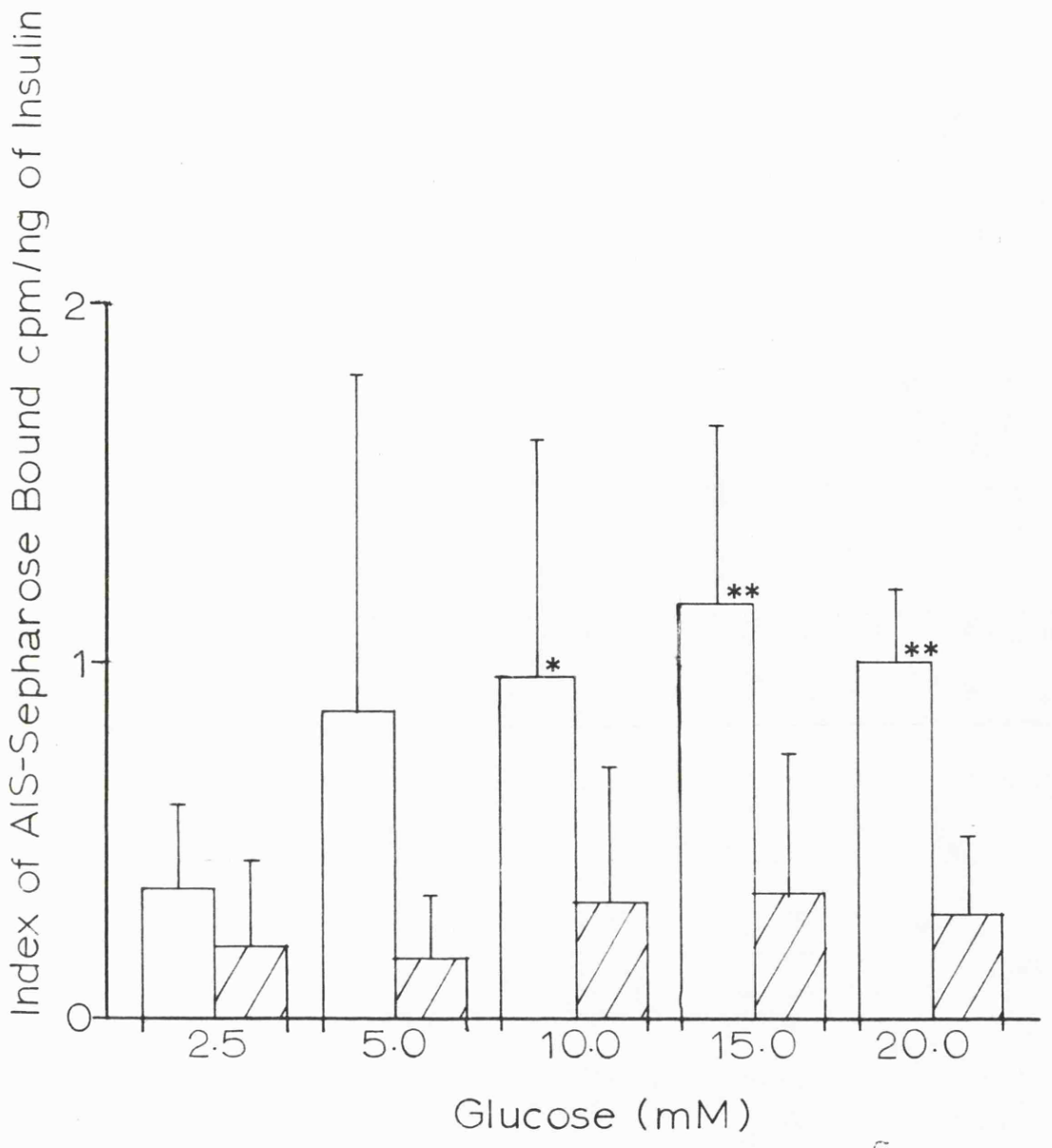

Index of AIS-Sepharose Bound cpm/ng of Insulin
2
1
0
*
**
**
2·5
5·0
10·0
15·0
20·0
Glucose (mM)

Figure 3.15

Figure 3.16

Effect of Actinomycin D on Total Protein Synthesis at Different Glucose Concentrations

Islets were incubated in medium containing various concentrations of glucose, in the presence or absence of actinomycin D, for 120 minutes. 60 minutes before the end of each incubation ^{3}H-leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for total protein synthesis and insulin content. The ratio of the rate of total protein synthesis to the islet insulin content was plotted against the glucose concentration of the medium for incubations carried out in the presence (cross hatched bars) and the absence (open bars) of actinomycin D.

The data points are the mean values from duplicate aliquots of eight samples with standard error bars.

** $P<0.005$ relative to the rate in the presence of actinomycin D.

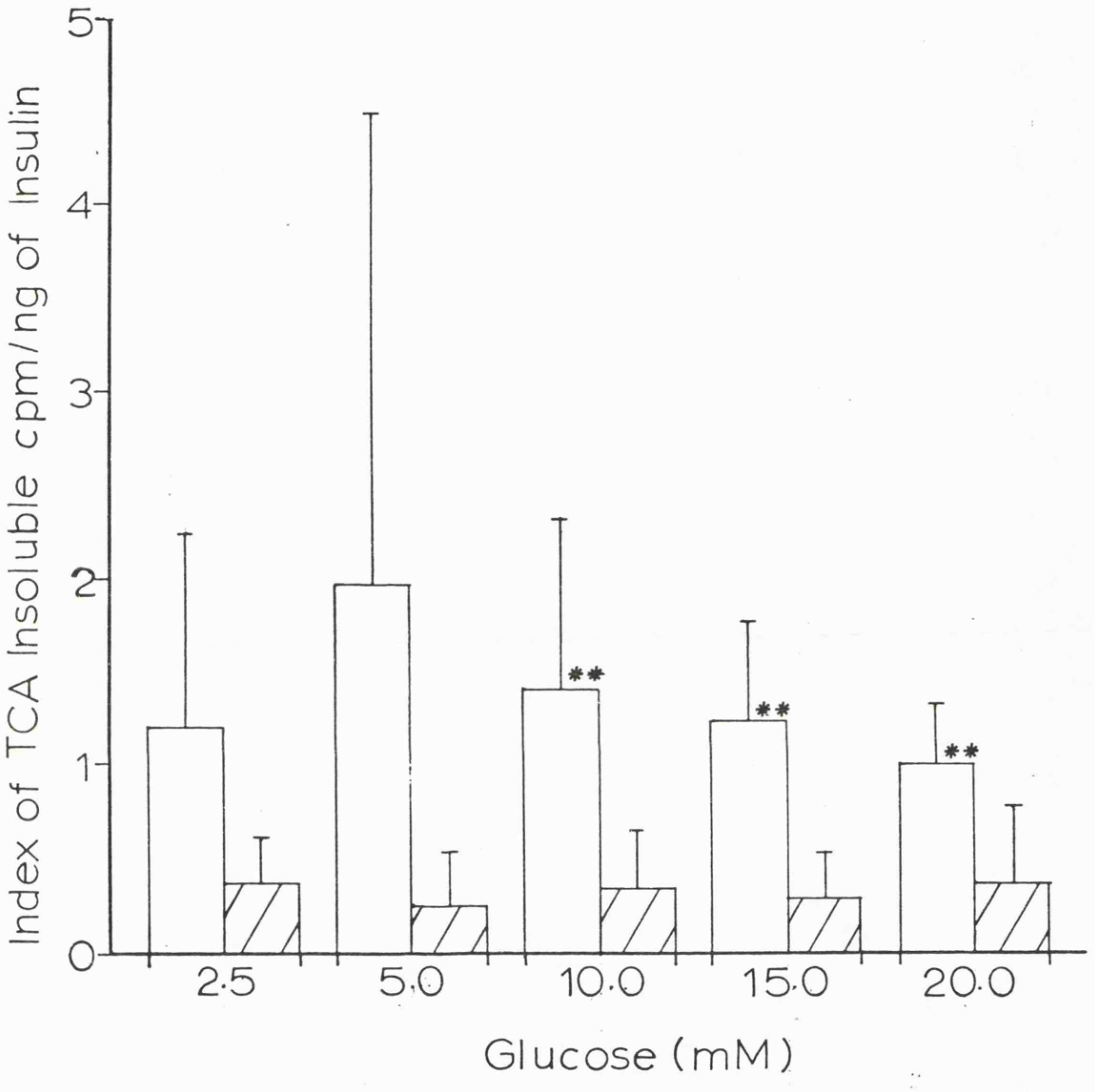

Index of TCA Insoluble cpm/ng of Insulin
0
1
2
3
4
5
**
**
**
2·5
5·0
10·0
15·0
20·0
Glucose (mM)

Figure 3.16

Figure 3.17

Effect of Actinomycin D on Fractional (Pro)Insulin Synthesis at Different Glucose Concentrations

Islets were incubated in medium containing various concentrations of glucose, in the presence or absence of actinomycin D, for 120 minutes. 60 minutes before the end of each incubation ^{3}H-Leucine was added. At the end of each incubation the islets were sonicated and aliquots of the sonicate were assayed for (pro)insulin and total protein synthesis. The index values of the fractional (pro)insulin synthesis rate were plotted against the glucose concentration of the medium for incubations carried out in the presence (cross hatched bars) and the absence (open bars) of actinomycin D.

The data points are the mean values from duplicate aliquots of eight samples with standard error bars.

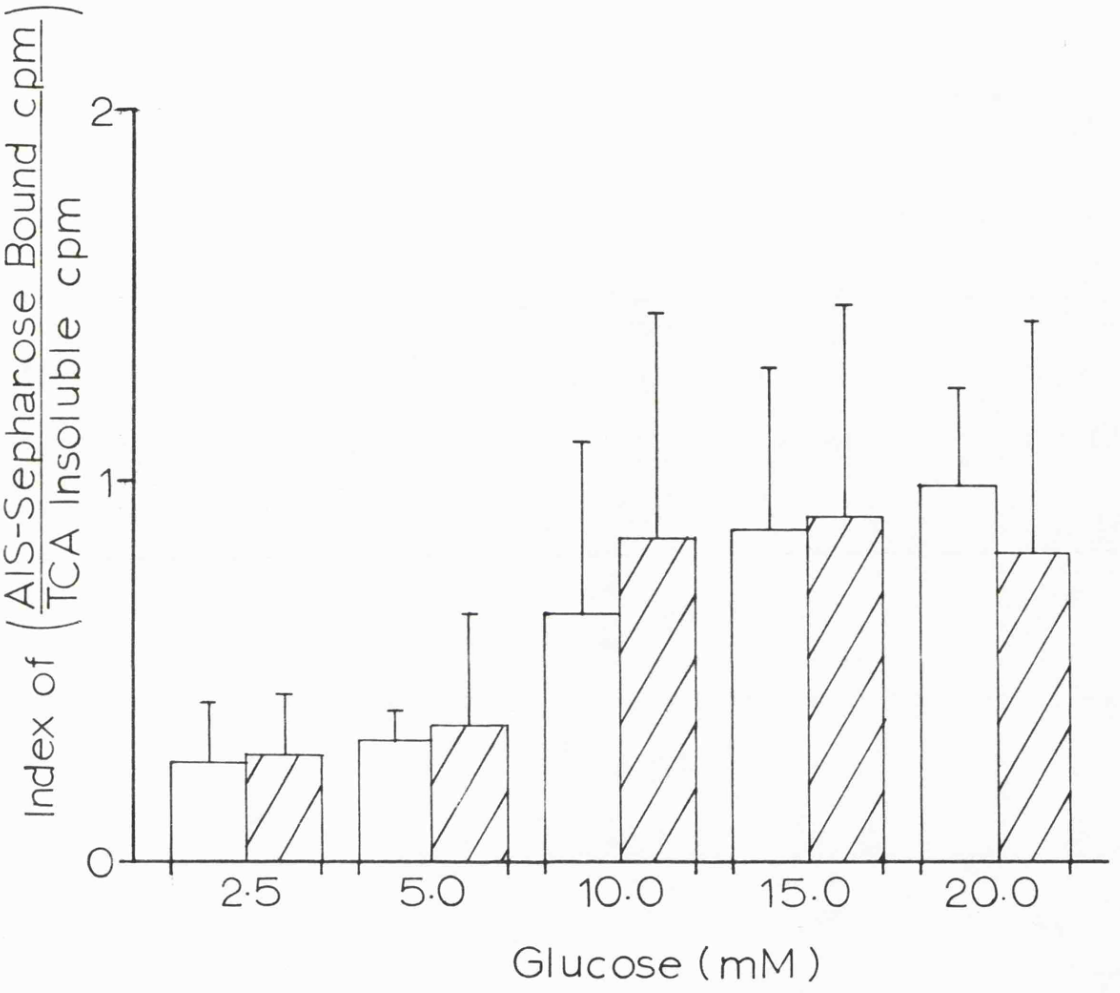

Index of (AIS-Sepharose Bound cpm / TCA Insoluble cpm)
2
1
0
2·5
5·0
10·0
15·0
20·0
Glucose (mM)

Figure 3.17

Discussion

(Pro)Insulin Synthesis Assay

The use of anti-insulin serum coupled to Sepharose gel was first used for extracting insulin from serum in a column method (Akanuma et al 1970) and later for the extraction of insulin from urine in a batch method (Crossley 1974). Berne was the first to apply anti-insulin serum coupled to Sepharose 4B in the study of (pro)insulin synthesis (Berne 1975a).

The binding capacity of the AIS-Sepharose, as measured with ^{125}I-bovine insulin, was 16 µg/g of gel (Figure 3.1) which is similar to that previously reported for AIS-Sepharose prepared in a similar manner (12.5 µg/g) (Berne 1975a). NGPS-Sepharose showed a low level of non-specific binding for ^{125}I-bovine insulin similar to that observed previously (Bone 1977). In the presence of islet sonicate the AIS-Sepharose column bound most of the ^{125}I-bovine insulin. The NGPS-Sepharose column bound none of the ^{125}I-bovine insulin loaded since it was completely eluted by the binding buffer wash (Figure 3.2). Thus the specific binding of ^{125}I-bovine insulin by AIS-Sepharose was not interfered with by islet sonicate and was of relatively high capacity. The quantity of AIS-Sepharose used per sample in the (pro)insulin synthesis assay protocol (20 mg) was sufficient to bind the insulin content of ~5 islets. This represented an excess in binding capacity, over the theoretical insulin content of the aliquots of islet sonicate treated, of at least two fold or greater.

Although the ^{125}I-bovine insulin binding properties of the AIS-Sepharose were adequate in terms of both specificity

and capacity its binding of *in vitro* ^{3}H-Leucine labeled (pro)insulin in islet sonicate was also tested. In order to obtain a high specific activity of ^{3}H-Leucine labeled (pro)insulin islets were incubated at a high glucose concentration (16.7 mM) for a relatively long period (180 minutes). The Sephadex G-50 fractionation profile of the resulting islet sonicate (Figure 3.3) shows that this aim was achieved; the results closely paralleled those previously reported for islets labeled under similar conditions (Maldonato et al 1976). The peak eluted at the void volume of the column (Peak 1) consisted of labeled proteins which were completely excluded from the gel and it had a small shoulder of slightly lower molecular weight material associated with it (Figure 3.3). This shoulder was also observed by Schatz et al (1973) and was shown to be probably due to acinar tissue contamination since it co-migrated with a peak from ^{3}H-Leucine labeled acinar tissue and ^{3}H-leucine incorporation into material in this shoulder was essentially independent of glucose concentration. The relative mobilities of the two intermediate peaks (Peaks 2 and 3) and their sizes were also similar to that previously reported (Maldonato et al 1976; Schatz et al 1973; Schatz et al 1972). The slower of the two intermediate mobility peaks (Peak 3) can confidently be identified as insulin since it co-migrated with the major peak in insulin immuno-reactivity (Figure 3.3) and had the same relative mobility as both bovine insulin and ^{125}I-bovine insulin standards (data not shown). Similarly the faster of the two intermediate mobility peaks (Peak 2) can be identified as proinsulin. It co-migrated with a small shoulder on the major insulin immuno-reactivity peak and was of a higher

molecular weight than the insulin peak (Figure 3.3). The K_{av} values for both the insulin (0.5) and proinsulin (0.30) peaks were in close agreement for values reported for rat insulin and proinsulin standards on similar Sephadex G-50 columns (Schatz et al 1973; Maldonato et al 1976; Permutt & Kipnis 1972a).

The Sephadex G-50 fractionation profiles for AIS-Sepharose and NGPS-Sepharose treated islet sonicate samples (Figures 3.4, 3.5 and 3.6) show that AIS-Sepharose bound both ^{3}H-Leucine labeled proinsulin and insulin (Peaks 2 and 3) as well as labeled material eluted in the void volume (Peak 1). NGPS-Sepharose only bound material eluted in the void volume (Peak 1). This probably represented the low level of non-specific binding seen in the ^{125}I-bovine insulin binding data (Figure 3.1). Thus the NGPS-Sepharose treated samples provided a good control for non-specific binding to AIS-Sepharose. A similar fractionation pattern for AIS-Sepharose and NGPS-Sepharose treated material has been previously reported (Bone & Howell 1977).

The ratio of proinsulin to insulin was higher in the AIS-Sepharose treated samples than in the original islet sonicate. This may have been due to either the AIS-Sepharose having a greater binding capacity for proinsulin than insulin or the AIS-Sepharose having a higher binding affinity for proinsulin. The later is more likely since increasing the sonicate to gel ratio increased the recovery of both proinsulin and insulin (Table 3.1).

The (pro)insulin synthesis assay protocol was designed so that the ratio of islet sonicate volume treated to the amount of gel closely approached that used in the 25 µl islet

sonicate sample (Figure 3.6) in order to maximize the recovery of ^{3}H-leucine labeled proinsulin and insulin. Although higher ratios may have given still higher recoveries, and hence increased sensitivity of the assay, they were not tested since any gain would have been offset by the reduction in sensitivity due to the lower absolute amounts of ^{3}H-leucine labeled material available per sample for liquid scintillation counting. The difference between the ratio of proinsulin to insulin in AIS-Sepharose treated samples and the original islet sonicate could effect the accuracy of the assay. Providing that any fluctuations in this ratio are minimal any error will be constant between samples. So this source of error can be disregarded as far as relative comparisons between synthesis rates are concerned. Since no significant conversion of labeled proinsulin to insulin was observed in islets incubated at 20 mM glucose for 75 minutes (Morris & Korner 1970a) this error is likely to be minimal. Any error due to the possiblity of non-binding of ^{3}H-leucine labeled C-peptide by the AIS-Sepharose is also averted by the use of conditions such as these which avoid significant rates of conversion of labeled proinsulin to insulin. Most of the experimental protocols used in this thesis meet these requirements. Subtraction of the NGPS-Sepharose treated control values from those of the AIS-Sepharose treated samples occasionally resulted in negative values. This was probably due to the discrepancy between the non-specific binding capacities of the AIS-Sepharose and NGPS-Sepharose. It would have been possible to correct this by titration of the two gels with ^{3}H-leucine labeled non-(pro)insulin proteins. This was not done, however, since the NGPS-Sepharose control values

remained constant between samples and experiments and as such were not subtracted from the experimental values.

In conclusion the (pro)insulin synthesis assay protocol that was adopted was considered to be adequate for relative comparisons between synthesis rates in the experimental systems used. It was not adequate for the measurement of absolute (pro)insulin synthesis rates. Since AIS-Sepharose bound both proinsulin and insulin the term (pro)insulin synthesis, rather than to insulin or proinsulin synthesis, is used throughout this thesis. The assay method used was much quicker than fractionation procedures (e.g. Sephadex G-50 gel chromotography, gel electrophoresis) and allowed a greater number of samples to be processed simultaneously. It also offered the advantage of easier washing procedures than double antibody precipitation or column AIS-Sepharose methods. Unlike fractionation techniques, however, it did not permit the resolution of the proinsulin and insulin components of (pro)insulin synthesis.

Dose Response of Glucose on (Pro)insulin Synthesis

Increasing the glucose concentration of the incubation medium during a 120 minute incubation increased the incorporation of ^{3}H-leucine into (pro)insulin in a sigmoidal fashion (Figure 3.7). This effect was preferential for (pro)insulin over total protein synthesis since the fractional (pro)insulin synthesis rate also increased with glucose concentration in a sigmoidal fashion (Figure 3.8). The pattern of response and the threshold and maximal glucose concentrations were identical to those previously reported

(Pipeleers et al 1973a; Lin et al 1972). On increasing the glucose concentration from a basal value of 2.5 mM to a maximal value of 20 mM there was a 5-fold increase in the incorporation of ^{3}H-leucine into (pro)insulin (Figure 3.7). Howell and Taylor (Howell & Taylor 1967) observed that at high glucose concentrations there was a marked increase in secretion of newly synthesised (pro)insulin in the second and third hours of incubation. Since the islets were incubated in the presence of ^{3}H-leucine for 120 minutes it is likely that loss of labeled (pro)insulin by secretion into the medium occurred at the higher glucose concentrations. Such losses would cause an underestimation of the (pro)insulin synthesis rates at the higher glucose concentrations. This would explain why other investigators using shorter incubation periods observed higher increases in (pro)insulin synthesis on raising the glucose concentration from 2.5 mM to 20 mM (~10-fold increase for a 90 minute incubation (Pipeleers et al 1973a) and ~20-fold for a 60 minute incubation (Lin et al 1972)).

Time Course of (Pro)insulin Synthesis

The incorporation of ^{3}H-leucine into the (pro)insulin fraction in islets incubated at 2.5 mM glucose was linear over the whole of the 180 minutes of incubation (Figure 3.9). The time course of incorporation into total islet protein (Figure 3.10) followed a similar pattern. Thus the fractional (pro)insulin synthesis rate at 2.5 mM glucose was constant at ~1.5% for the whole time course (Figure 3.11). Although the incorporation of ^{3}H-leucine into total islet protein at 20 mM glucose occurred at a linear rate almost identical to that at

2.5 mM glucose (Figure 3.10) the time course for incorporation into (pro)insulin differed. There was a sharp rise in incorporation rate at 20 mM glucose between 30 and 60 minutes (Figure 3.9). In terms of the fractional (pro)insulin synthesis rate, 20 mM glucose stimulated synthesis rate from 30 minutes onwards and this leveled off to 5% of total islet protein synthesis by 60-90 minutes (Figure 3.11). These observations are in broad agreement with those of previous studies (Ashcroft et al 1978; Permutt & Kipnis 1972a). The fractional (pro)insulin synthesis rates, at ~1.5% for 2.5 mM and 5% for 20 mM glucose were somewhat lower than previously reported. Morris & Korner (1970a) observed corresponding rates of 7% and 17% whilst Permutt & Kipnis (1972a) found rates of 6.1% and 21.8% at 2.8 mM and 15.3 mM glucose respectively.

Time Profile of (Pro)insulin Synthesis

At high glucose concentrations newly synthesised (pro)insulin is secreted from the β-cell from the second hour of incubation onwards (Howell & Taylor 1967). In the experiments discussed so far ^{3}H-Leucine has been present in the incubation medium for longer than 60 minutes and thus some loss of ^{3}H-labeled (pro)insulin from the islets by secretion into the medium was inevitable. This would cause an underestimation of the rate of (pro)insulin synthesis. Also since it was not known whether AIS-Sepharose bound C-peptide there existed the possibility that loss of ^{3}H-Leucine incorporated into the C-peptide was possible if conversion of ^{3}H-labeled proinsulin into ^{3}H-labeled insulin occurred. Rat proinsulin contains ten leucine residues whereas rat insulin

only contains six. This could have led to a further underestimation in the rate of (pro)insulin synthesis. To obviate these potential errors a pulse-labeling protocol was adopted for this and all subsequent (pro)insulin synthesis experiments in this thesis. In this revised protocol the ^{3}H-leucine was only present in the incubation medium for the last 30 minutes of each incubation period. This labeling period is short enough to prevent secretion of ^{3}H-labeled (pro)insulin due to secretion into the medium and also by conversion of ^{3}H-labeled proinsulin to ^{3}H-labeled insulin (Maldonato et al 1976). At the same time a 30 minute labeling period gave a sufficiently high specific activity of ^{3}H-(pro)insulin for study.

In general the ^{3}H-leucine incorporation rates, as well as the standard errors, were higher for islets incubated at 2.5 mM than 20 mM glucose (Figures 3.12 and 3.13). This variation can be attributed to one particular islet tissue preparation used in the 2.5 mM glucose incubations which gave much greater ^{3}H-leucine incorporation rates than the other tissue preparations used. The standard error values for the fractional (pro)insulin synthesis rates at 2.5 mM glucose were much lower as would be expected if this were the case. So only valid comparisons between the 2.5 mM and 20 mM glucose incubation data can be made using the fractional (pro)insulin synthesis rates.

The effect of glucose on (pro)insulin synthesis and total protein synthesis was the same indicating that glucose had an effect on protein synthesis in general and not just (pro)insulin synthesis in particular. This is in agreement with previous time course data for (pro)insulin and bulk

protein synthesis in the second hour of synthesis at 16.7 mM and total protein synthesis at 2.5 mM glucose remained constant throughout the incubation period. These data are consistent with the glucose dose response curve (Figure 3.8) since 2.5 mM is below the threshold for glucose stimulated (pro)insulin synthesis. Conversely 20 mM glucose lies at the other extreme of the dose response curve and represents a maximal rate of (pro)insulin synthesis. This was reflected in the time profile data as the rates of (pro)insulin, fractional (pro)insulin and total protein synthesis all rose steadily over the first 90 minutes of incubation at 20 mM glucose (Figures 3.12, 3.13 and 3.14). So it is apparent that 20 mM glucose stimulates the synthesis of islet proteins in general, and (pro)insulin in particular, within 30 to 60 minutes of the start of stimulus. There was a decline in rate from the 60-90 to the 90-120 minute time points in (pro)insulin and total protein synthesis. It is possible that this decline marks the start of the limit of the viability of isolated islets for *in vitro* (pro)insulin synthesis. By this time the islets have been subjected to a total incubation time similar to the 180 minute time point in the earlier time course experiment where a similar decline in the total protein synthesis rate was seen (Figure 3.10). Perhaps the β-cell pool of protein synthesis precursors was not sufficient to sustain the stimulated rates of *in vitro* protein synthesis that occurred at high glucose concentrations.

Actinomycin D, an inhibitor of DNA directed RNA synthesis (Goldberg et al 1962), has been shown to inhibit RNA synthesis in islets by 80% when used at a concentration of 10 µg/ml (Puchinger & Wacker 1972). It has been used in this study at

this concentration in order to ascertain the contribution made by RNA synthesis to glucose stimulated (pro)insulin synthesis. Actinomycin D treatment had no statistically significant effect on either (pro)insulin or total protein synthesis rates at 2.5 mM glucose. For islets incubated at 20 mM glucose, however, it caused a statistically significant reduction in (pro)insulin synthesis rates from the 30-60 minute time point onwards (Figure 3.12) and at all time points for total protein synthesis (Figure 3.13). In contrast to this general inhibition of synthesis rates actinomycin D treatment only caused a statistically significant reduction in the fractional (pro)insulin synthesis rate at the 60-90 minute time point (Figure 3.14). This pattern of actinomycin D action on protein synthesis rates can be explained by the inhibition of synthesis of two separate pools of RNA. One pool consists of RNA species required for general protein synthesis. Inhibition of synthesis of this RNA pool was apparent as early as the 0-30 minute time point and probably represented inhibition of synthesis of a labile RNA species. Since this form of inhibition applies equally to the total protein synthesis rate and the (pro)insulin synthesis rate it cancels itself out in the calculation of the fractional (pro)insulin synthesis rate (Figure 3.14). The residual inhibition by actinomycin D was apparent at the 60-90 minute time point for 20 mM glucose. This could have represented inhibition of synthesis of a second RNA pool consisting of RNA specific to the synthesis of (pro)insulin. The obvious candidate for such a RNA species is preproinsulin mRNA and this implies that synthesis of new preproinsulin mRNA is instrumental in glucose stimulated (pro)insulin synthesis over the short term (i.e. from 1 hour

of incubation onwards). There was no statistically significant reduction in the fractional (pro)insulin synthesis rate for 20 mM glucose at the 90-120 minute time point. This could have been due to masking by loss of islet viability for (pro)insulin synthesis which was discussed above.

Effect of Actinomycin D on (Pro)Insulin Synthesis at Different Glucose Concentrations

The results of the time profile experiment (Figures 3.12, 3.13 and 3.14) indicated that the inhibition of glucose stimulated (pro)insulin synthesis by actinomycin D occured at 20 mM but not at 2.5 mM glucose. In order to determine the glucose concentrations at which RNA synthesis contributed to protein synthesis a dose response experiment for glucose in the presence and absence of actinomycin D was carried out. Since it was believed that increased sensitivity of the (pro)insulin synthesis assay was necessary for this experiment so the specific activity of the ^{3}H-labeled (pro)insulin was increased by using a longer ^{3}H-leucine labeling period than 30 minutes (i.e. one hour). This period represented the maximum possible without incurring losses of ^{3}H-labeled (pro)insulin due to secretion into the medium (Howell & Taylor 1967). The labeling period was chosen for the second hour of incubation since the time profile experiment showed this to be the time of maximal actinomycin D inhibition (Figures 3.12, 3.13 and 3.14). In the absence of actinomycin D the glucose dose response curves for (pro)insulin (Figure 3.15) and fractional

(pro)insulin (Figure 3.17) synthesis followed the sigmoidal pattern precedented by the previous glucose dose response data (Figures 3.7 and 3.8). The rate of total protein synthesis in the second hour of incubation was independent of the concentration of glucose (Figure 3.16). Actinomycin D inhibited both (pro)insulin and total protein synthesis rates to a constant level at all glucose concentrations tested (Figures 3.15 and 3.16). Since this inhibition is only statistically significant at glucose concentrations of 10 mM or greater it is possible that at these concentrations the synthesis of RNA is rate limiting for general protein synthesis in the second hour of incubation.

In the case of fractional (pro)insulin synthesis (Figure 3.17) actinomycin D had no statistically significant effect on the glucose dose response curve. Applying the two RNA pool model for actinomycin D inhibition of protein synthesis discussed above it can be concluded from this that synthesis of RNA specific for (pro)insulin synthesis (i.e. preproinsulin mRNA) plays no part in the glucose stimulation of (pro)insulin synthesis at any of the concentrations tested. This, however, is in direct conflict with the time profile data where such an effect was seen at the 60-90 minute time point for the 20 mM glucose incubation (Figure 3.14). This can probably be explained by the choice of labeling period which included the 90-120 minute time point. The possible loss of (pro)insulin synthesis viability of the islets at this time point (as discussed above) could mask any effect of actinomycin D occuring at 60-90 minutes. In retrospect a labeling period of one hour from 30 minutes to 90 minutes could have avoided this problem.

Limitations of (Pro)Insulin Synthesis Measurements

Several limitations have to be borne in mind when studying *in vitro* (pro)insulin synthesis in isolated mammalian islets of Langerhans. The first of these is the trauma that the islets undergo during the collagenase digestion procedure. Ideally the freshly isolated islets should be tissue cultured for 24 hours in order to stabilize their protein synthetic abilities (Andersson 1978). However, this suffers from the inherent drawback of adaptation of the tissue to the prevailing culture conditions. Since the synthetic properties of freshly isolated islets are under study a shorter period of recovery is called for. Pilot labeling experiments in tissue culture medium (Ham's F12) resulted in poor ^{3}H-leucine incorporation rates in comparison to the BSA supplemented Gey and Gey bicarbonate buffered medium finally adopted (data not shown). Use of this type of medium necessarily limits the period of islet viability and so 45 minutes was believed to be the maximum practicable preincubation period. The time course and time profile data provided evidence that the limit of the islets viability for protein synthesis was being reached. This manifested itself as a tailing off in protein synthesis rates after 90 minutes of incubation. A similar reduction in (pro)insulin synthesis rate was observed after 75 minutes of incubation at high glucose concentration in a similar study using bicarbonate buffered medium supplemented with BSA only (Ashcroft et al 1978). This was not observed, however, under similar incubation conditions using bicarbonate buffered medium supplemented with amino acids at 30 to 100 μM (Morris & Korner 1970a; Baird & Bauer 1976). This would suggest that the

reduction in synthesis rates experienced after 90 minutes of incubation were due to limitations in amino acid pools for protein synthesis. Pilot studies showed that supplementing the incubation medium with amino acids (1 μM each except leucine) drastically reduced the incorporation of ^{3}H-Leucine into proteins (data not shown). Therefore this approach was abandoned.

This type of study assumes that the data obtained is an accurate reflection of the protein synthesis rates within the β-cell. This is not strictly valid since the β-cell at best only comprises ~80% of the cell mass of the islet, the remaining 20% being α-cells, D-cells or PP-cells (Orci et al 1976). This is aggravated by the fact that these other cell types are situated in the outer layers of the islet in closer contact with the medium than the β-cells. Autoradiographic studies of *in vitro* labeled islets have shown that incorporation of exogenous amino acids into the β-cells was not uniform. Peripheral non-beta endocrine cells and mesenchymal islet cells, however, were all uniformly labeled and this was not affected by changes in glucose concentration whereas that of the β-cells was (Logothetopoulos & Jain 1980a). This labeling pattern appeared to be due to irreversible anoxic injury of centrally located β-cells during the isolation/incubation procedures and not due to limited diffusion of amino acids to the central parts of the islet. All these factors lead to an underestimation of the rate of β-cell (pro)insulin synthesis and could also account for the variability in ^{3}H-Leucine incorporation experienced in this, and other, studies.

Summary

Increasing the glucose concentration of the incubation medium over the physiological range of 2 mM to 20 mM stimulates the rate of *in vitro* islet protein synthesis. This effect is preferential for (pro)insulin synthesis and follows a sigmoidal pattern. The threshold glucose concentration for stimulus was 2.5 mM to 5 mM and the maximal rate was at 15 mM to 20 mM. The synthesis of (pro)insulin was constitutive, and the rate was linear over 3 hours, at the sub-stimulatory level of 2.5 mM glucose. On raising the glucose concentration to the maximal level of 20 mM glucose there was a 5-fold increase in the (pro)insulin synthesis rate which was no longer linear over the two hour incubation period. The synthesis of RNA (as inhibited by actinomycin D) was required for (pro)insulin synthesis in two separate ways. RNA synthesis was required for general protein synthesis and this requirement was apparent as early as after 30 minutes. There was also a specific requirement of RNA synthesis for (pro)insulin synthesis which was apparent after 60 minutes. The first of these two requirements is thought to reflect synthesis of a labile RNA species involved in general protein translation and the second to reflect synthesis of a RNA species specific to (pro)insulin synthesis. This specific RNA species is thought to be preproinsulin mRNA and further data on this is presented in the next chapter.

Chapter 4

The Effect of Glucose on Islet Preproinsulin mRNA Content

Introduction

Since there was no specific assay for preproinsulin mRNA available early studies on the effect of glucose on insulin gene transcription were confined to indirect methods using radiolabeled RNA precursors, such as ^{14}C-orotic acid and ^{3}H-uridine. The rate of incorporation of these precursors into islet RNA was doubled by raising the glucose concentration of the incubation medium from 2.8 mM to 15.3 mM or greater (Jarrett et al 1967; Puchinger & Wacker 1972; Permutt & Kipnis 1972b). This effect of glucose was inhibited by up to 80% (Puchinger & Wacker 1972), or even completely abolished (Jarrett et al 1967), by actinomycin D, an inhibitor of RNA polymerase (Goldberg et al 1962). This increase in RNA synthesis was largely confined to high molecular weight RNA much of which was ribosomal precursor (Permutt & Kipnis 1972b). The information from this type of study was combined with that obtained from studying the effect of RNA synthesis inhibitors, such as actinomycin D, on glucose induced (pro)insulin synthesis discussed in the previous chapter. Data from both these experimental approaches led many investigators to postulate the existence of a glucose mediated control of insulin gene transcription for glucose stimulations of periods greater than 15-60 minutes (Ashcroft et al 1978; Permutt & Kipnis 1972a; Permutt 1974; Parman 1975; Gunnarsson 1977; Kaelin et al 1978). Not all such radiolabeling data, however, was in agreement with this hypothesis. In one study glucose was shown to stimulate incorporation of ^{3}H-uridine into RNA in islets from 48 hour fasted rats but not in islets from the fed control rats (Jahr et al 1980b). Furthermore actinomycin D and

α-amanitin (another inhibitor of RNA synthesis (Zylber & Pennman 1971)) failed to inhibit the stimulation of (pro)insulin synthesis by glucose during a 4 hour incubation.

The advent of cell free *in vitro* translation techniques enabled the hypothesis that glucose effects insulin gene expression to be investigated directly. Cell free *in vitro* translation, in a wheat germ derived system, of RNA purified from rat islets previously incubated for one hour at either 3.3 mM or 20 mM glucose failed to demonstrate any effect of glucose on islet preproinsulin mRNA content (Itoh et al 1978). The only effect on preproinsulin mRNA level demonstrable using cell free *in vitro* translation was in islets from 4 day fasted rats. The preproinsulin mRNA level, which had been reduced to 80% of the fed rat control level by fasting, was increased to 160% of the control level within 8 hours of refeeding (Giddings et al 1981).

The application of cell free *in vitro* translation systems in the assay of preproinsulin mRNA has been somewhat limited since it can only measure levels of translatable preproinsulin mRNA. The use of ^{32}P-labeled insulin gene or cDNA sequences as hybridization probes to measure hybridizable islet preproinsulin mRNA, however, has been more extensive since total levels of preproinsulin mRNA can be assayed. Once again this method was applied to *in vivo* studies with fasted rats just as cell free *in vitro* translation had been. In the same study on 4 day fasted rats as described above preproinsulin mRNA levels were assayed by ^{32}P-labeled DNA hybridization as well as by cell free *in vitro* translation. The restoration of translatable preproinsulin mRNA level from its 4 day fasted level to 160% of the fed rat control level was also paralleled

by that of hybridizable preproinsulin mRNA (Giddings et al 1981). A more direct effect of glucose on hybridizable preproinsulin mRNA level was demonstrated in islets isolated from rats that had been starved for 3 days (Giddings et al 1982). Fasting reduced the level to 15-20% of that of the fed control rats and upon injection of the rats with glucose there was a 3 to 4-fold increase in islet preproinsulin mRNA. This rise was detectable after 2 hours and complete within 12 hours.

Tissue culture of islets for up to 7 days has been the favoured method for the study of preproinsulin mRNA level *in vitro* (Brunstedt & Chan 1982; Welsh et al 1985; Nielsen et al 1985; Welsh et al 1986c). It was observed that when freshly isolated mouse islets were tissue cultured at 3.3 mM glucose the preproinsulin mRNA level dropped to 10% of that in freshly isolated islets within 24 hours (Brunstedt & Chan 1982). With subsequent tissue culture of the islets at 3.3 mM glucose the preproinsulin mRNA level remained constant. If, however, the islets were subsequently tissue cultured at 10 mM glucose the preproinsulin mRNA level was restored to that of freshly isolated islets within 3 days. Tissue culture of mouse islets for 7 days resulted in a 5-fold increase in preproinsulin mRNA in islets cultured at 20 mM as compared to 3.3 mM glucose (Welsh et al 1986c). Culture of the islets in medium containing 3.3 mM glucose and either L-leucine or 2-ketoisocaproate was as effective in maintaining a high preproinsulin mRNA level as was 20 mM glucose alone. L-Leucine and its deamination product 2-ketoisocaproate (which both stimulate (pro)insulin synthesis (Andersson 1976)) are thought to be non-glucogenic in islets since islets lack the enzyme

fructose 1,6-diphosphatase (Sener & Malaisse 1978). Thus it was postulated that the maintenance of high preproinsulin mRNA levels in islets is correlated with metabolic flux in the islets rather than the presence of the glucose molecule *per se* (Welsh et al 1986c). When rat islets were cultured in glucose free medium their preproinsulin mRNA content fell by 60% over 24 hours, however, culture of the islets at 17 mM glucose maintained the level of preproinsulin mRNA at a level similar to that of fresh islets (Nielsen et al 1985). ^{3}H-Uridine pulse-labeling studies of rat islets showed that tissue culturing in medium containing 17 mM, as opposed to 3.3 mM, glucose resulted in an almost 2-fold increase in the stability of preproinsulin mRNA (Welsh et al 1985) suggesting that glucose can regulate preproinsulin mRNA half-life.

To date all the studies which have used DNA/RNA hybridization techniques to measure preproinsulin mRNA have been on islets tissue cultured for 24 hours or longer. The only previous study of preproinsulin mRNA levels over short periods (i.e. 2 hours or less) reported an increase in insulin gene transcription in rat islets incubated at 17 mM, as opposed to 3.3 mM, glucose over a 90 minute incubation period (Nielsen et al 1985). In this study, however, no quantitative data was given. Thus in order to determine the role, if any, of glucose in the short term regulation of insulin gene transcription in islets isolated from animals under normal physiological conditions (i.e. not starved) a more detailed study is required. The studies detailed in this chapter were designed to achieve this aim. Levels of islet preproinsulin mRNA were measured using a dot blot RNA hybridization assay during short term incubations of 2 hours or during tissue

culture periods up to 24 hours. A dot blot assay for islet preproinsulin mRNA was specifically developed for use in this study from an existing assay for mouse hepatitis virus RNA in small quantities of cultured cells (Cheley & Anderson 1984). This was chosen as the basis of the assay because of its ability to use small quantities of ribonuclease rich tissue without the extensive RNA purification methodology associated with existing assay methods for islet preproinsulin mRNA (Brunstedt & Chan 1982; Giddings et al 1985).

Methods

Introduction

This section describes the methodology that was used to obtain the data presented in this chapter. This chapter is divided into two main sections. The first section deals with the validation of the dot blot assay for islet preproinsulin mRNA. The second section deals with the application of this assay to the measurement of preproinsulin mRNA content of islets.

(1) Validation of Islet Preproinsulin mRNA Assay

^{32}P-Labeling of DNA

(a) Nick Translation

(i) Time Course

Two nick translation reactions were set up, one containing pDI462 plasmid insert DNA and one containing phins214 plasmid insert DNA. Duplicate 1 µl aliquots were removed from each reaction mix every 45 minutes for the determination of α-^{32}P-dCTP incorporation. At the end of 315 minutes of incubation triplicate 1 µl aliquots were removed from each reaction for the determination of total radioactivity.

(ii) Titration of DNAse I

Five nick translation reactions were set up containing phins214 plasmid insert DNA with four different amounts of DNAse I (1 µl, 2 µl or 4 µl of 0.01 µg/ml or 1 µl of 0.1 µg/ml DNAse I stock solution) and one reaction containing no DNAse I. At the end of the incubation triplicate 1 µl aliquots were removed for determination of α-^{32}P-dCTP incorporation and triplicate 1 µl aliquots for the determination of total radioactivity. The incorporated α-^{32}P-dCTP was separated from the reaction mixes by gel filtration and aliquots of the purified ^{32}P-labeled DNA were subjected to

alkaline gel electrophoresis in the following manner. 100 µl aliquots were precipitated by the addition of 1/10 volume of 2.5 M sodium acetate and 2 volumes of ethanol and storage at -20°C for 2 hours. The precipitated DNA was recovered by centrifugation (MSE Micro Centaur microfuge, high speed, 10 minutes) and dissolved in 20 µl of alkaline loading buffer (50 mM NaOH containing 1 mM EDTA, 2.5% (w/v) Ficoll 400 and 0.025% (w/v) bromocresol green). The samples were then loaded onto a 2% (w/v) agarose gel in 30 mM NaOH containing 1 mM EDTA and electrophoresed at 60 volts for 5 hours. After electrophoresis the gel was soaked in 7% (w/v) TCA for 30 minutes. The gel was then placed on a sheet of cling-film and covered with a sheet of 3MM paper and a stack of paper towels 3-4 cm high. Finally a glass plate and a 500 g weight were placed on top and the gel left to blot dry overnight. The dried gel was wrapped in cling-film and autoradiographed for 2 days.

(b) Oligonucleotide Primer Extension and M13 Second Strand Synthesis

phins214, pDI462 and pKA30 plasmid insert DNA were ^{32}P-labeled by oligonucleotide primer extension as described in detail in chapter 2. ^{32}P-Labeling of M13 DNA was achieved by M13 second strand synthesis of the M13 rat insulin cDNA clone or the M13 α-1-microglobulin clone DNA as described in detail in chapter 2. The specific radioactivity of the DNA produced by each labeling protocol was estimated by measurement of the α-^{32}P-dCTP incorporation.

Purification of ^{32}P-Labeled DNA by Gel Filtration

Plasmid insert DNA from phins214 was ^{32}P-labeled by nick translation and subjected to Sephadex G-100 gel filtration. Thirty 100 µl fractions were collected from the column and duplicate 1 µl aliquots taken from each fraction for determination of α-^{32}P-dCTP incorporation and duplicate 1 µl aliquots for the determination of total radioactivity.

Northern Blotting of Poly A$^+$ RNA

10 µg of total RNA, 1 µg of poly A$^+$ and 1 µg of poly A$^-$ RNA isolated from rat pancreas and rat liver were Northern blotted. The blotted nitrocellulose paper was hybridized with M13 rat insulin cDNA clone DNA ^{32}P-labeled by M13 second strand synthesis. The nitrocellulose paper was then washed for 10 minutes in each of four changes of 250 ml of 1xSSC containing 0.1% (w/v) SDS at room temperature and then for 15 minutes in each of two changes of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 50°C. After air drying the nitrocellulose paper was autoradiographed for 2 days.

Hybridization Specificity of ^{32}P-Labeled DNA

The hybridization specificity of ^{32}P-labeled DNA, produced by oligonucleotide primer extension or M13 second strand synthesis was examined by assaying samples of poly A$^+$ RNAs for preproinsulin mRNA and washing the nitrocellulose papers at increasing stringencies as follows.

(i) Oligonucleotide Primer Extension

5 µg each of poly A^+ RNA isolated from rat pancreas, kidney and liver were assayed for preproinsulin mRNA. Eight such assays were set up. The nitrocellulose papers from four of the assays were hybridized with ^{32}P-labeled phins214 plasmid insert DNA and the other four with ^{32}P-labeled pDI462 plasmid insert DNA. Both plasmid insert DNAs were ^{32}P-labeled by the oligonucleotide primer extension method and each group of four nitrocellulose papers were hybridized together in the same hybridization chamber. After hybridization each nitrocellulose paper, in each group of four, was subjected to a different washing protocol. All the papers were first washed for 10 minutes in each of four changes of 250 ml of 2xSSC containing 0.1% (w/v) SDS at room temperature. Three papers in each group were then each subjected to a different second wash of 15 minutes in each of two changes of 250 ml of solution. For the first paper this solution was 0.5xSSC containing 0.1% (w/v) SDS at room temperature, for the second it was 0.1xSSC containing 0.1% (w/v) SDS at room temperature and for the third it was 0.1xSSC containing 0.1% (w/v) SDS at 42°C. During all washes the papers were agitated and turned occasionally. After washing the papers were air dried and autoradiographed together on the same X-ray film for 3 days.

In a similar manner to that above 5 µg each of poly A^+ RNA isolated from rat pancreas and liver were assayed for preproinsulin mRNA. Three such assays were set up. The nitrocellulose papers were hybridized together in the same

hybridization chamber with pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. After hybridization each of the nitrocellulose papers was subjected to a different washing protocol. All three were first washed for 10 minutes in each of four changes of 250 ml of 1xSSC containing 0.1% (w/v) SDS at room temperature. Two of the papers were then subjected to a second wash of 15 minutes each of two changes of 250 ml of solution. For the first paper this solution was 0.1xSSC containing 0.1% (w/v) SDS at room temperature and for the second it was 0.1xSSC containing 0.1% (w/v) SDS at 42°C. During all washes the papers were agitated and turned occasionally. After washing the papers were air dried and autoradiographed together on the same X-ray film for 3 days.

(ii) M13 Second Strand Synthesis

5 µg each of poly A$^+$ RNA isolated from rat pancreas and liver along with 200 freshly isolated rat islets were assayed for preproinsulin mRNA. Two such assays were set up and the nitrocellulose papers were hybridized with ^{32}P-labeled DNA prepared by second strand synthesis of either the M13 insulin cDNA clone or the M13 α-1-microglobulin clone. After hybridization the papers were subjected to five different sequential washing protocols. The first protocol consisted of four changes each of 250 ml of 1xSSC containing 0.1% (w/v) SDS at room temperature followed by four changes each of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at room temperature. Then there were two changes each of 250 ml of 0.1xSSC containing 0.1%

(w/v) SDS at 42°C. The second protocol was four changes each of 250 ml of 1xSSC containing 0.1% (w/v) SDS at 65°C. The third protocol was four changes each of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 65°C. The fourth protocol was four changes each of 250 ml of 0.05xSSC containing 0.1% (w/v) SDS at 65°C. The final protocol was two changes each of 250 ml of distilled water at 68°C. All washes were for 10 minutes each and the nitrocellulose papers were agitated and turned occasionally during washing. After each wash protocol the papers were air dried and autoradiographed on the same X-ray film before the next wash protocol was carried out. The exposure times were 1 day after the first and second protocols, 2 days after the third and fourth protocols and 3 days after the fifth protocol.

Relationship Between ^{32}P-Labeled DNA Bound and Autoradiograph Spot Density

For this experiment the three preproinsulin mRNA assays which were hybridized with ^{32}P-labeled pKA30 plasmid insert DNA described in the section above were used. Each dot on the papers was punched out with a hole punch (diameter 5 mm) into a scintillation vial as were representative samples of the spaces in between the dots from each of the three papers. The punched nitrocellulose circles were dissolved by standing for 2 hours in 0.5 ml of 2-methoxyethanol. The radioactivity (cpm) in each vial was determined by liquid scintillation counting in 4 ml of FisoFluor 2 scintillation fluid.

Relationship Between Islet Number and Autoradiograph Spot Density

Samples of 25, 50, 100 and 200 rat islets were preincubated in medium containing 2.5 mM glucose for 45 minutes and then assayed for preproinsulin mRNA content. Two such assays were carried out, one using ^{32}P-labeled phins214 plasmid insert DNA and one using ^{32}P-labeled pDI462 plasmid insert DNA. Both plasmid insert DNAs were ^{32}P-labeled by oligonucleotide primer extension. After hybridization the nitrocellulose papers were washed for 10 minutes in each of four changes of 250 ml of 2xSSC containing 0.1% (w/v) SDS at room temperature and then for 15 minutes in each of two changes of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 42°C. After air drying the papers were autoradiographed for 7 days.

Effect of Homogenization Buffer and Carrier RNA on Islet Preproinsulin mRNA Recovery

Eight samples of 50 rat islets were each homogenized in different ways and then assayed for preproinsulin mRNA content. The samples were split into two groups of four samples each, one group of islets was preincubated in medium containing 2.5 mM glucose for 45 minutes and the other group of islets was used fresh from the isolation protocol. Two samples from each group were homogenized in 1 ml of 7.6 M guanidine HCl containing 0.1 M potassium acetate pH 5.0. 5 µg of total RNA isolated from rat kidney was then added to one of these samples as a carrier. Then 0.6 ml of 95% (v/v) ethanol

was added to both samples. The other two islet samples in each group were homogenized in 1 ml of 4 M stock guanidinium thiocyanate solution. 5 µg of total RNA isolated from rat kidney was then added to one of the samples as a carrier. Then 0.75 ml of ethanol and 25 µl of acetic acid was added to both of the samples. The RNA from all eight samples was then precipitated and dot blotted in the same manner as for the preproinsulin mRNA assay from this point onwards. The nitrocellulose paper was hybridized with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. After hybridization the paper was washed for 10 minutes in each of four changes of 250 ml of 2xSSC containing 0.1% (w/v) SDS at room temperature and then for 15 minutes in each of two changes of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 42°C. The paper was air dried and autoradiographed for 8 days.

DNAse I and RNAse Action on Islet Preproinsulin mRNA

Three samples of 100 rat islets were each treated in different ways and assayed for preproinsulin mRNA. A sample of pancreatic acinar tissue hand picked from the same islets tissue preparation, and roughly equal in size to the islet samples, was also assayed along with the islet samples. One islet sample was syringe homogenized in 0.5 ml of 20 mM Tris-HCl pH 7.6 containing 10 mM $MgCl_2$ in a 15 ml Corex tube, 2 µg DNAse I added and then incubated at 37°C for 60 minutes. Another other islet sample was syringe homogenized in 0.5 ml of sterile water in a 15 ml Corex tube, 2 µg of RNAse added and then incubated at 37°C for 60 minutes. To both of these samples 50 µl of 2 M potassium acetate pH 5.0 and 0.7 g of

solid guanidine HCl was added. The salt was then dissolved by vortexing the tube and the volume made up to 1 ml with sterile water. From this point onwards the RNA in these samples was precipitated and dot blotted as in the preproinsulin mRNA assay. The third islet sample and the acinar tissue sample were assayed in the normal fashion along with the treated islet samples. The blotted nitrocellulose paper was hybridized with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. After hybridization the paper was washed for 10 minutes in each of four changes of 250 ml of 2xSSC containing 0.1% (w/v) SDS at room temperature and then for 15 minutes in each of two changes of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 42°C. The paper was air dried and autoradiographed for 7 days.

(2) Measurement of Preproinsulin mRNA in Islets

Effect of Glucose on Preproinsulin mRNA in Incubated Islets

Ten samples of 50 rat islets each were preincubated in medium containing 2.5 mM glucose for 45 minutes. Two samples were then assayed for preproinsulin mRNA and the remaining samples split into two groups of four samples. One group was incubated in medium containing 2.5 mM glucose and the other in medium containing 20 mM glucose. After one hour incubation two samples from each group were assayed for preproinsulin mRNA and after two hours incubation the remaining two samples were assayed. The nitrocellulose paper was hybridized with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. After hybridization the paper was washed for 10 minutes in each of four changes of 250 ml of 2xSSC containing 0.1% (w/v) SDS at room temperature and then for 15 minutes in each of two changes of 250 ml of 0.1xSSC containing 0.1% (w/v) SDS at 42°C. The paper was air dried and autoradiographed for 3 to 7 days. The whole experiment was repeated with 10 µg/ml actinomycin D present in both the preincubation and the incubation mediums.

Effect of Glucose on Preproinsulin mRNA in Tissue Cultured Islets

Groups of ~1000 islets were placed in tissue culture medium containing either 2.5 mM or 20 mM glucose, and either with or without 10 µg/ml actinomycin D, for 24 hours. At 0, ~6, ~18 and 24 hours a sample of 50 islets was hand picked and

assayed for preproinsulin mRNA using phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. A 0.5 ml aliquot of the culture medium was also removed at each time point, any particulate matter was sedimented by centrifugation (MSE Micro Centaur microfuge, high speed, 20 minutes) and duplicate 100 µl aliquots of the supernatant assayed for insulin content.

Results

(1) Validation of Preproinsulin mRNA Assay

^{32}P-Labeling of DNA

(a) Nick Translation

(i) Time Course

Figure 4.1 shows the time course of α-^{32}P-dCTP incorporation into phins214 and pDI462 plasmid insert DNA by nick translation. Incorporation of α-^{32}P-dCTP into phins214 plasmid insert DNA increased steadily during the first 90 minutes of incubation and after 180 minutes had reached a plateau level of 32.5% (equivalent to a specific radioactivity of $\sim 1 \times 10^8$ cpm/µg of DNA). Incorporation into pDI462 plasmid insert DNA was negligible and never rose above 3% incorporation for the whole of the 315 minutes of the incubation (equivalent to a specific radioactivity of 9×10^6 cpm/µg of DNA).

Figure 4.1

Time Course of Nick Translation

Nick translation reactions containing phins214 plasmid insert DNA (open triangles and dashed line) and pDI462 plasmid insert DNA (closed triangles and solid line) were set up. Duplicate aliquots were removed at various time points for the determination of α-^{32}P-dCTP incorporation.

The data points are the mean values of duplicate aliquots expressed as a percentage of the total radioactivity (cpm) incorporated into TCA insoluble material. The results are also expressed as the equivalent specific radioactivity of the ^{32}P-labeled DNA.

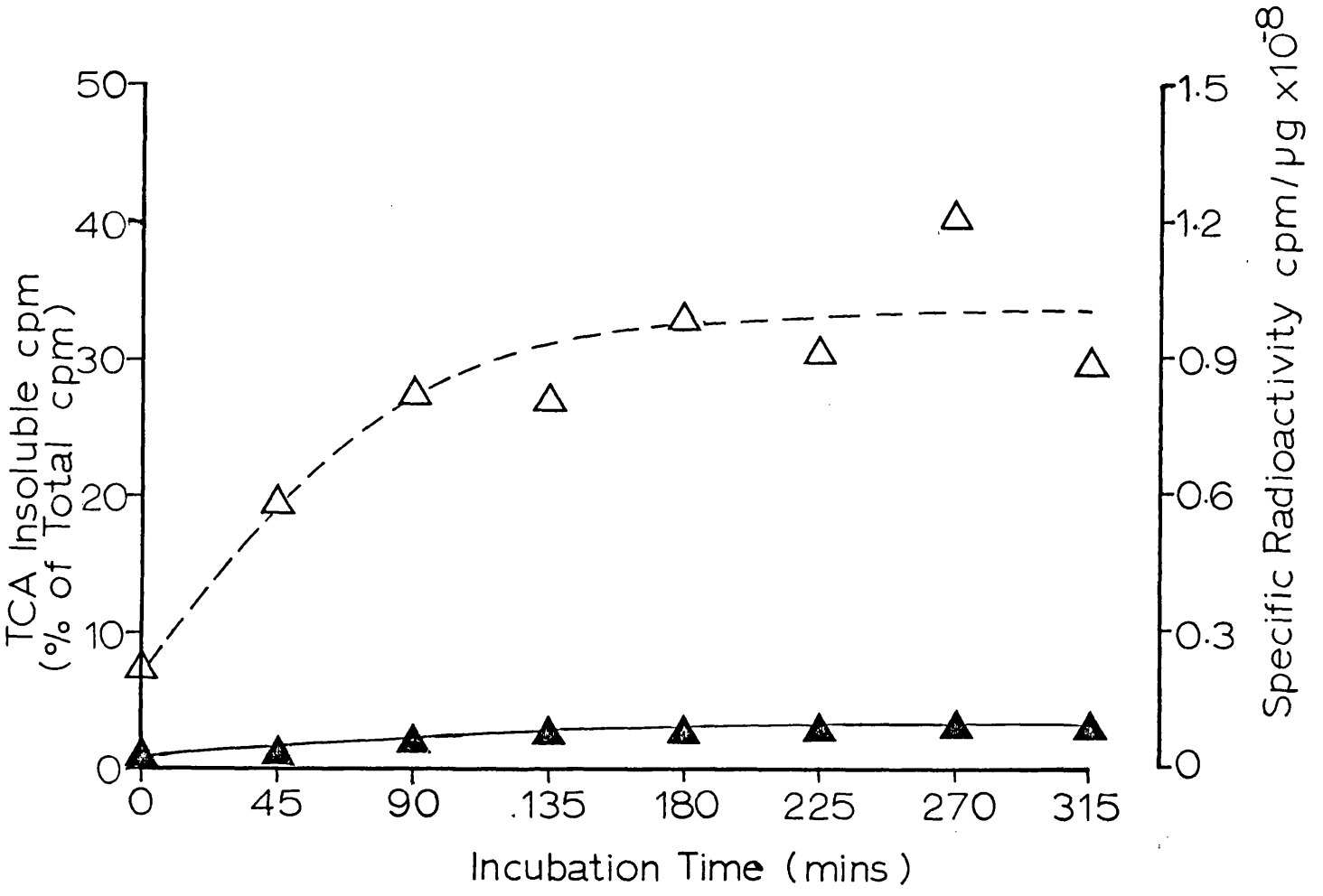

TCA Insoluble cpm
(% of Total cpm)
50
40
30
20
10
0
0
45
90
135
180
225
270
315
Incubation Time (mins)
Specific Radioactivity cpm/µg x10⁻⁸
1.5
1.2
0.9
0.6
0.3
0

Figure 4.1

(ii) Titration of DNAse I

Table 4.1 shows the effect of various amounts of DNAse I on the incorporation of α-^{32}P-dCTP into TCA insoluble material by nick translation. The incorporation of α-^{32}P-dCTP into TCA insoluble material was slightly higher in the presence of DNAse I than in the absence. The highest incorporation was seen with 1 µl of 0.01 µg/ml DNAse I stock solution but there was no obvious relationship between the amount of DNAse I added and the percentage of α-^{32}P-dCTP incorporation.

Figure 4.2 is an autoradiograph of the nick translated samples electrophoresed on an alkaline agarose gel. Of the five samples the one treated with 4 µl of 0.01 µg/ml DNAse I produced the furthest migrating, and hence the smallest, ^{32}P-labeled material. There was some ^{32}P-labeled material of high molecular weight near the sample well in all five samples. The sample treated with 4 µl of 0.01 µg/ml DNAse I exhibited the least amount of smearing of ^{32}P-labeled material from the sample well. There appeared to be small amounts of ^{32}P-labeled material in the empty lanes flanking the sample lanes.

Amount of Stock DNAse I (µl)		
0.01 µg/ml	0.1 µg/ml	% α-^{32}P-dCTP Incorporation
0	0	4.1%
1	0	7.1%
2	0	4.5%
4	0	5.1%
0	1	5.5%

Table 4.1

Effect of DNAse I on α-^{32}P-dCTP incorporation into phins214 plasmid insert DNA by nick translation.

Nick translation reactions containing phins214 plasmid insert DNA were set up with different amounts of DNAse I stock solutions. At the end of the incubations aliquots of the reaction mixes were removed for the determination of α-^{32}P-dCTP incorporation and total radioactivity.

The data points are the mean values of duplicate aliquots expressed as α-^{32}P-dCTP incorporation as a percentage of the total radioactivity (cpm).

Figure 4.2

Autoradiograph of Alkaline Electrophoresis of Nick Translated phins214 Plasmid Insert DNA

Nick translation reactions containing phins214 plasmid insert DNA were set up with different amounts of DNAse I stock solutions. The ^{32}P-labeled DNA was purified from each reaction mix by Sephadex G-100 fitration and aliquots were run on an alkaline electrophoresis gel. The gel was then dried and autoradiographed.

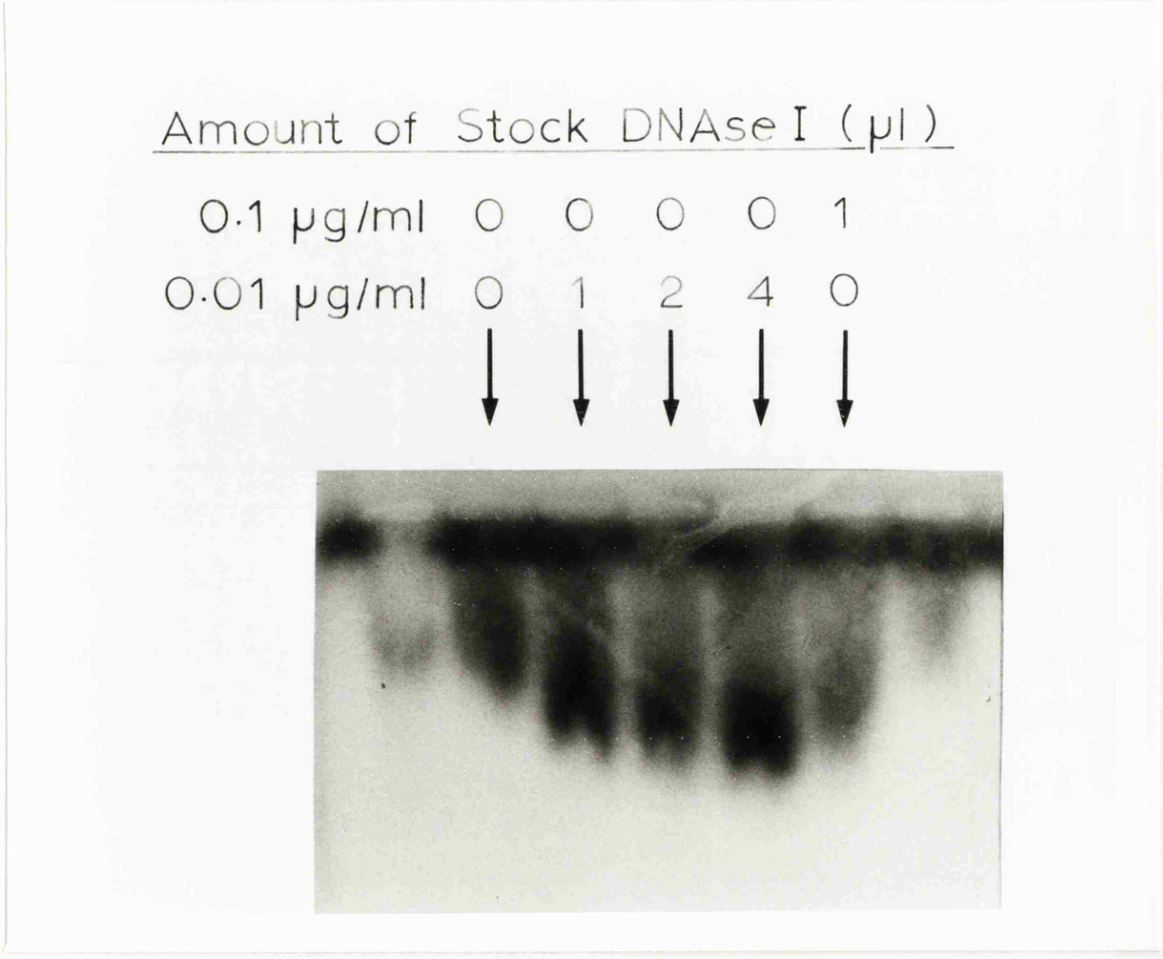


Figure 4.2

(b) Oligonucleotide Primer Extension and M13 Second Strand Synthesis

^{32}P-Labeling of both pDI462 and phins214 plasmid insert DNA by oligonucleotide primer extension was consistent. Between 50% and 80% of the α-^{32}P-dCTP was incorporated into TCA insoluble material, this was equivalent to a specific radioactivity of 1-4 x 10^8 cpm/µg of DNA (data not shown). Both the M13 rat insulin cDNA and the M13 α-1-microglobulin clone DNAs were ^{32}P-labeled by M13 second strand synthesis to an estimated specific radioactivity of 1-2 x 10^8 cpm/µg of DNA in a consistent manner (data not shown).

Purification of ^{32}P-Labeled DNA by Gel Filtration

Figure 4.3 shows the G-100 gel filtration profile of phins214 plasmid insert DNA ^{32}P-labeled by nick translation. There was only one peak of TCA insoluble radioactivity which was eluted between fractions 5 and 14 and only one peak of TCA soluble radioactivity which was eluted between fractions 16 and 30. Both peaks were well defined and separated from each other.

Figure 4.3

Sephadex G-100 Filtration of phins214 Plasmid Insert DNA ^{32}P-Labeled by Nick Translation

phins214 plasmid insert DNA was nick translated and loaded onto a Sephadex G-100 column. The column was washed and 100 µl fractions collected. Duplicate aliquots were taken from each fraction for the determination of α-^{32}P-dCTP incorporation and total radioactivity. The TCA insoluble radioactivity (cpm) (dashed line) and the TCA soluble radioactivity (cpm) (solid line; calculated by subtracting the TCA insoluble radioactivity from the total radioactivity) are plotted against the fraction number.

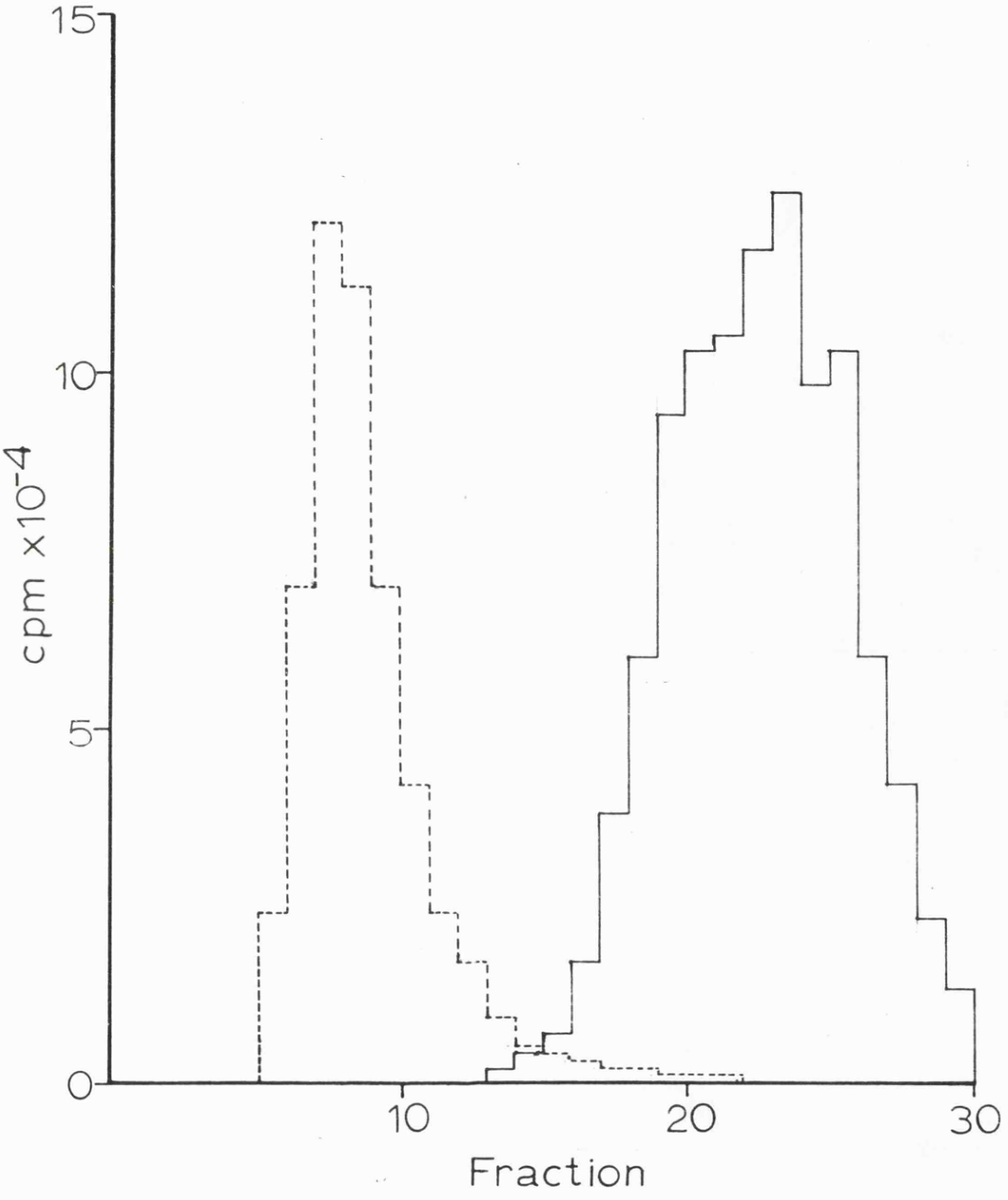


Figure 4.3

Isolation of Total and Poly A^+ RNA

Table 4.2 shows the yields and absorbance ratios of both total and poly A^+ RNA isolated from rat pancreas, liver and kidney. The yield of total kidney RNA was ~25 fold lower than for pancreas or liver. The percentage yield of poly A^+ RNA, however, was similar for all three tissues at ~5% of the total RNA.

	Total RNA		Poly A^+ RNA		
Tissue	Yield	Absorbance Ratio	Yield	Absorbance Ratio	% of Total RNA
Pancreas	10.99	2.0	0.37	2.0	3.4%
Liver	6.87	1.8	0.47	1.6	6.9%
Kidney	0.44	1.4	0.02	1.4	5.6%

Table 4.2

Isolation of total RNA and Poly A^+ RNA From Rat Pancreas, Liver and Kidney

Total RNA was isolated from rat pancreas, liver and kidney by the guanidinium thiocyanate/hot phenol method. Poly A^+ RNA was isolated from total RNA by oligo(dT)cellulose affinity chromotography.

Yields are shown as mg of RNA per gram of original starting tissue. Yield of Poly A^+ RNA is also quoted as the percentage of total RNA recovered after oligo(dT)cellulose chromotography. Absorbance ratios are given as the ratio of the sample absorbance at 260 nm to that at 280 nm.

Northern Blotting of Poly A^+ RNA

Figure 4.4 is an autoradiograph of a Northern blot of total, poly A^+ and poly A^- RNA isolated from rat pancreas and liver. There were no bands evident for any of the liver RNA samples but there was a single band in each of the pancreatic RNA sample lanes. The total pancreatic RNA band had a sharply defined start at ~550 bp with a tail of lower molecular weight material. The pancreatic poly A^+ RNA band was sharply defined at ~550 bp in size whilst the pancreatic poly A^- RNA band was diffuse with an average size smaller than 550 bp.

Figure 4.4

Autoradiograph of Northern Blot

Samples of total, poly A^+ and poly A^- RNA isolated from rat pancreas and liver were Northern blotted. The nitrocellulose paper was hybridized with M13 rat insulin cDNA clone DNA ^{32}P-labeled by M13 second strand synthesis and then autoradiographed.

Sizes of DNA fragments (in Kb) were calculated from the positions of size markers (Lambda phage DNA digested with HindIII).

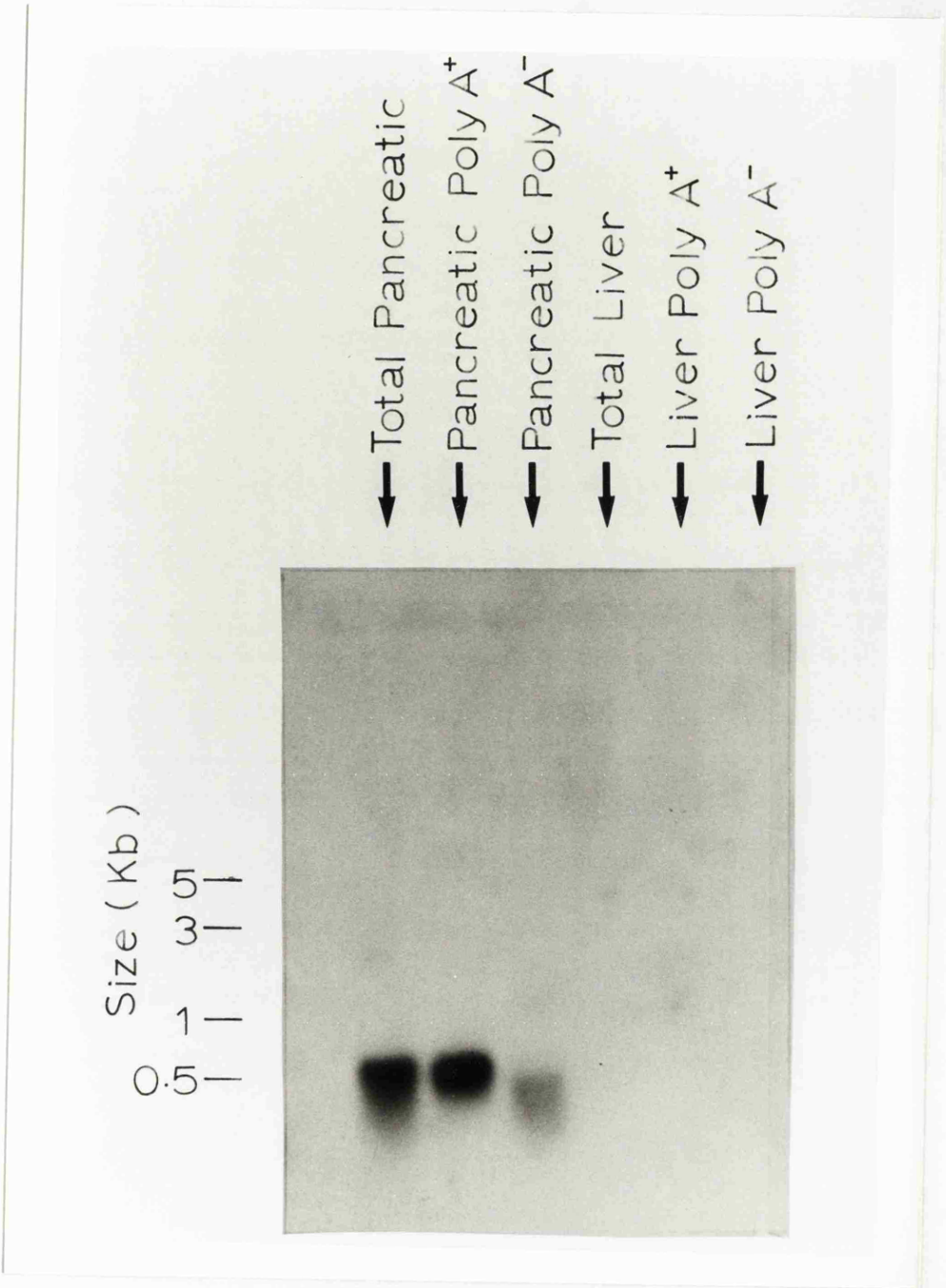

Size (Kb)
0.5
1
3
5
Total Pancreatic
Pancreatic Poly A+
Pancreatic Poly A-
Total Liver
Liver Poly A+
Liver Poly A-

Figure 4.4

Hybridization Specificity of ^{32}P-Labeled DNA

(i) Oligonucleotide Primer Extension

Figures 4.5a and 4.5b show the dot blot spot densities of poly A^+ RNA isolated from rat pancreas, liver and kidney after hybridization with phins214 and pDI462 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension respectively. In the case of hybridization with ^{32}P-labeled phins214 plasmid insert DNA (Figure 4.5a) the spot density of pancreatic poly A^+ RNA was unaffected by the increased stringency of the post-hybridization washes. Both the liver and kidney poly A^+ RNA spot densities, however, were reduced to values of 0.11 and 0.33 times that of the pancreatic poly A^+ RNA by the most stringent of the the post-hybridization washes. In the case of hybridization with pDI462 plasmid insert DNA (Figure 4.5b) increasing the stringency of the post-hybridization washes reduced the spot densities of poly A^+ RNA isolated from pancreas, liver and kidney. The relative spot densities of liver and kidney poly A^+ RNA were reduced from 1.81 and 2.31, after the lowest stringency post-hybridization wash, to 0.79 and 1.35 respectively after the most stringent post-hybridization wash.

Figure 4.5c shows the dot blot spot densities of poly A^+ RNA isolated from rat pancreas and liver after hybridization with pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. The spot densities of both pancreatic and liver poly A^+ RNA decreased with

increasing stringency of the post-hybridization washes. The relative density of the liver poly A^+ RNA spots was reduced to a level 0.21 times that of pancreatic poly A^+ RNA by the most stringent post-hybridization wash.

Figure 4.5

Hybridization specificity of DNA ^{32}P-Labeled by Oligonucleotide Primer Extension

(4.5A and 4.5B) - Samples of poly A$^+$ RNA isolated from rat pancreas (1), kidney (2) and liver (3) were assayed for preproinsulin mRNA with either phins214 (4.5A) or pDI462 (4.5B) plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. The hybridized nitrocellulose papers were then washed with washes of increasing stringencies (wash 1 being the lowest stringency) and autoradiographed.

The spot densities (in Gelscan program units) are plotted for each sample after each wash protocol. The spot densities are also normalized to that of the pancreatic poly A$^+$ RNA sample after each wash (shown as figures above each bar).

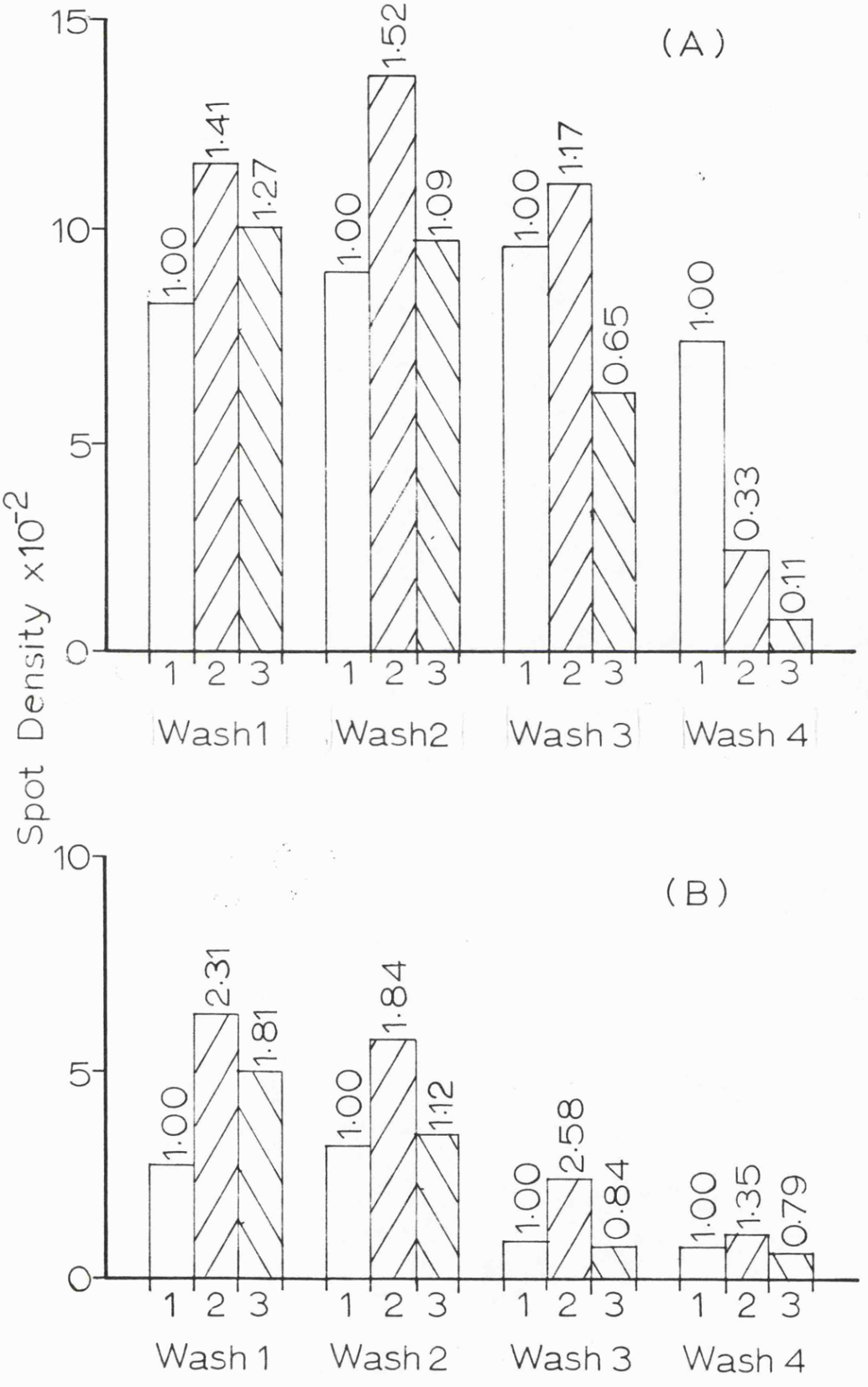

(A)
Spot Density x10⁻²
15
10
5
0
1.00
1.41
1.27
1.00
1.52
1.09
1.00
1.17
0.65
1.00
0.33
0.11
1 2 3
Wash1
Wash2
Wash 3
Wash 4
(B)
10
5
0
1.00
2.31
1.81
1.00
1.84
1.12
1.00
2.58
0.84
1.00
1.35
0.79
1 2 3
Wash 1
Wash 2
Wash 3
Wash 4

Figure 4.5

Figure 4.5

Hybridization specificity of DNA ^{32}P-Labeled by Oligonucleotide Primer Extension

(4.5C) - Samples of poly A$^+$ RNA isolated from rat pancreas (1) and liver (2) were assayed for preproinsulin mRNA with pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. The hybridized nitrocellulose paper was then washed with washes of increasing stringencies (wash 1 being the lowest stringency) and autoradiographed.

The spot densities (in Gelscan program units) are plotted for each sample after each wash protocol. The spot densities are also normalized to that of the pancreatic poly A$^+$ RNA sample after each wash (shown as figures above each bar).

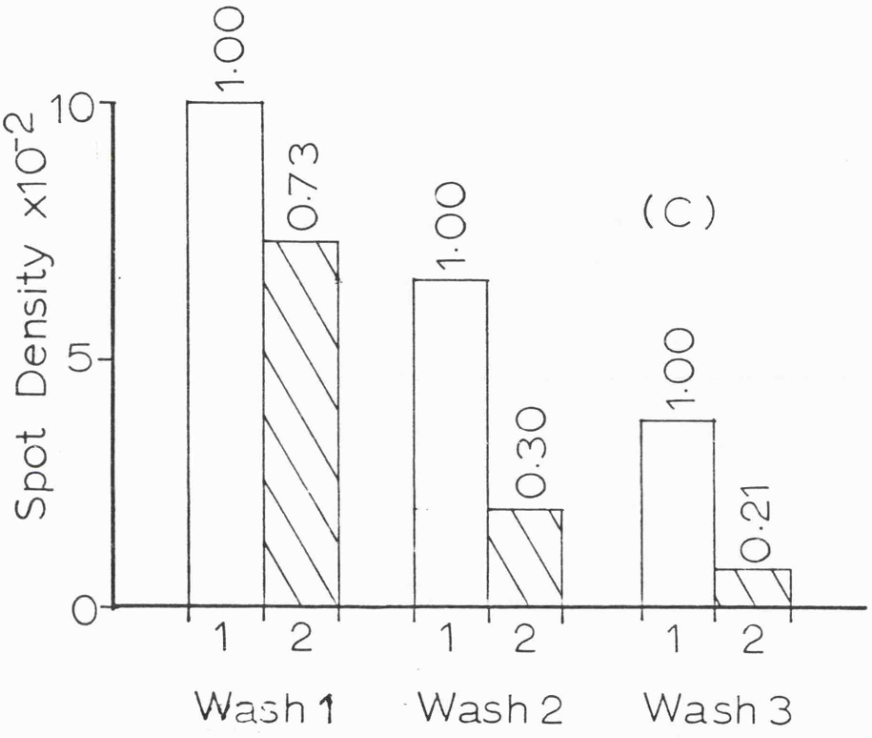

Spot Density x10⁻²
10
5
0
1.00
0.73
1.00
0.30
(C)
1.00
0.21
1
2
1
2
1
2
Wash 1
Wash 2
Wash 3

Figure 4.5

Figure 4.6 is the autoradiograph of the four nitrocellulose papers which were hybridized with ^{32}P-labeled phins214 plasmid insert DNA (see Figure 4.5a). The level of background hybridization decreased with the increasing stringency of the post-hybridization wash protocols.

Figure 4.7 is a plot of the dot blot spot densities of the serial dilutions of rat pancreatic and liver poly A^+ RNA from the experiment shown in Figure 4.5c (after the second post-hybridization wash protocol). For both pancreatic and liver poly A^+ RNA samples spot density increased with the amount of RNA in a linear fashion. The gradient of the line for pancreatic poly A^+ RNA was ~3-fold greater than that of liver poly A^+ RNA.

Figure 4.8 is a sample densitometer trace of the spots of the serial dilutions of pancreatic poly A^+ RNA after the lowest stringency post-hybridization wash protocol of the experiment shown in Figure 4.5c. The peaks seen were evenly shaped and spaced. They were well defined and their size decreased systematically with the dilution of the sample.

Figure 4.6

Autoradiograph of Hybridization Specificity Test

Serial dilutions (2/3 fold) of poly A^+ RNA isolated from rat pancreas, kidney and liver were assayed for preproinsulin mRNA with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. The hybridized nitrocellulose paper was then washed and autoradiographed.

The identity of the poly A^+ RNA samples are shown along with the log of the dilution for each sample spot.

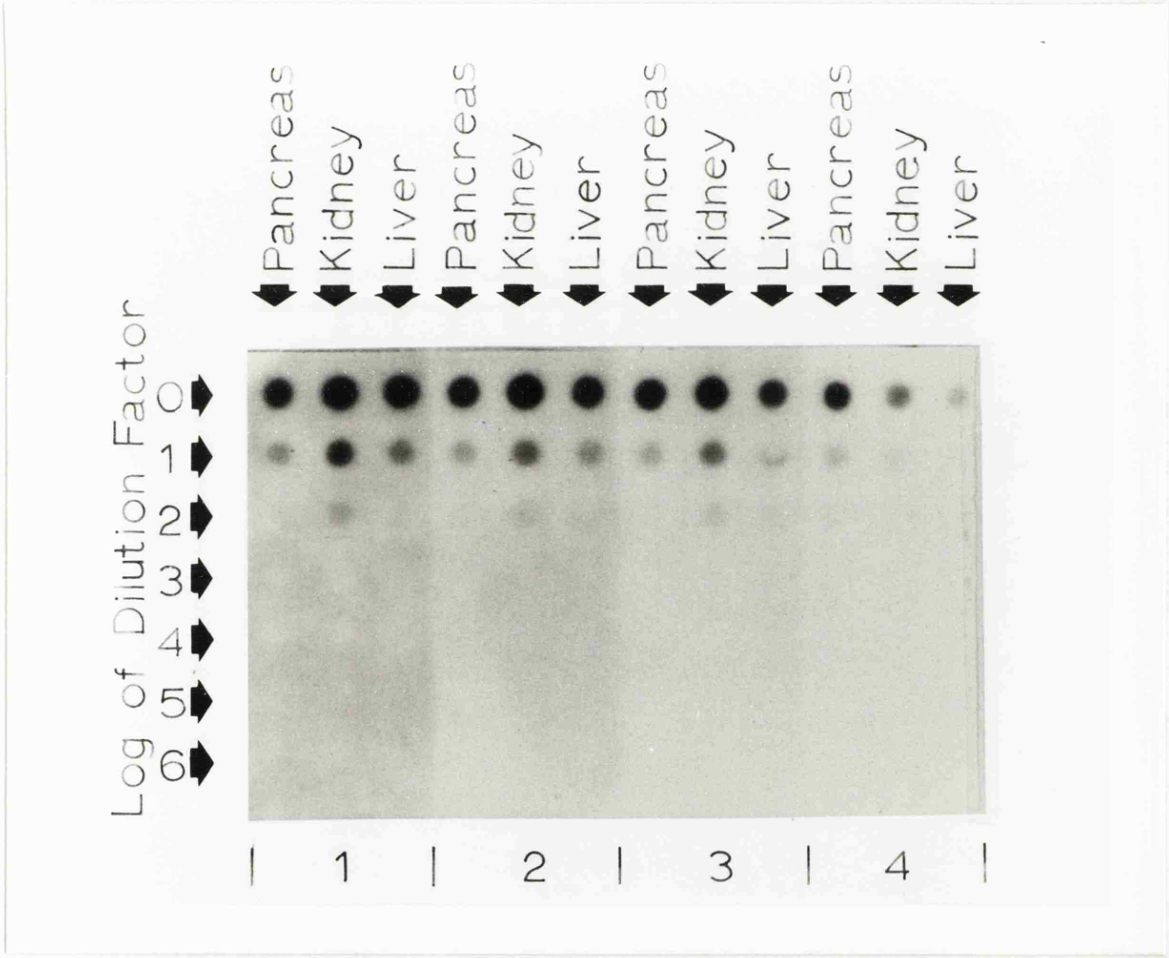

Log of Dilution Factor
0
1
2
3
4
5
6
Pancreas
Kidney
Liver
Pancreas
Kidney
Liver
Pancreas
Kidney
Liver
Pancreas
Kidney
Liver
1
2
3
4

Figure 4.6

Figure 4.7

Relationship Between Dot Density and Amount of RNA Standards

Serial dilutions (2/3 fold) of pancreatic poly A^+ RNA (closed triangles and solid line) and liver poly A^+ RNA (open triangles and dashed line) were assayed for preproinsulin mRNA. The hybridization probe was pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension. After washing and autoradiograph exposure the autoradiograph was scanned and the optical densities of the dots were plotted against the amounts of poly A^+ RNA.

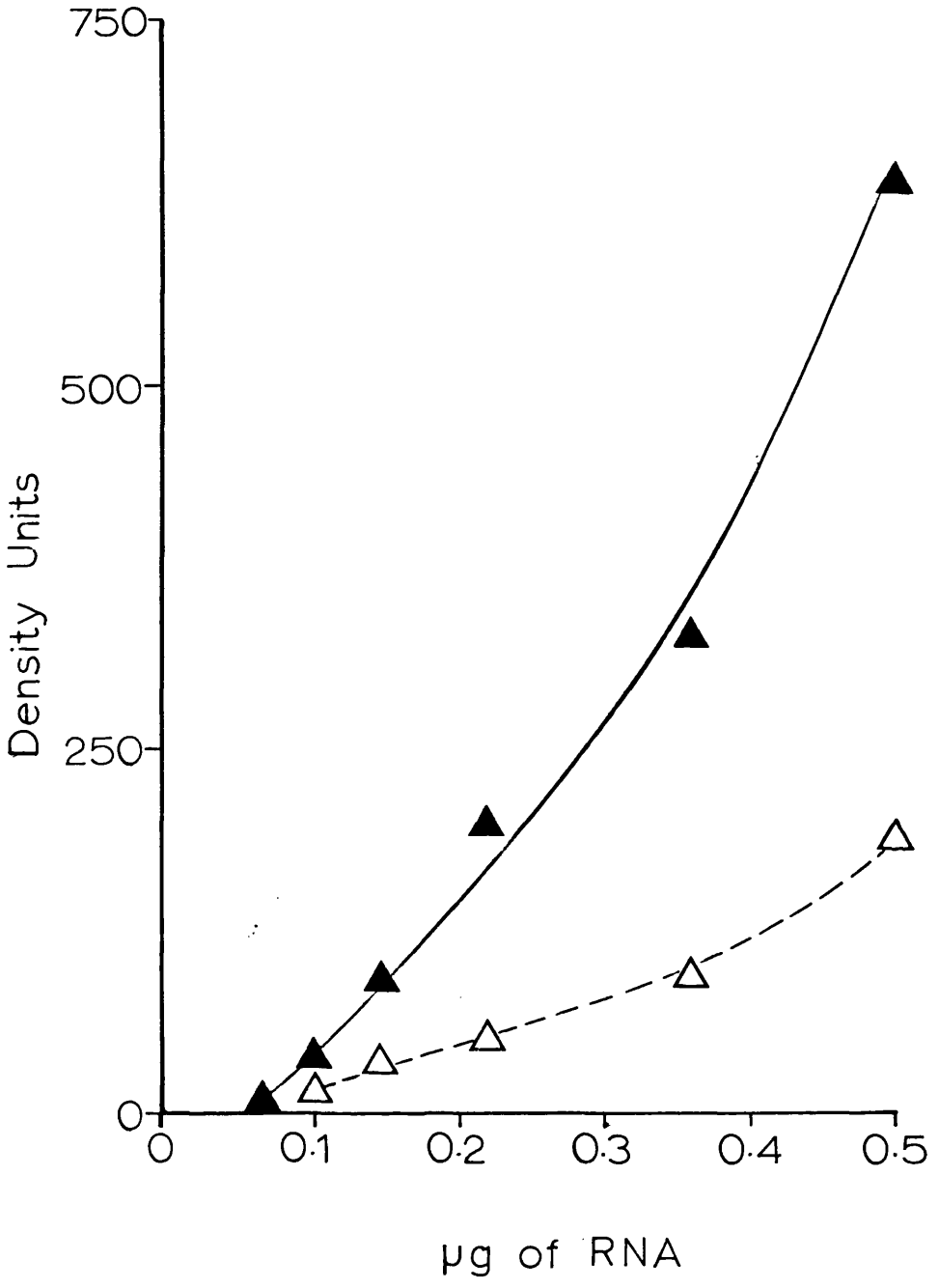


Figure 4.7

Figure 4.8

Sample Densitometer Trace Produced by the Gelscan Program

Serial dilutions (2/3 fold) of pancreatic poly A^+ RNA were assayed for preproinsulin mRNA with pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. After washing and autoradiograph exposure three separate densitometer scans were made of the sample dots and averaged using the Gelmerge program.

A plot of the averaged scan is shown as distance along the autoradiograph against the optical density (scaled from 0 to 10). The dilution of the samples increases from right to left.

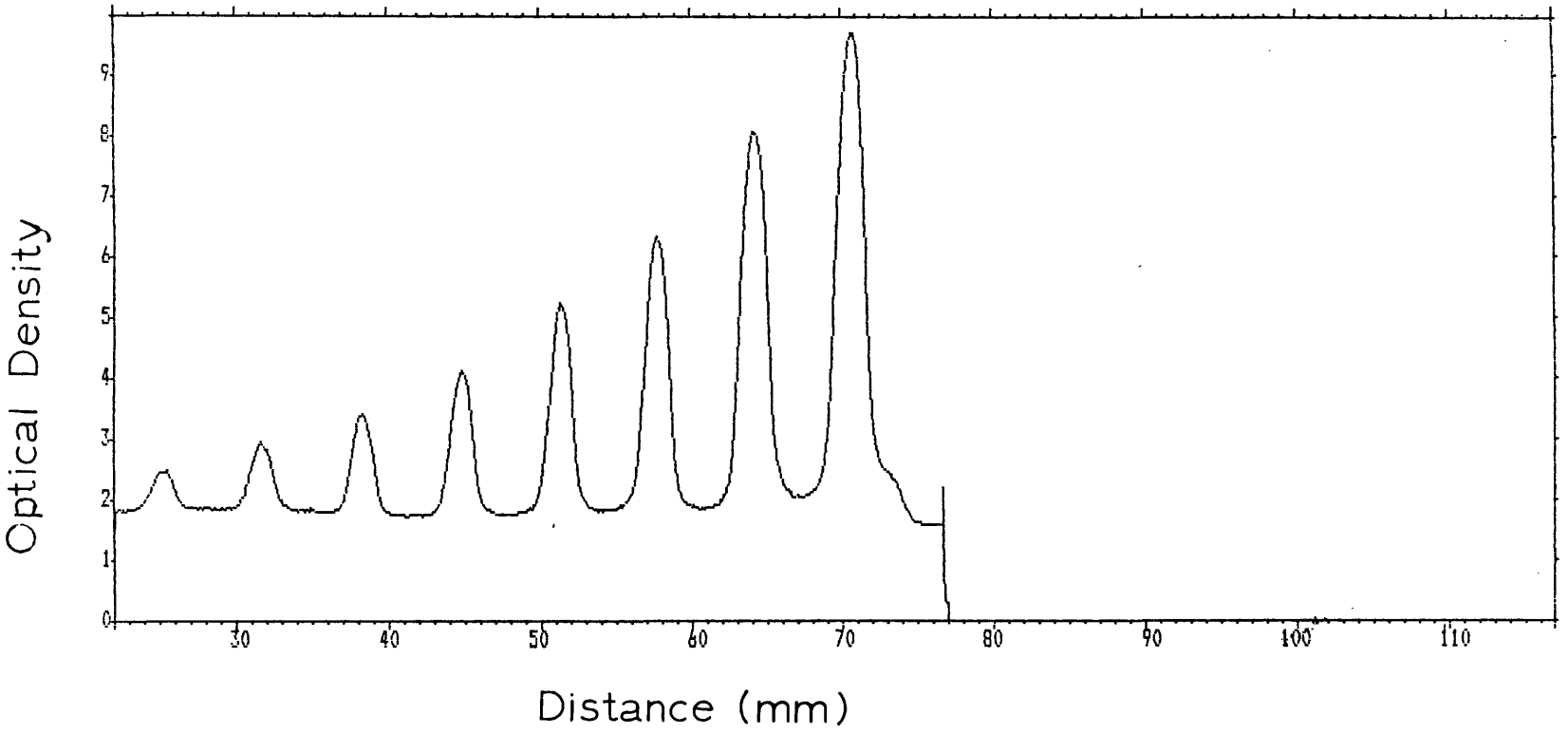

Optical Density
0
1
2
3
4
5
6
7
8
9
30
40
50
60
70
80
90
100
110
Distance (mm)

Figure 4.8

(ii) M13 Second Strand Synthesis

Figures 4.9a and 4.9b show the dot densities of poly A^+ RNA isolated from rat pancreas and liver, and a dot blotted sample of 200 islets, after hybridization with DNA ^{32}P-labeled by second strand synthesis of the M13 insulin cDNA clone and the M13 α-1-microglobulin clone respectively. In the case of hybridization with ^{32}P-labeled DNA synthesised from the M13 insulin cDNA clone (Figure 4.9a) increased stringency of the post-hybridization wash reduced the relative density of the islet sample spot to 0.5 after the highest stringency wash. The relative density of the liver poly A^+ RNA spot, however, was only slightly reduced to a level almost equal to that of the pancreatic poly A^+ RNA after the same wash.

In the case of hybridization with ^{32}P-labeled DNA synthesised from the M13 α-1-microglobulin clone (Figure 4.9b) increased stringency of the post-hybridization wash had little effect on the relative density of the liver poly A^+ RNA spot which remained at ~1.5 after all the washes. No spot corresponding to the islet sample could be detected after any of the washes.

Figure 4.9

Hybridization Specificity of DNA ^{32}P-Labeled by M13 Second Strand Synthesis

Samples of pancreatic (1) and liver (2) poly A$^+$ RNA, along with a sample of islets (3), were assayed for preproinsulin mRNA with DNA ^{32}P-labeled by M13 second strand synthesis of either the M13 insulin cDNA clone (4.9A) or the M13 α-1-microglobulin clone (4.9B) as the hybridization probe. The nitrocellulose paper was then subjected to five sequential washes of increasing stringencies (wash 1 being of the lowest stringency) and autoradiographed after each wash.

The dot densities, relative to that of the pancreatic poly A$^+$ RNA sample, are plotted for each sample after each wash.

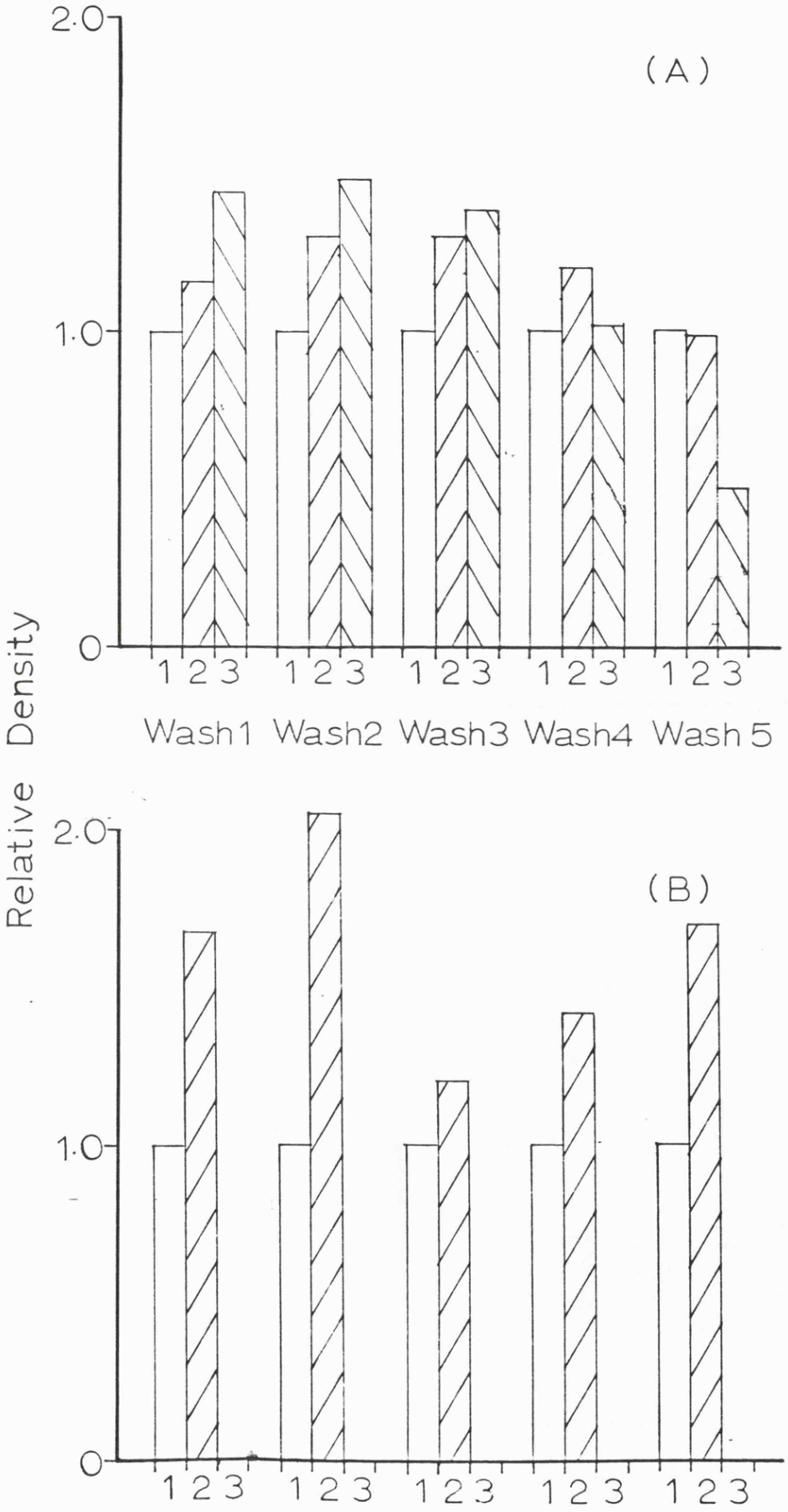

(A)
2·0
1·0
0
Relative Density
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
Wash1 Wash2 Wash3 Wash4 Wash 5
(B)
2·0
1·0
0
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

Figure 4.9

Relationship Between ^{32}P-Labeled DNA Bound and Autoradiograph Spot Density

Figure 4.10 shows the relationship between ^{32}P-labeled DNA bound to the nitrocellulose paper and the density of the corresponding autoradiograph spot. The data was fitted to a linear regression line with a correlation coefficient of 0.833 and a slope of 1.75 density units/cpm.

Figure 4.10

Relationship Between ^{32}P-Labeled DNA Bound and Autoradiograph Spot Density

Serial dilutions of pancreatic poly A^+ RNA were assayed for preproinsulin mRNA with pKA30 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. Three such assays were carried out with post-hybridization washes of different stringencies. After autoradiography portions of the nitrocellulose, corresponding to the dots on the autoradiograph, were punched out and their radioactivities (cpm) determined and plotted against their corresponding optical densities.

The data was fitted to the line shown by linear regression.

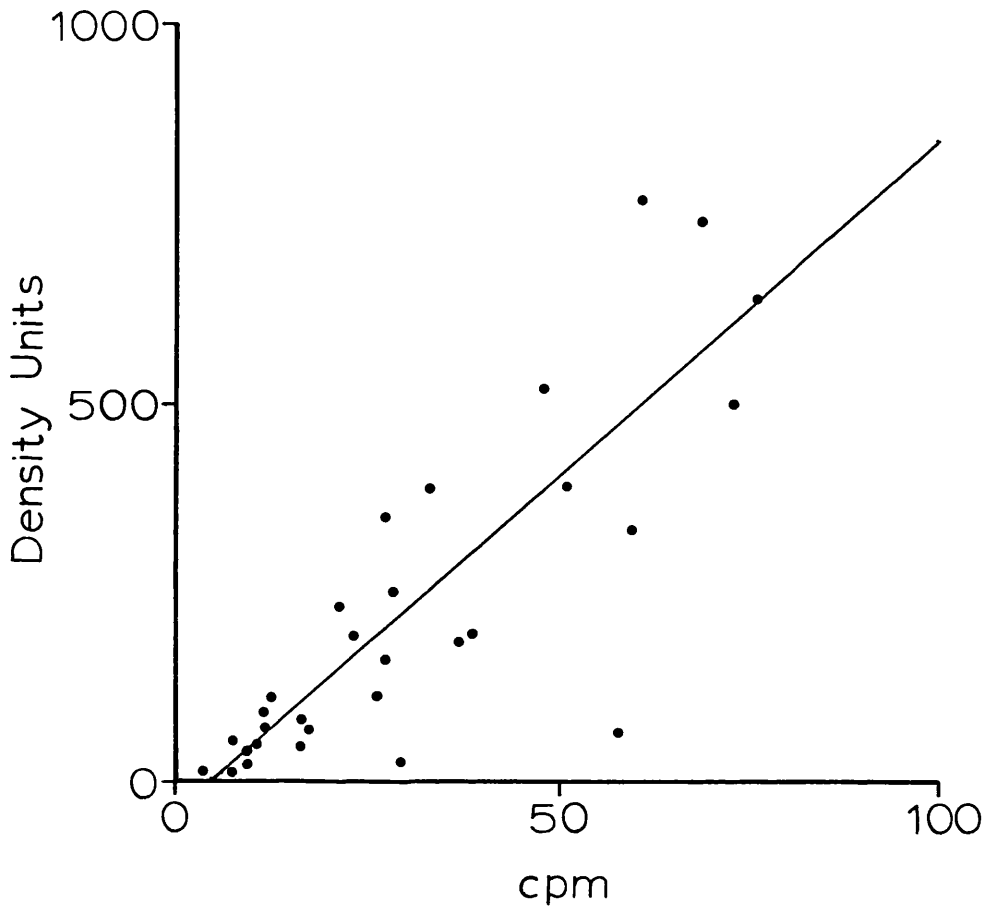


Figure 4.10

Relationship Between Islet Number and Autoradiograph Spot Density

Figure 4.11 shows the relationship between the number of islets in a sample and the density of the autoradiograph spot for preproinsulin mRNA assays with either phins214 or pDI462 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. The data for both ^{32}P-labeled phins214 and pDI462 plasmid insert DNA were of a similar pattern. No spots were detected for samples of 25 islets and the curve was steepest between 25 and 100 islets and leveled off for samples of 100 islets or greater.

Figure 4.11

Relationship Between Islet Number and Autoradiograph Spot Density

Samples of 25, 50, 100 and 200 islets were assayed for preproinsulin mRNA with either phins214 plasmid insert DNA (closed triangles and solid line) or pDI462 plasmid insert DNA (open triangles and dashed line) ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe. The spot density, relative to that of a pancreatic poly A^+ RNA control sample, of each sample was plotted against the number of islets in the sample.

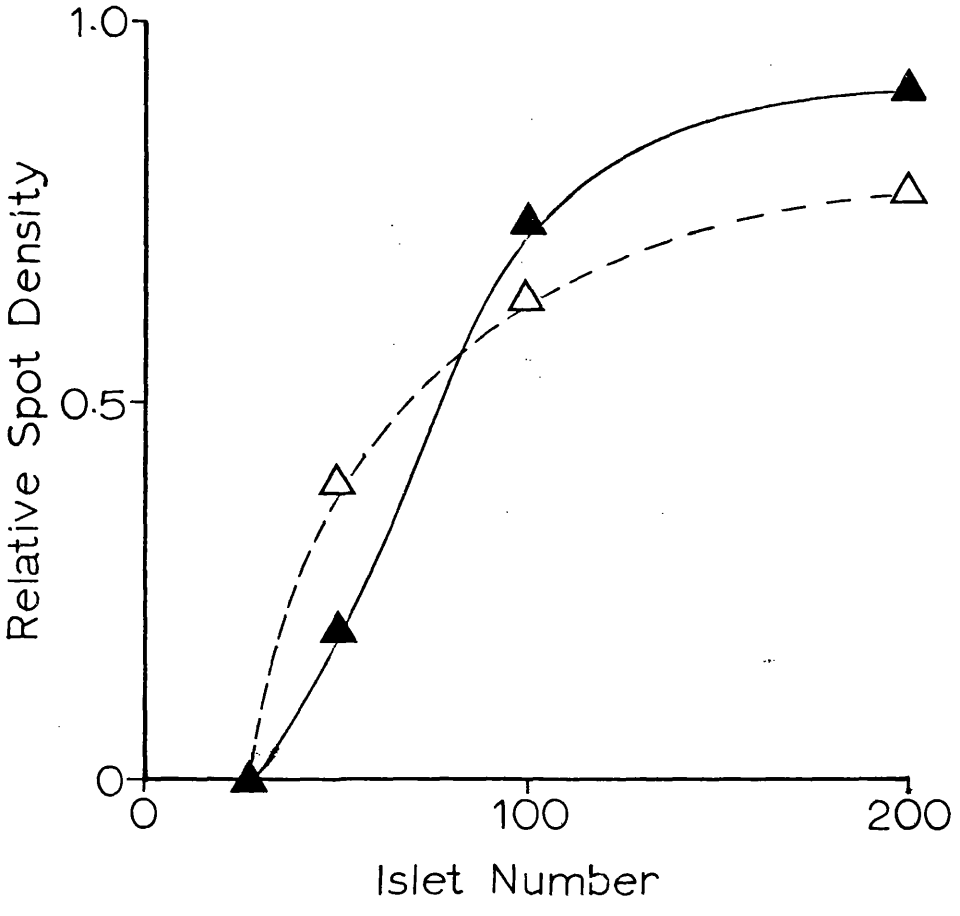


Figure 4.11

Effect of Carrier RNA and Homogenization Buffer on Islet Preproinsulin mRNA Recovery

Table 4.3 shows the effect of two different homogenization buffers, and the use of total kidney RNA as a carrier, on the recovery of preproinsulin mRNA from freshly isolated islets and islets preincubated for 45 minutes. Homogenization in guanidinium thiocyanate resulted in recoveries ~3 to 4-fold higher than in guanidine HCl but this was only true when carrier RNA was present since without it no sample spots were detected. When carrier RNA was used the recovery from preincubated islets was 0.5 to 2-fold greater than from fresh islets but this pattern was reversed in the absence of carrier RNA. Carrier RNA on its own had a recovery roughly equal to that of freshly isolated islets with carrier RNA and preincubated islets without carrier RNA.

Islet Sample	Carrier RNA	Homogenization Buffer: Guanidine HCl	Homogenization Buffer: Guanidinium Thiocyanate
Freshly isolated	YES	0.15	0.64
	NO	0.28	0.0
Preincubated	YES	0.30	1.00
	NO	0.18	0.0

Carrier RNA only = 0.19

Table 4.3

Effect of Carrier RNA and Homogenization Buffer on Islet Preproinsulin mRNA Recovery

Samples of 50 islets, either freshly isolated or preincubated in medium containing 2.5 mM glucose for 45 minutes, were homogenized in either 1 ml of 7.6 M guanidine HCl containing 0.1 M potassium acetate pH 5.0 or 1 ml of 4 M guanidinium thiocyanate stock solution. 5 µg of total RNA isolated from rat kidney was added as a carrier to those samples indicated after homogenization.

The results are shown as the densities of the autoradiograph spots relative to that of preincubated islets homogenized in guanidinium thiocyanate stock solution with carrier RNA. The density of the autoradiograph spot due to 5 µg of total rat kidney RNA on its own is also given.

DNAse I and RNAse Action on Islet Preproinsulin mRNA

Table 4.4 shows the effect of DNAse I and RNAse treatment on the recovery of islet preproinsulin mRNA. No recovery of preproinsulin mRNA could be detected after RNAse treatment. DNAse I treatment reduced the amount of preproinsulin mRNA recovered to a third of that of untreated islets. The acinar tissue sample contained a fifth of the preproinsulin mRNA content of the untreated islets.

Sample	µg Poly A^+ RNA Equivalents
100 islets	4.21
100 islets/DNAse I treated	1.43
100 islets/RNAse treated	0.0
Acinar tissue	0.84

Table 4.4

DNAse I and RNAse Action on Islet Preproinsulin mRNA

Samples of 100 islets were digested at 37°C for 60 minutes with either DNAse I or RNAse before assaying for preproinsulin mRNA with phins214 plasmid insert DNA labeled by oligonucleotide primer extension as the hybridization probe. A sample of acinar tissue and an untreated sample of 100 islets from the same tissue preparation were also assayed. Results are shown as the equivalent in rat pancreatic poly A^+ RNA in µg.

The amount of preproinsulin mRNA is expressed as the equivalent amount in µg of pancreatic poly A^+ RNA per islet.

(2) Measurement of Preproinsulin mRNA in Islets

Effect of Glucose on Preproinsulin mRNA in Incubated Islets

Figure 4.12 shows the effect of incubation of islets, for 120 minutes, in medium containing 2.5 mM or 20 mM glucose, in the presence or absence of 10 µg/ml actinomycin D, on islet preproinsulin mRNA content. For islets incubated in medium containing 2.5 mM glucose in the absence of actinomycin D (Figure 4.12a) there was no change in preproinsulin mRNA content over the 120 minutes of incubation. The addition of 10 µg/ml actinomycin D had no statistically significant effect on preproinsulin mRNA content of islets incubated at 2.5 mM glucose (Figure 4.12b). Incubation in medium containing 20 mM glucose in the absence of actinomycin D resulted in an increase in islet preproinsulin mRNA content from the same level as islets incubated in medium containing 2.5 mM, at zero time, to a level ~2.5-fold higher after 120 minutes of incubation (Figure 4.12a). These differences between preproinsulin mRNA content of islets incubated in medium containing 2.5 mM as opposed to 20 mM glucose, however, were not statistically significant. The presence of 10 µg/ml actinomycin D had no statistically significant effect on islet preproinsulin mRNA content for islets incubated in medium containing 20 mM glucose (Figure 4.12b).

Figure 4.12

Effect of Glucose on Preproinsulin mRNA in Incubated Islets

(4.12A) - Samples of 50 islets were preincubated in medium containing 2.5 mM glucose for 45 minutes and then transferred to medium containing either 2.5 mM (open triangles and dashed line) or 20 mM (closed triangles and solid line) glucose for incubation for various times up to 120 minutes. At the end of each incubation the islets were assayed for preproinsulin mRNA with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe.

(4.12B) - Samples of 50 islets were preincubated in medium containing 2.5 mM glucose, and 10 µg/ml actinomycin D, for 45 minutes and then transferred to medium containing 10 µg/ml actinomycin D and either 2.5 mM (open triangles and dashed line) or 20 mM (closed triangles and solid line) glucose for incubation for various times up to 120 minutes. At the end of each incubation the islets were assayed for preproinsulin mRNA with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe.

The data points are the mean values of a number of samples (indicated by each point) with standard error bars. The amount of preproinsulin mRNA is expressed as the equivalent amount in µg of pancreatic poly A$^+$ RNA per islet.

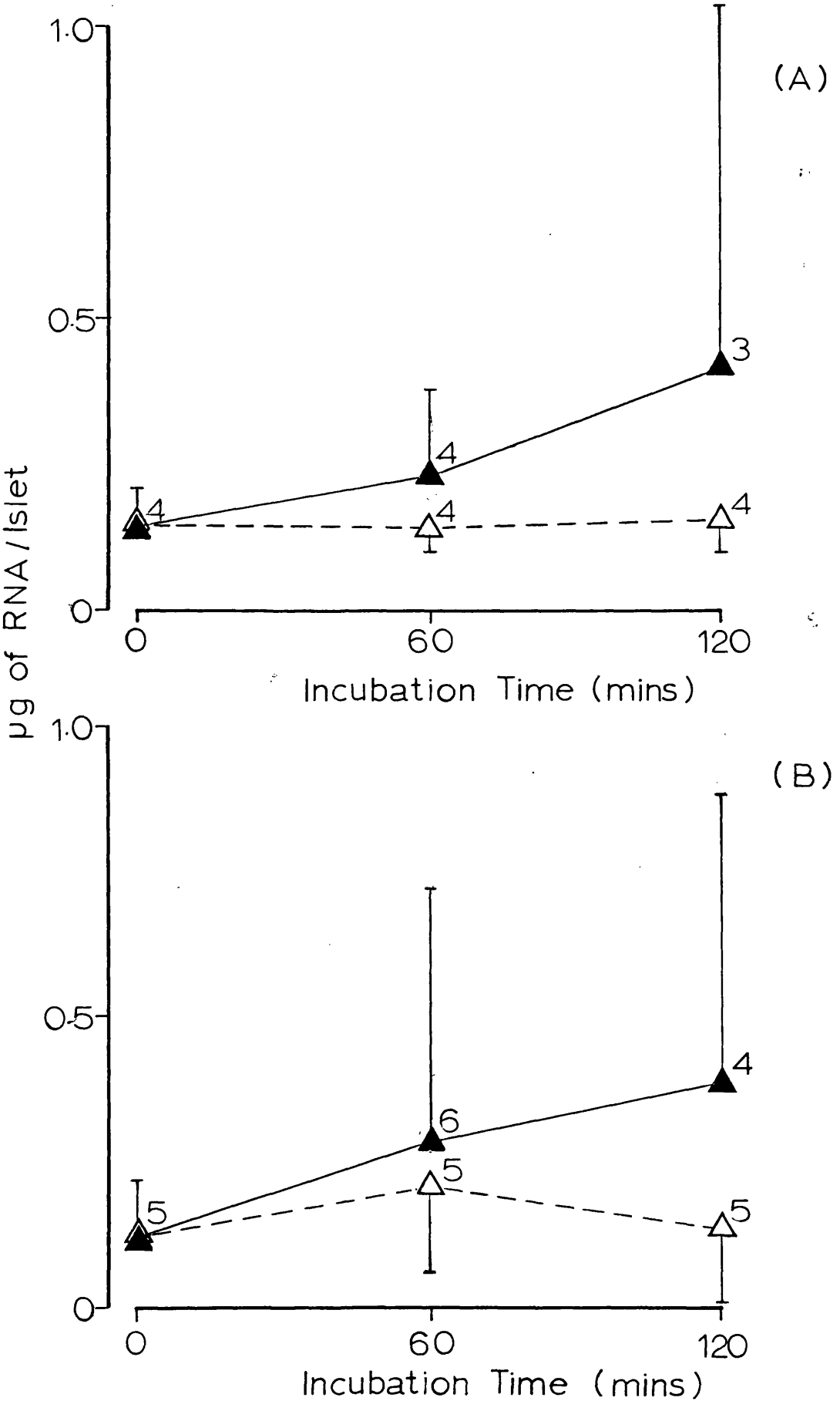

1·0
0·5
0
μg of RNA / Islet
(A)
4
4
4
3
0
60
120
Incubation Time (mins)
1·0
0·5
0
(B)
5
6
5
4
5
0
60
120
Incubation Time (mins)

Figure 4.12

Effect of Glucose on Preproinsulin mRNA in Tissue Cultured Islets

Figures 4.13a and 4.13b show the effect of 24 hour tissue culture in medium containing either 2.5 mM or 20 mM glucose, in the presence or absence of 10 µg/ml actinomycin D, on islet preproinsulin mRNA level. The data varied greatly between experiments so the data shown in figures 4.13a and 4.13b are each from one representative experiment with one islet sample per data point. Tissue culturing in medium containing 2.5 mM glucose (Figure 4.13a) resulted in a slight overall increase in islet preproinsulin mRNA content over the 24 hours of culture. The addition of 10 µg/ml actinomycin D resulted in an overall decrease in preproinsulin mRNA level. Tissue culturing in medium containing 20 mM glucose (Figure 4.13b) resulted in a steady decline in preproinsulin mRNA level over the 24 hours of culture. The addition of 10 µg/ml actinomycin D had little effect on this pattern. In general preproinsulin mRNA levels were higher in the presence of 10 µg/ml actinomycin D and 20 mM glucose.

Figures 4.14a and 4.14b show the accumulation of insulin in the tissue culture medium over the 24 hours of culture. The pattern of insulin accumulation in the medium was the same both in the presence (Figure 4.14b) and absence (Figure 4.14a) of 10 µg/ml actinomycin D. During culture at 2.5 mM glucose there was no accumulation of insulin in the medium whereas during culture at 20 mM glucose insulin accumulated to a level ~10-fold higher than at 2.5 mM glucose after 24 hours of culture.

Figure 4.13

Effect of Glucose on Preproinsulin mRNA in Tissue Cultured Islets

Samples of ~1000 islets were tissue cultured in medium containing either 2.5 mM (4.13A) or 20 mM (4.13B) glucose in either the presence (open triangles and dashed line) or the absence (closed triangles and solid line) of 10 µg/ml actinomycin D. After various times samples of 50 islets were removed and assayed for preproinsulin mRNA with phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension as the hybridization probe.

The data points are the mean values of duplicate samples from the same experiment. The amount of preproinsulin mRNA is expressed as the equivalent amount in µg of pancreatic poly A^+ RNA per islet.

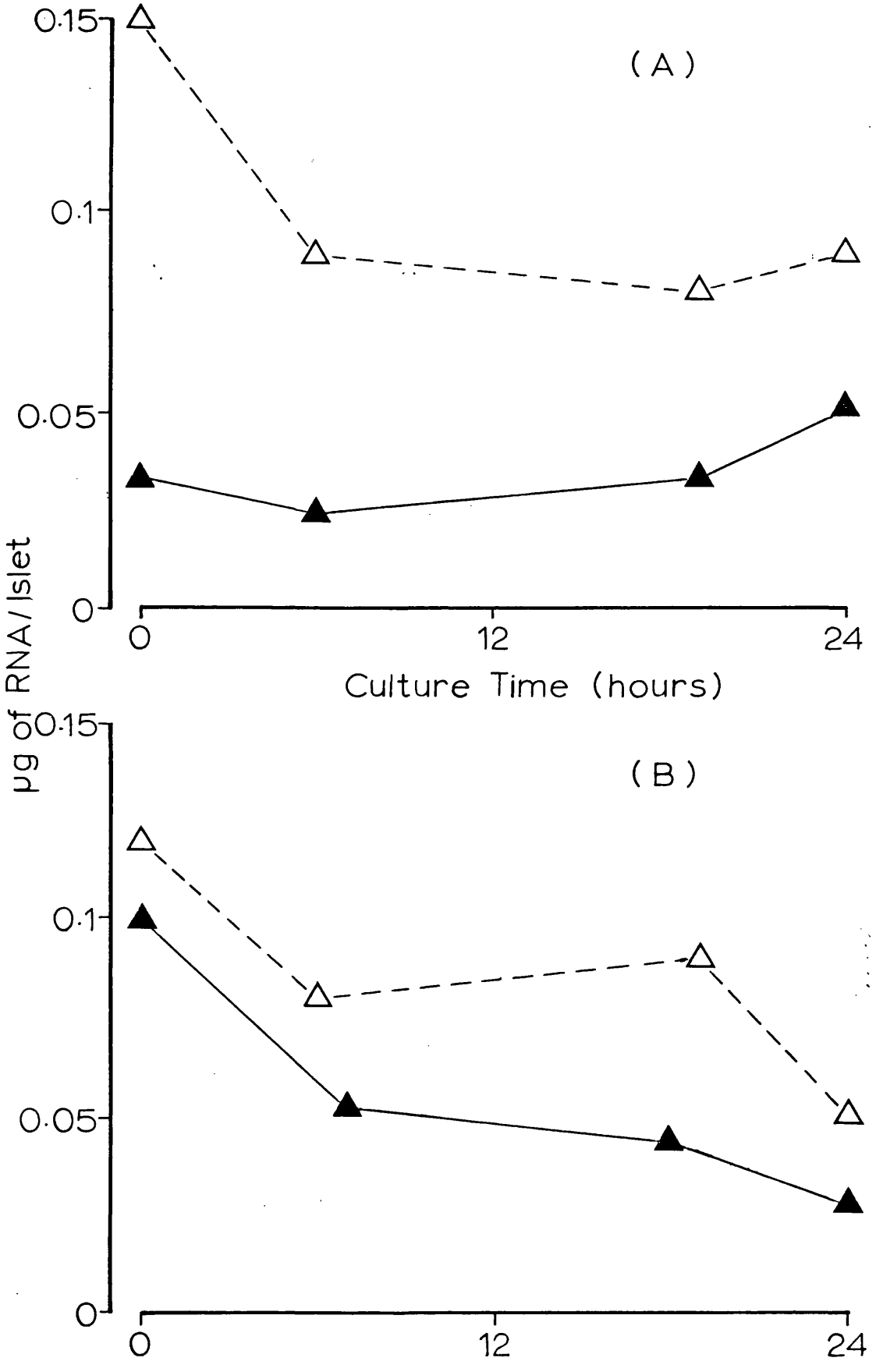

0·15
0·1
0·05
0
(A)
0
12
24
Culture Time (hours)
µg of RNA/Islet
0·15
0·1
0·05
0
(B)
0
12
24
Culture Time (hours)

Figure 4.13

Figure 4.14

Accumulation of Insulin in the Medium During Tissue Culture of Islets

Samples of ~1000 islets were tissue cultured in medium containing either 2.5 mM (open triangles and dashed line) or 20 mM (closed triangles and solid line) glucose in either the absence (4.14A) or the presence (4.14B) of 10 µg/ml actinomycin D. After various times samples of the culture medium were removed and assayed for insulin content.

The data points are the mean values of duplicate samples from the same experiment.

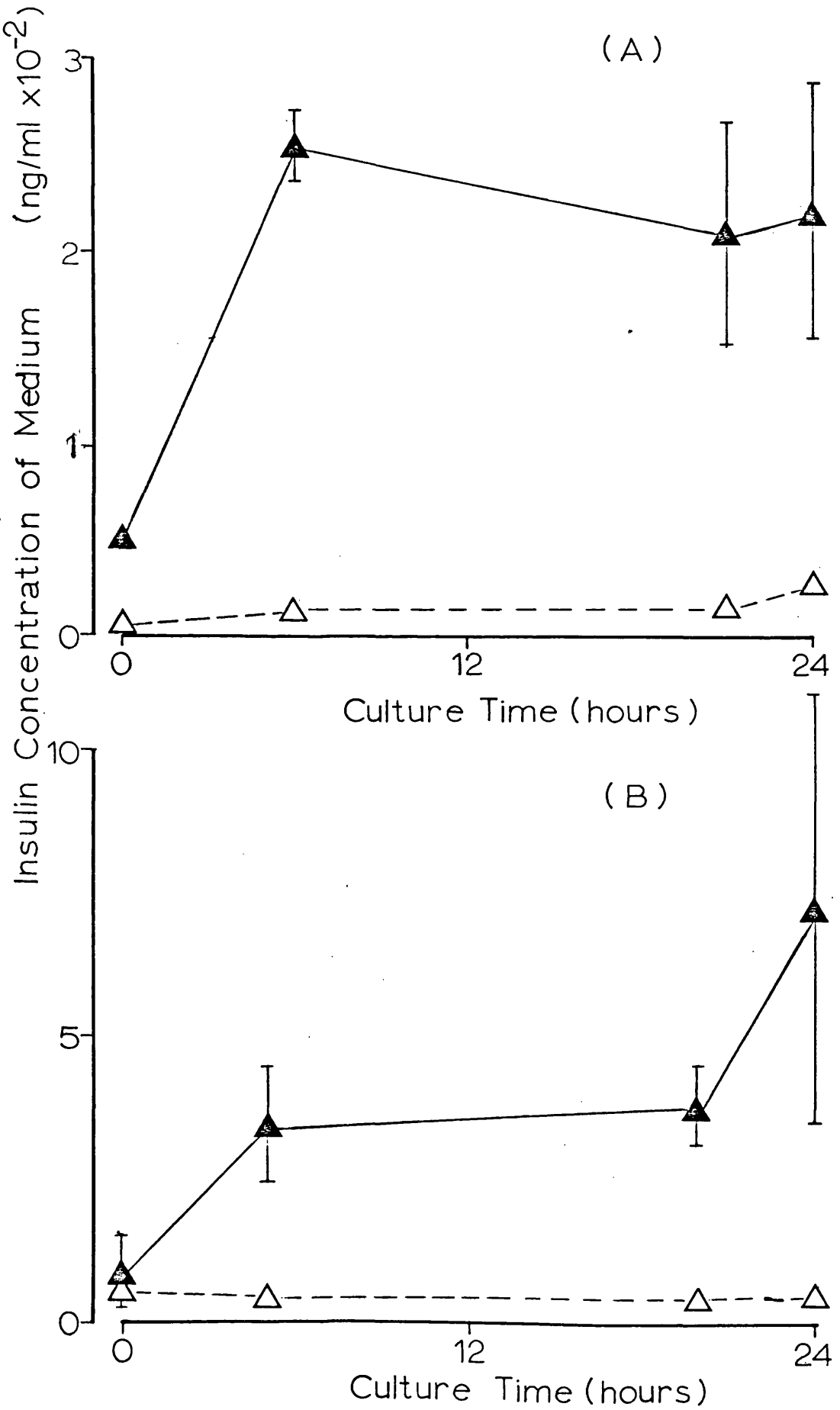

(A)
Insulin Concentration of Medium (ng/ml x10^{-2})
3
2
1
0
0
12
24
Culture Time (hours)
(B)
10
5
0
0
12
24
Culture Time (hours)

Figure 4.14

Discussion

(1) Validation of Preproinsulin mRNA Assay

^{32}P-Labeling of DNA

Two methods for ^{32}P-labeling double stranded DNA to a high specific radioactivity for use as preproinsulin mRNA hybridization probes were investigated, nick translation and oligonucleotide primer extension.

(i) Nick Translation

The principle of this method is as follows. Single stranded nicks are introduced into the double stranded template DNA by the action of DNAse I which produces randomly distributed 3'-hydroxyl termini. The nucleotide residue on the 5' side of the nick is removed by the 5' to 3' exonuclease activity of *E. coli* DNA polymerase I. At the same time nucleotide residues, from nucleotide triphosphates precursors, complementary to the residue on the opposite DNA strand, are added by the 5' to 3' polymerase activity of the same enzyme on to the hydroxyl group on the 3' side of the nick. The sequential addition and removal of nucleotide residues at each side of the nick results in a movement of the nick (nick translation) along one strand of the DNA molecule in a 5' to 3' direction (Kelly et al 1970). Replacement of one, or more, of the four nucleotide triphoshate precursors with its α-^{32}P-labeled form results in the synthesis of DNA,

complementary in sequence to the template DNA, which is ^{32}P-labeled, in the phosphate groups of the sugar-phosphate backbone of the molecule, to a high specific radioactivity (Rigby et al 1977). The particular α-^{32}P-labeled nucleotide triphosphate used in this study was α-^{32}P-dCTP.

The time course of nick translation of phins214 plasmid insert DNA (Figure 4.1) showed a maximal incorporation of α-^{32}P-dCTP into DNA of 30% after ~3 hours. This is a similar result to that previously reported (Rigby et al 1977). The time course of nick translation of pDI462 plasmid insert DNA (Figure 4.1) showed no appreciable incorporation of α-^{32}P-dCTP into DNA over the 315 minutes of the incubation. The reason for this is not known but one possiblity is the inhibition of the DNAse I activity by agarose contamination of the plasmid insert DNA preparation (Bouche 1982). This failure of the nick translation reaction to incorporate α-^{32}P-dCTP into DNA occurred a number of times with both pDI462 and phins214 plasmid insert DNA during this study (data not shown).

Under the conditions of excess DNA polymerase I activity used in the nick translation reaction the rate of nucleotide replacement is dependent upon the number of single stranded nicks introduced by the DNAse I (Rigby et al 1977). In the presence of magnesium ions (as in the protocol used in this study) DNAse I attacks each strand of double stranded DNA independently and the resultant sites of cleavage are distributed throughout the DNA molecule in a statistically random fashion (Maniatis et al

1982). The optimal length of DNA for use in hybridization is 500-1500 bp (Meinkoth & Wahl 1984). Therefore in order to ensure maximal α-^{32}P-dCTP incorporation and optimal ^{32}P-labeled DNA fragment length it was necessary to titrate the DNAse I against the incorporation of α-^{32}P-dCTP and the size of the ^{32}P-labeled DNA fragments produced. Since appropriate gel markers were not available at the time it was not possible to determine the absolute sizes of the ^{32}P-labeled DNA fragments produced by the nick translation reactions (Figure 4.2). It was possible, however, to observe the relative size distributions of the ^{32}P-labeled DNA fragments. ^{32}P-Labeled DNA probes longer than ~1500 bp have been correlated with high backgrounds in filter hybridization experiments (Meinkoth & Wahl 1984). Thus the amount of DNAse I that gave the lowest amount of high molecular weight ^{32}P-labeled DNA was chosen for all subsequent nick translation reactions (i.e. 4 µl of 0.01 µg/ml DNAse I). Although this amount did not give the highest incorporation of α-^{32}P-dCTP into DNA (Table 4.1) it was not much lower than the highest value observed. The small amounts of ^{32}P-labeled material seen in the empty sample lanes of the alkaline DNA gel (Figure 4.2) can be attributed to leakage of sample from the adjacent loaded slots into the empty lane sample slots. All the values of α-^{32}P-dCTP incorporation in the DNAse I titration experiment (Table 4.1) were lower than would be expected from the results of the nick translation time course (Figure 4.1). This may possibly be due to agarose inhibition of DNAse I activity as discussed above.

(ii) Oligonucleotide Primer Extension

The principle of this method is as follows. Double stranded template DNA is heat denatured into single strands and annealed, by slow cooling, with oligonucleotide primers. These primers are hexamers of random nucleotide sequences produced by size fractionation of DNAse I digested calf thymus DNA. The large (Klenow) fragment of *E. coli* DNA polymerase I then catalyses the sequential addition of nucleotide residues, complementary to the corresponding nucleotide residue on the DNA template strand, from nucleotide triphosphate precursors to the 5'-hydroxyl termini of the oligonucleotide primers. If one, or more, of the four nucleotide triphosphate precursors is α-^{32}P-labeled the product of the reaction is DNA, complementary to the template DNA, ^{32}P-labeled in the sugar-phosphate backbone of the molecule which is of high specific radioactivity (Feinberg & Vogelstein 1983). The large fragment of *E. coli* DNA polymerase I (Klenow fragment) is produced by the limited proteolysis of the holoenzyme by subtilisin (Jacobsen et al 1974). Klenow fragment consists of a single polypeptide chain of 76,000 daltons which retains the 5' to 3' polymerase and the 3' to 5' exonuclease activities but lacks the 5' to 3' exonuclease activity of the holoenzyme.

^{32}P-Labeling of both pDI462 and phins214 plasmid insert DNA by oligonucleotide primer extension was consistent. The specific radioactivity of the ^{32}P-labeled DNA product was typically ~5-fold higher than that of nick translation of phins214 plasmid insert DNA and ~50-fold

higher for that of pDI462 plasmid insert DNA.

Oligonucleotide primer extension was seen to have a number of advantages over nick translation for the routine preparation of ^{32}P-labeled plasmid insert DNA for hybridization probes. Firstly a higher proportion of the α-^{32}P-dCTP is incorporated into the template DNA, 80-90% (Feinberg & Vogelstein 1983) as opposed to 20-40% for nick translation (Rigby et al 1977). These figures are reflected by the data from this study. The DNA ^{32}P-labeled by oligonucleotide primer extension is thus of a higher specific radioactivity, especially since a smaller quantity of DNA is labeled (25 ng as opposed to 100 ng for nick translation), and therefore provides greater sensitivity for the preproinsulin mRNA assay. Oligonucleotide primer extension was also more reliable than nick translation and was able to ^{32}P-label plasmid insert DNA from both pDI462 and phins214 to comparable specific radioactivities. The unreliability of nick translation can most probably be attributed to inhibition of the reaction by agarose. This highlights the ability of oligonucleotide primer extension to utilize unpurified DNA cut straight out of a low melting point agarose gel as its substrate (Feinberg & Vogelstein 1984). This was the technique used in this study. Therefore any losses of DNA associated with purification of DNA from agarose gels, as is required for nick translation, are avoided. This also means that the protocol for oligonucleotide primer extension is much simpler. Loss of ^{32}P-label from ^{32}P-labeled DNA is possible during nick translation, this is probably due to the 5' to 3' exonuclease activity of

the DNA polymerase I (Rigby et al 1977). Because of this instability of the reaction product it was necessary to follow the time course of the nick translation reaction in order to determine the optimal α-^{32}P-dCTP incorporation time. The DNA product of oligonucleotide primer extension, however, is stable. This is presumably due to the lack of the 5' to 3' exonuclease activity of the Klenow fragment (Feinberg & Vogelstein 1983) and therefore it was not considered necessary to follow the time course of the oligonucleotide primer extension reaction. The incorporation of α-^{32}P-dCTP into DNA by oligonucleotide primer extension reaches a maximum after 1 to 10 hours of incubation (Feinberg & Vogelstein 1983) and it was found to be experimentally expedient to incubate this reaction overnight.

In conclusion, oligonucleotide primer extension was chosen, in preference to nick translation, for the production of ^{32}P-labeled double stranded DNA since the methodology of this method was simpler and the reliability greater than that of nick translation.

(iii) M13 Second Strand Synthesis

The protocol for this method was supplied by Dr. W. Kastern (personal communication) and the principle of the method is as follows. A 17-base oligonucleotide primer (sequencing primer), complementary to the M13 sequence just upstream of the multiple cloning sites of the M13 vector, is annealed with the M13 clone. Klenow fragment is then used to extend the 3'-hydroxyl end of the sequencing

primer and thus synthesise a DNA strand complementary to the DNA sequence downstream of the site of the sequencing primer. This downstream sequence includes any DNA that has been cloned into the multiple cloning site of the M13 vector. The conditions of the reaction and length of incubation are chosen so that the majority of any second strand synthesis proceeds only as far as the 3' boundary of the cloned insert. If one of the four nucleotide triphosphate precursors used in the synthesis of the DNA by the Klenow fragment is replaced with its α-^{32}P-labeled form (in this case α-^{32}P-dCTP) then the product of the reaction is ^{32}P-labeled single stranded DNA complementary in sequence to the cloned insert. Since both the rat insulin cDNA and the α-1-microglobulin M13 clone inserts were of the coding strands of the DNA the ^{32}P-labeled DNAs produced were complementary in sequence to their respective mRNAs.

As with oligonucleotide primer extension this method proved to be a reliable method for producing ^{32}P-labeled single stranded DNA to a high specific radioactivity.

Purification of ^{32}P-Labeled DNA

The use of Sephadex G-100 gel filtration chromotography to separate ^{32}P-labeled DNA from unincorporated α-^{32}P-dCTP proved to be both adequate and simple. The resolution between the peaks of the incorporated and the unincorporated material (Figure 4.3) was sufficient to allow the elution of the ^{32}P-labeled DNA from the column to be followed with a hand held β-radiation monitor. One of the advantages of this method

over phenol extraction/ethanol precipitation methods is that the yield of purified ^{32}P-labeled DNA is high. Also the contamination of experimental materials with ^{32}P, and the time taken for purification, are kept to a minimum thus reducing the exposure of the operator to any harmful radiation. This gel filtration method for purification of DNA worked equally well for DNA ^{32}P-labeled by oligonucleotide primer extension or M13 second strand synthesis (data not shown).

Isolation of Total and Poly A$^+$ RNA

The pancreas is a problematical tissue for RNA isolation as it is particularly rich in ribonuclease (as much as 200 µg/g in rat pancreas (Beintema et al 1973)). Ribonuclease, and other digestive enzymes in the form of zymogens, are normally contained within secretory granules but are released when the cell is disrupted and they are then free to attack cellular RNA and proteins. Thus upon disruption of pancreatic tissue it is necessary to inactivate ribonuclease as quickly as possible to preserve the integrity of the RNA. Chaotropic agents such as guanidine hydrochloride (Cox 1968) and denaturants such as phenol (even when used with ribonuclease inhibitors such as heparin, iodoacetate and detergent) normally used for RNA isolation are not rapid enough in their action for the isolation of undegraded RNA from rat pancreas (Chirgwin et al 1979). 4 M Guanidinium thiocyanate, whose anion and cation both reversibly inhibit ribonuclease, in conjunction with the reductant β-mercaptoethanol (which breaks the disulphide bonds essential to the activity of ribonuclease (Sela et al 1956)) have been successfully used in the

isolation of intact RNA from rat pancreas (Chirgwin et al 1979). Rapid disruption of the tissue in the homogenization buffer by a Tissuemizer, or similar (i.e. the Polytron homogenizer used in this study), is essential to avoid RNA degradation. In this study a modification of the method previously used to isolate RNA from rat pancreas (Chirgwin et al 1979) which employs phenol at 68°C in conjunction with 4 M guanidinium thiocyanate was adopted (Feramisco et al 1982) since it gave higher yields of RNA than the original method (data not shown). The glycogen content of liver and kidney tissue can be a problem since it coprecipitates with RNA (Giddings & Rotwein 1986). In order to minimize this problem when isolating RNA from liver or kidney the rats were fasted overnight before sacrifice to reduce endogenous glycogen levels.

The yields of total RNA (Table 4.2) were 30 to 50% of that previously reported (Chirgwin et al 1979) for pancreas and liver and ~2.5% for kidney. This reduction of yield was probably related to the contamination of the RNA preparations since the absorbance ratios of the preparations decreased with yield (Figure 4.2). The percentage yields of the poly A^+ RNA, however, were the same as previously reported (Giddings & Rotwein 1986). Poly A^+ RNA isolated in a similar manner to the method used in this study was shown to be structurally intact and fully active in cell free *in vitro* translation, hybridization and recombinant DNA experiments (Chirgwin et al 1979).

Northern Blotting of Poly A^+ RNA

The single band of ~550 bp in size seen in the pancreatic poly A^+ RNA sample (Figure 4.4) is in agreement with the size quoted for fully processed preproinsulin mRNA in other studies (Giddings et al 1985; Duguid et al 1976; Muglia & Locker 1984; Itoh et al 1979). This roughly corresponds with 330 coding and 110 non-coding nucleotides and a poly A^+ tail of 150 nucleotides as previously reported for preproinsulin mRNA (Cordell et al 1979). Since the mRNAs for both rat preproinsulins (I and II) are of the same size only a single band was expected. Thus from the size of the mRNA and the sharpness of the band it is apparent that the RNA isolation techniques used in this study produce full length preproinsulin mRNA. The diffuse band in the pancreatic poly A^- RNA sample probably represented non-polyadenylated degradation products of preproinsulin mRNA since it did not bind to the oligo(dT)cellulose and was of lower molecular weight than the polyadenylated preproinsulin mRNA. The diffuse nature of this band could also be accounted for by a varying degree of partial polyadenylation of undegraded preproinsulin mRNA. The longer, fully length, polyadenylated tails of fully processed preproinsulin mRNA would have a higher affinity for the oligo(dT)cellulose and may have excluded the binding of partially polyadenylated transcripts. The band in the total pancreatic RNA sample was of the position and profile that one would expect from summing the poly A^+ and poly A^- RNA bands and it in fact represents the sum of both these species of RNA. None of the liver RNA samples hybridized to the ^{32}P-labeled M13 rat insulin cDNA clone DNA. Therefore it

seemed feasible that liver poly A^+ RNA could be used as a negative control for the preproinsulin mRNA assay. This proposal was examined further in the series of experiments discussed in the following section.

Hybridization Specificity of ^{32}P-Labeled DNA

The stability of DNA/RNA hydrids depends upon the ionic strength of the solution, base composition of the hybrid, the concentration of helix destabilizing agents (e.g. formamide) and the length of the shortest chain of the DNA-RNA duplex. The stabilty of the duplex is measured as its melting temperature (T_m) which is given by the following equation (Meinkoth & Wahl 1984);

$$T_m = 81.5^{\circ}C + 16.6 \log M + 0.41 (\%G+C)$$

Where M is the ionic strength (in mol/litre) and %G+C is the percentage guanosine and cytidine nucleotide content of the duplex. Since the T_m of a duplex decreases for every mismatched base pair it is necessary to perform the hybridization reaction at the highest stringency possible to minimize any hybridization to non-identical but related sequences. Stringency can be adjusted either during the hybridization reaction or the post-hybridization wash. It was most convenient to carry out all hybridization reactions in this study at a constant low stringency and then wash the hybridized nitrocellulose papers at the appropriate higher stringency. The stringency of the post-hybridization wash was increased by decreasing the ionic strength of the wash

solution and/or increasing temperature of the wash. One advantage of this approach is that the relative homologies between various ^{32}P-labeled DNA probes and RNA samples can be measured by post-hybridization washing of the nitrocellulose papers at increasing stringencies and analysing the results after each wash. This allows the choice of the most suitable ^{32}P-labeled DNA probe and poly A$^+$ RNA control samples (both positive and negative controls) for the preproinsulin mRNA assay to be made empirically.

The criterion used to choose the most suitable probe was the highest signal to noise ratio. In practise this meant the probe that gave the highest ratio of the positive (pancreatic poly A$^+$ RNA) to the negative (liver and kidney poly A$^+$ RNA) control sample autoradiograph spot densities. Poly A$^+$ RNA was chosen for the RNA controls rather than total RNA since nitrocellulose has a limited binding capacity for RNA (~80 µg/cm^2 (Thomas 1980)). Poly A$^+$ RNA contains a higher fraction of preproinsulin mRNA than total RNA and thus should result in a greater sensitivity.

Increased stringency of post-hybridization washing resulted in an increase of the signal to noise ratio for the ^{32}P-labeled DNA probes prepared from phins214 (Figure 4.5a) and pKA30 (Figure 4.5c) plasmid insert DNA but not for pDI462 plasmid insert DNA (Figure 4.5b). The signal to noise ratio for the phins214 plasmid insert DNA probe was better than that of the pKA30 plasmid insert DNA probe at the highest stringency of post-hybridization wash used. Also with the phins214 plasmid insert DNA probe the spot density of the positive control (pancreatic poly A$^+$ RNA) was not reduced to as great an extent as with the pKA30 plasmid insert DNA probe

by the increase in post-hybridization wash stringency. Taken together these results indicate a higher degree of sequence homology between the phins214 plasmid insert DNA and pancreatic poly A^+ RNA and a lower degree of homology with liver poly A^+ RNA than with pDI462 or pKA30 plasmid insert DNA. Therefore phins214 plasmid insert DNA ^{32}P-labeled by oligonucleotide primer extension was chosen as the most suitable double stranded DNA probe for use in the preproinsulin mRNA assay. Kidney poly A^+ RNA gave consistently lower signal to noise ratios than liver poly A^+ RNA with all three probes used. The reason for this is not clear but may be related to contamination of the RNA preparation which was less pure than the liver poly A^+ RNA (Table 4.2). Thus pancreatic poly A^+ RNA was chosen as the positive control and liver poly A^+ RNA as the negative control for hybridizations with double stranded DNA probes in this study.

The higher homology of the positive RNA control with phins214 as compared to the pDI462 plasmid insert DNA was as expected. The phins214 and pDI462 plasmids contain cloned inserts containing the entire human and the entire canine insulin genes respectively. The nucleotide sequence homology between the human and rat insulin genes is ~70% (Bell et al 1980). Although no figure for the homology between the canine and rat insulin genes has been published it is likely to be lower since the homology at the amino acid level is lower (e.g. ~60% for the canine and rat C-peptide as opposed to ~70% for the human and rat C-peptide (Kwok et al 1983)). The observed higher homology between the positive RNA control and phins214 as compared to pKA30 plasmid insert DNA was unexpected. The cloned insert of the pKA30 plasmid is a

400-450 bp fragment of rat preproinsulin cDNA and as such should be identical in sequence to rat preproinsulin mRNA. This cloned insert, however, contains an unknown length of a poly GC tail introduced by homopolymeric tailing during the cloning procedure (W. Kastern - personal communication). It is possible that the length of this poly GC tail could reduce the length of the preproinsulin mRNA/pKA30 plasmid insert DNA duplex to sufficiently destabilize it in relation to the preproinsulin mRNA/phins214 plasmid insert DNA duplex. This unexpected result emphasizes the necessity of undertaking an empirical determination of hybridization probe specificity as was done in this study.

The single stranded DNA probe ^{32}P-labeled by second strand synthesis of the M13 rat insulin cDNA clone did not give as good a signal to noise ratio (Figure 4.9a) as the double stranded ^{32}P-labeled phins214 plasmid insert DNA (Figure 4.5a). The ability of single stranded DNA to act as a hybridization probe is not compromised by any self-annealing between the complementary DNA strands which can occur with double stranded DNA probes. This advantage over double stranded probes, however, may be abrogated by the ability of double stranded probes to form networks or "hyperpolymers" by complementary hybridization of the two strands (Meinkoth & Wahl 1984). This can increase the hybridization sensitivity of double stranded DNA probes. A disadvantage of single stranded DNA hybridization probes produced by the method used in this study is that some second strand synthesis of M13 sequences downstream of the cloned insert is inevitable in a small proportion of the ^{32}P-labeled DNA molecules. This would reduce the hybridization specificity of the probe. The poor relative

performance of the single stranded M13 rat insulin cDNA probe, as compared to the double stranded phins214 plasmid insert DNA, could have been due to one or both of these factors. The high homology of the liver poly A^+ RNA sample with the M13 rat insulin cDNA probe (Figure 4.9a) was unexpected. This could have been due to the presence of insulin-like growth factor mRNA. Human insulin-like growth factors I and II are peptides, of 70 and 73 amino acid residues respectively, which have growth stimulating effects. These factors are expressed in the liver (Rotwein 1986) (the major site of their synthesis (Philips et al 1976)) and they have an overall homology, at the nucleotide level, of 53% with human preproinsulin (Jansen et al 1983). This, however, is not entirely consistent with the Northern blot data since the M13 rat insulin cDNA probe did not cross-hybridize with any sequences in the poly A^+, poly A^- or total liver RNA samples (Figure 4.4).

The M13 α-1-microglobulin clone was intended to be used as a negative control for hybridizations with the M13 rat insulin cDNA probe. α-1-Microglobulin is a glycoprotein of a molecular weight of 30,000 daltons first reported in human urine and serum (Ekström et al 1975). It has immunosuppressive properties and inhibits the migration of peripheral blood leukocytes (Lögdberg & Akerström 1979). Immuno-cytochemistry has revealed it to be present extracellularly in many rat tissues, including pancreas, in association with immunoglobulin A (Bouic et al 1984). However synthesis of α-1-microglobulin has only been reported in rat liver (Akerström & Landin 1985) and not in rat pancreas (W. Kastern - personal communication). Thus the M13 α-1-microglobulin DNA probe exhibited high homology with liver poly A^+ RNA and no

homology with kidney poly A^+ RNA as expected. The high homology with pancreatic poly A^+ RNA (Figure 4.9b) was unexpected. This could suggest that rat pancreas expresses α-1-microglobulin, or a closely related peptide. However, since the high homology with liver poly A^+ RNA (Figure 4.9a) was not reflected in the liver poly A^+ RNA sample in the Northern blot (Figure 4.4) these unexpected high homologies may have been artifactual. Since the single stranded DNA probe ^{32}P-labeled by second strand synthesis of the M13 rat insulin cDNA clone gave a poor hybridization signal to noise ratio and because of the possiblity of artifactually high homologies with the negative controls the use of M13 single stranded hybridization probes for the preproinsulin mRNA assay was abandoned in favour of double stranded DNA probes.

Preproinsulin mRNA Assay

In order for this assay to be useful for the study of insulin gene expression in islets of Langerhans a number of experimental requirements have to be met. Firstly the assay must be sensitive enough to allow measurement of preproinsulin mRNA in the limited amounts of islet tissue available from the collagenase digestion isolation procedure. A hybridization signal was detectable in as few as 50 islets (Figure 4.11) which is adequate since up to 1000 islets were available per islet tissue preparation. In fact since only a 50 µl aliquot of the 250 µl islet RNA sample was applied to the nitrocellulose paper the sensitivity could be effectively increased 5-fold by applying all the sample to one spot rather than as a series of serial dilutions. However, the need for

linearity of the assay restricts this possibility (see below). High background hybridization could reduce the sensitivity of the assay but this was not a problem with the high stringencies of the post-hybridization washes used (Figure 4.6). Since guanidinium thiocyanate was found to be more effective than guanidine hydrochloride in the isolation of pancreatic RNA (Chirgwin et al 1979) its use as an alternative homogenization buffer for the assay was investigated. Despite an increased recovery of hybridizable islet preproinsulin mRNA, when used with carrier RNA (Table 4.3), guanidinium thiocyanate was not adopted in place of guanidine hydrochloride as the homogenization buffer. This was because the carrier RNA used (5 µg of total RNA isolated from rat kidney) had a high hybridization signal of its own which would have compromised the sensitivity of the assay. Also when guanidinium thiocyanate was used without carrier RNA no hybridizable islet preproinsulin mRNA was recovered.

Another essential requirement of the assay is that any hybridization signal is due to islet RNA and not to any contaminating DNA or non-islet tissue. Although digestion of an islet sample with DNAse I reduced the preproinsulin mRNA recovery by 66% digestion with RNAse completely abolished the recovery suggesting that the hybridization signal was in fact due to islet RNA and not DNA (Table 4.4). The reduction in recovery by the DNAse I digestion could have been due to contaminating RNAse since the DNAse I preparation used was not pretreated to remove such contamination. Such pretreatment is possible by digesting the DNAse I in the presence of Ca^{2+} which protects DNAse I but not RNAse from proteolytic action (Tullis & Rubin 1980). The acinar tissue sample had a

preproinsulin mRNA recovery of 20% of that of the untreated islet sample. This was considered to be satisfactory since the recovery could have in fact been due to contaminating islet tissue. Also if the recovered preproinsulin mRNA was indeed due to the acinar tissue in practise the content of any likely acinar tissue contamination of an islet sample would be much lower than seen for the pure acinar tissue sample used here.

Another requirement is that of linearity. For the assay to be linear (i.e. the density of the dot blot spots must be proportional to the preproinsulin mRNA present) many of the individual assay conditions must be optimized for linearity. One experimental factor that may cause non-linearity is the response of the X-ray film to exposure by the β-radiation emitted by the ^{32}P-labeled DNA probe. Another factor is the measurement of the optical density of the dot blot spots. Since the optical density varied across the width of the spot, the most dense part being in the center, it was necessary to measure the optical density across the whole width of each spot and integrate the area under the resulting density curve. This was carried out with an LKB Ultroscan 2202 laser densitometer to scan the optical densities of the spots. The data thus generated was collected and stored on floppy disk, for later analysis, by an Apple IIe computer, connected to the densitometer, running the LKB Gelscan program. Each set of sample serial dilutions was scanned in a single line three times at slightly different positions across the center of the spots. The data from these three scans was then arithmetically averaged to minimize any inaccuracy due to misalignment of the scanning position over the spots. The data averaging was carried out using the Gelmerge program, which was specifically

written for this purpose (see Appendix). The averaged scan data (Figure 4.8) gave evenly shaped peaks at the expected spacing thus allowing the precise demarcation of each sample peak for analysis. The LKB Gelscan program was used to estimate the area under each peak.This was done by approximating each curve to a Gaussian curve. All spot densities quoted in this study are in the arbitrary units of density used within the LKB Gelscan program. The relationship between the area under each sample peak and the amount of ^{32}P-labeled DNA probe bound to each spot was linear (Figure 4.10). Furthermore the spot densities showed a linear relationship to the amount of blotted poly A^+ RNA standard (Figure 4.7). Thus spot density could be assumed to be directly proportional to the amount of hybridizable RNA present in the sample. Assuming an islet RNA content of 120 ng (Giddings et al 1981) and a nitrocellulose RNA binding capacity of 80 $\mu g/cm^2$ (Thomas 1980) then each spot, which is 3 mm in diameter, can bind the equivalent RNA content of a 200 islet sample. So the tailing off in the response of spot density to islet number for samples of greater than 100 islets (Figure 4.11) was probably due to a limitation in the RNA binding capacity of the nitrocellulose. This limits the sensitivity of the assay since a maximum sample size of 100 islets can be used. A sample size of 50 islets was chosen for the assay to ensure that the sample spot density fell well within the linear portion of the response which lay between 25 and 100 islets (Figure 4.11).

As well as being of adequate sensitivity and linearity the assay had also to be consistent between experiments. This was achieved by running the same positive and negative

controls in each assay. These controls were pancreatic and liver poly A^+ RNA respectively and they were assayed as a set of serial dilutions in the same manner as the islet samples. If in any particular assay the negative control spot densities were equal to or higher than the positive control ones then those assay results were discarded, in practise this seldom occured. A standard curve was constructed from the spot densities of the serial dilutions of the positive RNA control for each assay. A further curve was plotted from the spot densities of the serial dilutions of each islet sample and one spot density was chosen from the most linear portion of this curve. This value was then used with the standard curve to calculate the amount of preproinsulin mRNA present in the islet sample as the equivalent amount of pancreatic poly A^+ RNA. This method of calculation ensured that the linearity of the assay was maintained and that variation between assays due to differences in ^{32}P-labeled DNA probe specific activity, autoradiograph exposure and hybridization conditions was minimized. Variation between islet samples was minimized as far as possible by the random assignment of islets to each sample at the isolation stage. Since the preproinsulin mRNA content of the pancreatic poly A^+ RNA sample standard was not known no estimate of the islet preproinsulin mRNA content could be made and therefore this assay was restricted to estimates of relative preproinsulin mRNA contents. Other investigators, using cloned rat preproinsulin I cDNA as a standard, have estimated the concentration of preproinsulin mRNA to be 1000-1500 pg/µg of islet RNA (Giddings et al 1985). Assuming an islet RNA content of 120 ng (Giddings et al 1981) this corresponds to a copy number for preproinsulin mRNA of

50-150,000 molecules per cell (Giddings et al 1985). This figure is similar to that for other abundant mRNAs in differentiated tissues such as ovalbumin in chick oviduct (McKnight & Palmiter 1979) and casein in mouse mammary gland (Ashcroft et al 1978).

In summary the dot blot assay developed in this study was shown to be of adequate sensitivity and linearity for the purposes of relative measurements of islet preproinsulin mRNA content.

(2) Measurement of Preproinsulin mRNA in Islets

Over a 2 hour incubation period increased glucose concentration (from 2.5 mM to 20 mM) had no statistically significant effect on islet preproinsulin mRNA content (Figure 4.12). The trend in the data, however, indicated that for islets incubated in medium containing 20 mM, rather than 2.5 mM, glucose there was a 2-fold increase in preproinsulin mRNA content after 2 hours. The preproinsulin mRNA content of islets incubated in medium containing 2.5 mM glucose remained constant throughout the 2 hours of the incubation. The data for the 20 mM glucose incubations had larger standard variations than those of the 2.5 mM glucose incubations suggesting that there was a variation in the response of the islet preproinsulin mRNA content to incubation at the higher glucose concentration. This could account for why no statistically significant differences were observed. On the basis of the data presented here it was not possible to distinguish whether the increased islet preproinsulin mRNA content of the 20 mM glucose incubated islets reflected an

increase in the rate of preproinsulin mRNA synthesis or a decrease in the rate of degradation. The inhibition of RNA synthesis by actinomycin D had no effect on preproinsulin mRNA content for islets incubated at either 2.5 mM or 20 mM glucose (Figure 4.12). Similar data has previously been reported where actinomycin D at a concentration of 5 µg/ml had no effect on the preproinsulin mRNA content of islets tissue cultured for 24 hours at various glucose concentrations (Welsh et al 1986c). It has been suggested that the degradation of preproinsulin mRNA may require the continuous synthesis of RNA factors (Welsh et al 1985). This could explain why actinomycin D had no effect on islet preproinsulin mRNA content in this study. Since if both the synthesis and degradation of the preproinsulin mRNA could be inhibited to a similar degree by actinomycin D there would be no net effect on islet preproinsulin mRNA content. It is likely that the degradation of preproinsulin mRNA plays an important role in the control of islet preproinsulin mRNA content since tissue culturing of islets at 17 mM, as opposed to 3.3 mM, glucose stabilized preproinsulin mRNA by almost 2-fold relative to other poly A^+ RNAs (Welsh et al 1985). Stabilization of mRNA has also been shown to be involved in the control of ovalbumin (McKnight & Palmiter 1979), prolactin (Laverrier et al 1983) and β-interferon (Raj & Pitha 1983) gene expression.

Studies outlined in the previous chapter on the rate of glucose stimulated (pro)insulin synthesis showed that 2 hours was the practical limit for the viability of incubated islets. Therefore it was decided to use tissue culture to extend the period of islet viability to observe the effect of glucose on islet preproinsulin mRNA content for periods longer than 2

hours. Unfortunately the data from the tissue culture experiments varied so greatly as to make any statistical analysis meaningless. Therefore only representative single sets of data have been presented (Figures 4.13a, 4.13b and 4.14). In the presence of actinomycin D the islet preproinsulin mRNA contents were in general higher (Figure 4.13). This, however, was probably due to variation between the islet tissue preparations since otherwise one would not expect the difference at zero time as was seen here. The overall pattern of the change in preproinsulin mRNA content for islets tissue cultured at either 2.5 mM or 20 mM glucose was unaffected by the presence of actinomycin D. This was also seen in the 2 hour islet incubations and therefore the discussion of these observations presented above also apply here. In the light of the data from the 2 hour islet incubations the slight increase in preproinsulin mRNA content of the islets tissue cultured in medium containing 2.5 mM glucose and the decrease in content for those tissue cultured in medium containing 20 mM glucose was unexpected. This unexpected pattern of islet preproinsulin mRNA content was not likely to have been due to any necrosis of the β-cells since they appeared to secrete insulin in the expected manner. Immunoreactive insulin accumulated in the medium of the islets tissue cultured at 20 mM glucose but not in that of those incubated at 2.5 mM glucose (Figure 4.14). Since 2.5 mM glucose is below the threshold for insulin secretion and 20 mM glucose is a maximal rate for insulin secretion this pattern is expected for normally secreting islets. The presence of actinomycin D in the tissue culture medium had no effect on the secretory ability of the islets (Figure 4.14b). However,

due to the lack of statistical analysis of the islet preproinsulin mRNA contents it is inadvisable to place too much importance on any interpretation of this islet tissue culture data. Further standardization of the data, for example by DNA content of the islets (Hopcroft et al 1985), may have allowed statistical analysis of this data.

Summary

In summary the conclusions from the data presented in this chapter were that increasing the glucose concentration from 2.5 mM to 20 mM may enhance islet preproinsulin mRNA content within 2 hours of incubation. It was not possible to distinguish whether this was due to a stimulation of the rate of insulin gene transcription or an inhibition of the rate of preproinsulin mRNA degradation. Studies with actinomycin D suggested that the changes in the rate of degradation of preproinsulin mRNA may play an important role in the control of islet preproinsulin mRNA content. The use of short term tissue culture of islets proved to be impracticable in the study of the effect of glucose on islet preproinsulin mRNA due to excessive variation in the data between islet tissue preparations.

Chapter 5

The Regulation of Insulin Gene Expression in Pancreatic Islets by Glucose: A General Discussion

Introduction

The regulation of insulin gene expression in pancreatic islets by glucose is mediated by a number of different mechanisms. They can, however, be divided into two main classes; those which effect islet preproinsulin mRNA content and those which effect the rate of translation of preproinsulin mRNA. Each of these will be discussed in turn.

Regulation of Islet Preproinsulin mRNA Content by Glucose

The data presented in this thesis supports that of previous studies which found that glucose increased the preproinsulin mRNA content of mouse (Brunstedt & Chan 1982; Brunstedt 1982) and rat islets (Nielsen et al 1985) *in vitro*. Another study reported that a high glucose concentration was necessary for the maintenance of a high preproinsulin mRNA content in mouse islets *in vitro* (Welsh et al 1986c). The addition of dbcAMP to rat islets was shown to partially mimic this effect of glucose on insulin gene transcription (Nielsen et al 1985). Also in the same study insulin gene transcription in an insulin producing cell line (RIN-5F cells) was transiently stimulated by the addition of cholera toxin, a stimulator of adenylate cyclase (Hellman et al 1974a). This data implies that cAMP has a role in the regulation of insulin gene transcription, and since glucose increases islet cAMP content (Green et al 1978; Charles et al 1973) it is possible that the effect of glucose on insulin gene transcription may be mediated, at least in part, by cAMP.

(i) cAMP and Gene Transcription

The stimulation of transcription by cAMP in higher eukaryotes has been reported for the tyrosine amino-transferase (Noguchi et al 1978), prolactin (Maurer 1981), phosphoenolpyruvate carboxykinase (Lamers et al 1982) and plasminogen activator genes (Nagamine et al 1983). Nagamine and Reich (1985) postulated that this stimulation of gene transcription by cAMP is mediated by a protein kinase, possibly type II mammalian protein kinase. The proposed mechanism is similar to that controlling the induction and repression of specific bacterial genes by cAMP (reviewed in de Crombrugghe et al 1984). In the Nagamine and Reich model the protein kinase consists of two types of subunit, the regulatory subunit and the catalytic subunit. cAMP binds to a site on the regulatory subunit causing it to dissociate from the catalytic subunit. The dissociated cAMP/regulatory subunit complex can then bind to specific regulatory DNA sequences, associated with the inducible genes, to promote their transcription. A 29 nucleotide consensus was proposed for this regulatory sequence. The consensus sequence was derived from sequences shown to be involved in cAMP regulation in the rat tyrosine aminotransferase, prolactin, phosphoenolpyruvate carboxykinase and porcine plasminogen activator genes. This consensus sequence has some homology with sequences upstream of prokaryotic genes implicated in cAMP regulation of transcription by DNAse footprinting studies (Nagamine & Reich 1985). The consensus sequence in the rat tyrosine aminotransferase, prolactin, phosphoenolpyruvate carboxykinase genes occur between 107 bp

and 488 bp upstream of their transcriptional promoters. It is not known whether similar sequences are present upstream of the rat insulin I or II genes. Nagamine and Reich proposed that concurrent with the activation of the regulatory subunit by cAMP binding, the catalytic subunit of the protein kinase becomes active and phosphorylates a variety of cellular substrates thus providing scope for further stimulation of gene expression. This will be discussed later in this chapter.

(ii) Glucose and cAMP Metabolism in Islets

Preincubation of islets with 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase, raised islet cAMP and also islet protein kinase activity (Montague & Howell 1972). Glucose stimulated the adenylate cyclase activity of rat islets (Howell et al 1973). This stimulation reached a peak after 4 to 8 hours and was due to the activation of existing enzyme, rather than the synthesis of new enzyme, since it was unaffected by the inhibition of protein synthesis by cycloheximide or the inhibition of RNA synthesis, by Actinomycin D. Since pyruvate and mannose, which are metabolized by islets, raise adenylate cyclase activity, whereas galactose and 2-deoxyglucose, which are not metabolized by islets, do not, this stimulation is probably mediated by the metabolism of glucose. The identity of the glucose metabolite responsible is not known but purine nucleotides are possible candidates since they have

been shown to modulate adenylate cyclase activity in acute dosage experiments in islets (Howell & Montague 1973) as well as in liver (Rodbell et al 1971) and thyroid (Wolff & Cook 1973). In general a close correlation between islet protein kinase activity and islet cAMP content exists. One notable exception to this, however, is glucose which raises islet cAMP but not islet protein kinase activity (Sharp 1979).

It has been suggested that the stimulation of insulin gene transcription by glucose is in part due to an increased islet metabolic flux. Both leucine and 2-ketoisocaproate (the deamination product of leucine) increase islet catabolic flux and are capable, in conjunction with a low glucose concentration, of maintaining a high islet preproinsulin mRNA content (Welsh et al 1986c). However, since there are reports that leucine activates adenylate cyclase in an islet cell adenoma (Levey et al 1972) and that 2-ketoisocaproate elevates islet cAMP (Campbell & Taylor 1981) it is possible that the effect of these compounds on insulin gene transcription is also mediated by cAMP.

From the evidence discussed above it can be proposed that the effect of glucose on insulin gene transcription is mediated by cAMP in a manner similar to that postulated by Nagamine & Reich (1985). Cortisol and human growth hormone have also been shown to increase the preproinsulin mRNA content of mouse islets (Brunstedt 1982). Although no effect of human growth hormone on islet cAMP has been reported it is conceivable that the elevation of islet cAMP by stimulation of adenylate cyclase, mediated by the binding of human growth

hormone to a receptor, can occur in the β-cell. The control of gene transcription by glucocorticoids, such as cortisol, however, is thought to be mediated by intracellular steroid hormone receptor binding. The steroid hormone/receptor complex binds to a specific regulatory DNA sequence which modulates the transcription of the appropriate genes (Rousseau 1984). It is possible that the stimulation of insulin gene transcription by cortisol is mediated by such a mechanism.

The concentration threshold for the glucose effect on islet cAMP lies between 4.2 mM and 5.5 mM (Maldonato et al 1977). Therefore in the proposed model of cAMP mediation of glucose stimulation of insulin gene transcription glucose stimulations below this concentration should not elicit a transcriptional response. This is substantiated by a study on the rates of turn off of (pro)insulin synthesis in islets incubated in the presence or absence of an RNA synthesis inhibitor (actinomycin D). The conclusion of this study was that 20 mM glucose but not 5.5 mM glucose stimulated insulin gene transcription (Kaelin et al 1978).

Glucose also plays a part in the stabilization of preproinsulin mRNA against degradation. Incubation of islets at 17 mM glucose stabilized islet poly A^+ RNA but was selective for preproinsulin mRNA which had a half-life twice as long as that in islets incubated at 3.3 mM glucose (Welsh et al 1985). In the same study treatment of RIN-5F cells with cholera toxin, a stimulator of adenylate cyclase, also stabilized preproinsulin mRNA suggesting that this effect of glucose may be mediated by cAMP. In another study glucose was shown to inhibit the degradation of total islet RNA. Once again cAMP was implicated as a possible mediator of the effect

since theophylline, an inhibitor of cyclic nucleotide phosphodiesterase, increased the half-life of islet RNA (Welsh et al 1986b).

In summary the stimulation of insulin gene transcription by glucose and other compounds can be accounted for by the cAMP-dependent protein kinase model of Nagamine and Reich (1985). In this model glucose concentrations above ~5 mM raise islet cAMP which then binds to the regulatory subunit of an unidentified protein kinase causing it to dissociate from its catalytic subunit. The dissociated regulatory subunit is then free to bind to a specific regulatory DNA sequence upstream of the rat insulin gene(s) and thereby promote their transcription. Concurrent with this cAMP induces an increase in the stability of existing preproinsulin mRNA to further raise islet preproinsulin mRNA content. Glucose, mediated by cAMP, also inhibits the degradation of total islet RNA. The glucose induced stabilization of both total RNA and preproinsulin mRNA can be accounted for in either one of two ways. Firstly the regulatory subunit of the protein kinase, with its bound cAMP, could bind to further DNA regulatory sequences to modulate the production of a gene product(s) involved in the stabilization of RNA. Secondly the catalytic subunit of the protein kinase, once activated by dissociation from the regulatory subunit by cAMP binding, could phosphorylate a cellular protein(s) involved in the stabilization of RNA and thus modulate its activity. Similarly the glucose stimulation of total RNA synthesis could be accounted for by binding of the regulatory subunit to DNA sequences or the phosphorylation of a protein(s) involved in the modulation of general gene transcription.

The stimulation of insulin gene transcription by glucocorticoids, such as cortisol, may be accounted for by the intracellular steroid hormone receptor model (Rousseau 1984).

Regulation of Preproinsulin mRNA Translation by Glucose

It has been shown that stimulation of islet (pro)insulin synthesis by glucose is possible without increasing islet preproinsulin mRNA content (Itoh & Okamoto 1980) and in the presence of the RNA synthesis inhibitor actinomycin D (Permutt & Kipnis 1972a). Therefore in addition to its effect on transcription glucose also exerts an effect on preproinsulin mRNA translation. This effect is not confined to (pro)insulin synthesis since total islet protein synthesis is also stimulated.

Raising the glucose concentration preferentially stimulated the synthesis of islet peptides on trisomes or larger polysomes; if a coding length of ~250 bp is assumed for preproinsulin mRNA then a maximum of 3 ribosomes can be accommodated on preproinsulin mRNA (Permutt & Kipnis 1972). This may be accounted for by the stimulation of the rate of islet peptide chain initiation by glucose (Permutt 1974). Studies on the *in vitro* translation of preproinsulin mRNA with aurintricarboxylic acid, a blocker of peptide chain initiation, and competing mRNAs (Lomedico & Saunders 1977) led to the proposal that glucose modulated the translation of preproinsulin mRNA by the kinetic model of Lodish (1971). In this model an overall increase in the rate of peptide chain initiation allows mRNAs with low affinities for chain initiation factors to compete more efficiently for these

factors and hence be translated at a greater rate. It was proposed that preproinsulin mRNA has a lower affinity for translation initiation factors than other islet mRNAs so that at low glucose concentrations only a fraction of it is engaged on active ribosomes. An increase in glucose concentration increases the overall rate of peptide chain initiation and hence the rate of preproinsulin mRNA translation is increased so that a higher proportion of it is engaged on active ribosomes. Welsh et al (1986a) have observed that the overall rate of peptide chain initiation *in* islets is stimulated by glucose concentrations over 3.3 mM. Glucose increases both the thiol (Anjaneyulu et al 1982) and the glucose phosphate content (Ashcroft et al 1972c) of islets and these may mediate the effect of glucose on peptide chain initiation. These two factors act co-operatively to prevent the early inhibition of peptide chain initiation in a rabbit reticulocyte lysate *in vitro* translation system to maintain maximum rates of protein synthesis (Jackson et al 1983). It is thought that sugar phosphates have a role as an activating cofactor for peptide chain initiation and that the reducing power of the thiol system activates a protein kinase which phosphorylates the initiation factor eIF-2 thus modulating its activity. Phosphorylation of eIF-2 has not, as yet, been successfully demonstrated in islets (Welsh et al 1986a) but this mechanism remains plausible for the glucose regulation of islet peptide initiation rate.

In conflict with an earlier report (Permutt 1974) Welsh et al (1986a) observed that increases in glucose concentration up to 5.6 mM stimulated the rate of preproinsulin peptide elongation. Although this was closely paralleled by glucose

induced changes in the islet energy state (Malaisse et al 1979; Hellman et al 1969) such a general mechanism could not account for such a specific stimulation of the elongation rate of a particular peptide unless it was coupled to transduction of the energy in some way.

A glucose stimulated shift of preproinsulin mRNA from cytosolic ribosomes to membrane bound ribosomes in islets has been observed (Itoh & Okamoto 1980; Welsh et al 1986a). To account for this Welsh et al (1986a) proposed that the transfer of the nascent preproinsulin/ribosome complex from the cytosol to the endoplasmic reticulum is mediated by signal recognition particle (SRP) binding. Formation of the ribosome-membrane junction, which is crucial in the synthesis of secreted peptides such as proinsulin, is dependent on the SRP-particle (Walter & Blobel 1981) and a membrane associated protein, of the rough endoplasmic reticulum, called the SRP-receptor (Gilmore et al 1982), or the docking protein (Meyer et al 1982). The SRP binds to the nascent prepeptide as it emerges from the ribosome and arrests further translation until the SRP/prepeptide/ribosome complex binds to the SRP-receptor. This causes dissociatation of the SRP which permits peptide chain translation to proceed to completion (Gilmore & Blobel 1983). This mechanism prevents the synthesis of secretory proteins in the cell cytosol since translation can only proceed once the initiated prepeptide is at its correct synthesis site (i.e. the rough endoplasmic reticulum). Support for this model comes from inhibition of *in vitro* translation of preproinsulin mRNA by purified SRP (Eskridge & Shields 1983; Welsh et al 1986a). Also the run-off incorporation of ^{125}I-tyrosine-tRNA, from preinitiated

ribosomes, into preproinsulin is greater upon the addition of SRP to islets preincubated in 16.7 mM, rather than 3.3 mM, glucose (Welsh et al 1986a). It is possible that glucose increases the affinity of the SRP-receptor for the SRP/preproinsulin/ribosome complex, possibly by some form of modification. This could be by phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase. This would result in a lower rate of SRP-mediated translational arrest of preproinsulin mRNA and a higher ratio of preproinsulin mRNA in membrane bound polysomes, as opposed to cytosolic polysomes, at high glucose concentrations as observed.

Effect of Glucose on β-Cell Intracellular Insulin Degradation

The intracellular degradation of insulin plays a very important role in the homeostasis of β-cell insulin content. During a 24 hour chase period islets, which had been prelabeled with ^{3}H-Leucine at 8.3 mM glucose for 3 days, exhibited an inverse relationship between the rate of intracellular insulin degradation and the glucose concentration of the chase medium (Halban & Wollheim 1980). With a chase medium containing 2.8 mM glucose 37% of the labeled insulin was degraded and with a chase medium containing 16.7 mM glucose the rate of degradation was insignificant. In a similar study the effect of glucose on the rate of insulin degradation during a 24 hour chase period was measured in islets which had been prelabeled with ^{3}H-Leucine at different glucose concentrations (Halban & Renold 1983). Those islets preincubated at 2.8 mM and 16.7 mM glucose had a

much lower immunoreactive insulin content and exhibited lower rates of degradation at all the chase glucose concentrations than those preincubated at 8.3 mM glucose. It was therefore concluded that the rate of intracellular insulin degradation is correlated with β-cell insulin content rather than with the glucose concentration of the medium or the rates of insulin synthesis or secretion. It was proposed that two pools of insulin secretory granules exist, one available for secretion and one for degradation. There is, however, no morphological evidence for the existence of these two granule pools. Autoradiographic and immunocytochemical evidence has shown that insulin secretory granules are degraded via a lysosomal pathway (Orci et al 1984). The presence of non-lysosomal insulin degrading enzymes in islets (Kohnert et al 1976) suggests that this may not be the only pathway by which intracellular β-cell insulin is degraded.

In summary islet insulin is degraded by a lysosomal mechanism the rate of which is thought to be modulated by the β-cell insulin content. Thus it seems that glucose only exerts indirect control over β-cell intracellular insulin degradation by modulating the relative rates of insulin secretion and synthesis to regulate the β-cell insulin content. This mechanism ensures that the β-cell content of insulin does not become depleted by excessive rates of intracellular insulin degradation. As well as having a homeostatic role the intracellular degradation of insulin may play a role in removing old and damaged insulin from the β-cell. Evidence in favour of this has come from studies in which proinsulin modified by the incorporation of lysine and arginine analogues is degraded rapidly by islet homogenates (Halban et al 1984).

Recognition of the Glucose 'Signal' by the β-Cell

The β-cell requires some form of 'glucoreceptor' which can assess and respond to the extracellular glucose concentration and regulate the expression of the insulin gene accordingly. The ability of a compound to stimulate islet (pro)insulin synthesis is closely correlated with its metabolism by the β-cell. Therefore this glucoreceptor has been identified with a rate limiting step in islet glucose metabolism and evidence suggests that this may be the phosphorylation of glucose (see Ashcroft 1980 for review). Studies on extracts of mouse and rat islets have identified a D-glucose-ATP-phosphotransferase activity, distinct from hexokinase, which is similar to liver glucokinase (Ashcroft & Randle 1970; Matschinsky & Ellermann 1968; Malaisse et al 1976). This enzyme has been proposed as the islet glucoreceptor since its kinetic properties and inhibition by mannoheptulose are similar to that of islet glucose metabolism. This may be generally applicable since glucokinase is present in human, rat, mouse and hamster islet tissue (Bedoya et al 1986). Glucokinase occupies a key position in hepatic glucose metabolism (Hers & Hue 1983) and is only expressed, in significant quantities, in the liver and islets of the rat (Iynedjian et al 1986). Therefore it is possible that glucose exerts its effect on islet (pro)insulin synthesis by the action of the product of the glucokinase reaction, glucose-6-phosphate, to prevent the early inhibition of peptide chain initiation, as discussed in the previous section. Glucose increases the rat islet $NADPH/NADP^+$ ratio (Ashcroft 1980). Therefore it is also plausible that

glucose-6-phosphate acts indirectly via an increase in the islet $NADPH/NADP^+$ ratio caused by the catabolism of glucose-6-phosphate through the pentose phosphate pathway. The increased $NADPH/NADP^+$ ratio could act via the islet glutathione intermediary redox couple (Ammon et al 1977) and the thiol reduction of a protein kinase (discussed in the previous section) to phosphorylate the peptide initiation factor eIF-2.

If the stimulation of islet insulin gene transcription by glucose is mediated by cAMP, as is proposed in this thesis, then the most likely candidate for the glucoreceptor for this process is the islet adenylate cyclase system. The existence of islet receptors coupled to adenylate cyclase has been demonstrated for glucagon and β-adreneregic agents (Sharp 1979). Although no such receptor has been reported for human growth hormone it is likely that the stimulation of islet insulin gene transcription by this substance (Brunstedt 1982) is by a similar mechanism.

In summary the identification of glucokinase as the islet glucoreceptor could account for the stimulation of (pro)insulin and total protein synthesis in islets by glucose. This is achieved by the action of glucose-6-phosphate and changes in the $NADPH/NADP^+$ ratio on islet peptide chain initiation. The glucoreceptor for the cAMP mediated stimulation of islet insulin gene transcription by glucose is the metabolism of glucose; the precise location within glucose metabolism is unknown. For the stimulation of transcription by human growth hormone the receptor is most likely an islet receptor coupled to adenylate cyclase.

Summary: The Regulation of Islet Insulin Gene Expression by Glucose and its Physiological Significance

In the previous sections of this chapter a model for the regulation of islet insulin gene expression by glucose has been proposed. It is mostly based on the model of Nagamine and Reich (1985) for cAMP mediated gene transcription and the observations of Welsh et al (1986a) on the translation of islet preproinsulin mRNA. In this model the inhibition of preproinsulin mRNA degradation, as well as the stimulation of preproinsulin mRNA synthesis, by glucose plays an important role in the regulation of islet preproinsulin mRNA content. The basis of the model is as follows.

Glucose, at concentrations greater than ~5 mM, raises islet cAMP by the stimulation of islet adenylate cyclase activity. The cAMP binds to the regulatory subunit of an islet cAMP-dependent protein kinase thus causing it to dissociate from the catalytic subunit. The regulatory subunit is then free to bind to a DNA regulatory sequence upstream of the insulin gene and thus promote its transcription. Concurrent with this the dissociated catalytic subunit is activated to phosphorylate certain target proteins which may be further involved in the regulation of insulin gene expression. This rise in islet cAMP also stabilizes existing preproinsulin mRNA thus further contributing to an increased islet preproinsulin mRNA content. This stabilization may be mediated by phosphorylation of proteins involved in mRNA stabilization, by the catalytic subunit of the cAMP-dependent protein kinase. Hormones, such as human growth hormone, increase insulin gene transcription by binding to islet receptors which are coupled

to the islet adenylate cyclase system thus raising islet cAMP. Steroid hormones, such as cortisol, effect transcription via the cAMP-independent mechanism of intracellular steriod hormone receptors (Rousseau 1984).

The effect of glucose on the rate of translation of islet preproinsulin mRNA is mediated by its metabolism in the β-cell. The enzyme glucokinase is at a rate limiting step in β-cell glucose utilization and therefore the rate of synthesis of glucose-6-phosphate the product of the reaction catalysed by this enzyme is proportional to the extracellular glucose concentration. This compound, and the increase in islet $NADPH/NADP^+$ ratio caused by its catabolism through the pentose phosphate pathway, are necessary for the prevention of early inhibition of peptide chain initiation. This effect is responsible for the preferential stimulation of preproinsulin mRNA translation by the kinetic model of Lodish (1971). In this model the increase in the overall rate of islet peptide chain initiation favours the translation of preproinsulin mRNA since it has a lower affinity for peptide chain initiation factors than other islet mRNAs. This effect on peptide chain initiation occurs at glucose concentrations over 3.3 mM. Specific stimulation of preproinsulin peptide elongation rate occurs at glucose concentrations greater than 5.6 mM, the mechanism of this is not known. The complete translation of preproinsulin mRNA in the islet cytosol is prevented by SRP-mediated translational arrest of the nascent prepeptide. This translational arrest is relieved by binding of the SRP/preproinsulin ribosome complex to the SRP-receptor which is situated on the rough endoplasmic reticulum. Glucose reduces the degree of SRP-mediated translational arrest by

increasing the affinity of the SRP-receptor for the SRP/preproinsulin/ribosome complex possibly by phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase.

Intracellular β-cell insulin stores are regulated by lysosomal degradation of secretory granules and the rate of this is inversely proportional to the β-cell insulin content. Therefore glucose only exerts an indirect effect of the rate of lysosomal intracellular insulin degradation by controlling the relative rates of insulin synthesis and secretion.

This model can account for several observed physiological phenomena. In islets isolated from pregnant rats the rate of (pro)insulin synthesis is higher than in islets isolated from normal female rats at all glucose concentrations tested (Bone & Howell 1977). The adenylate cyclase activity, cAMP content and protein kinase activity of the islets from pregnant rats was higher than of islets from normal female rats (Green et al 1973). The model under discussion would predict a higher endogenous rate of insulin gene transcription mediated by the increased activity of the cAMP-dependent protein kinase to account for this. Since the fasting serum glucose concentration of pregnant rats is lower than in normal rats (Green & Taylor 1972) the higher adenylate cyclase activity is presumably due to hormonal factors. The rate of (pro)insulin synthesis in islets isolated from 48 hour starved rats was lower than in islets isolated from normal rats at all glucose concentrations tested (Bone & Howell 1977). Since the adenylate cyclase activity of these islets was lower than of islets from fed rats (Howell et al 1973; Selawry et al 1972; Selawry et al 1973) this can be accounted for by a lower endogenous rate of insulin gene transcription mediated by a

lower cAMP-dependent protein kinase activity. This is supported by the observation that treatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine increased the glucose dose response of (pro)insulin synthesis in islets from normal female rats almost to that of islets from pregnant rats. The same treatment also partially restored the glucose dose response of (pro)insulin synthesis of islets from 48 hour starved rats to that of islets from fed rats (Bone & Howell 1977). The preproinsulin mRNA content of islets isolated from starved rats is lower than that of fed rats (Giddings et al 1981; Giddings et al 1982).

The potentiation of (pro)insulin synthesis by glucagon (Schatz et al 1973; Schatz et al 1974) can be explained by a stimulation of adenylate cyclase, following the binding of glucagon to islet cell surface receptors associated with adenylate cyclase leading to an increase in cAMP-dependent protein kinase mediated insulin gene transcription. Compounds such as glucagon, dibutyryl cAMP and methylxanthines can only stimulate (pro)insulin synthesis in the presence of glucose (Campbell et al 1982). Presumably this is because although they increase the rate of insulin gene transcription the rate of translation of preproinsulin mRNA is limited by glucose.

Normal fasting serum glucose is 4 to 5 mM or greater in the rat (Logothetopoulos & Jain 1980a). This means that any increase of serum glucose over this level would stimulate insulin gene transcription. Since the glucose stimulated increase in adenylate cyclase activity only reaches a maximum after 4 to 8 hours of stimulation (Howell et al 1973) short term increases in serum glucose, for example after feeding, should not significantly increase the rate of insulin gene

transcription. Only prolonged periods of hyperglycemia would be effective in increasing this rate.

The model proposed in this chapter can account for the majority of experimental observations made to date, however, further work is required in order to substantiate it. Firstly the glucose dose response of insulin gene transcription, in parallel with islet cAMP content, adenylate cyclase and phosphodiesterase activity, needs to be determined. The glucose threshold for the stimulation of islet cAMP content should correspond to that of insulin gene transcription. Changes in insulin gene transcription rate should correlate with cAMP content and protein kinase activity. For these measurements it should be ensured that the period of glucose stimulation is long enough to allow for the full stimulation of adenylate cyclase activity (i.e. 4 to 8 hours or longer (Howell et al 1973)). Since β-cells possess receptors for glucagon and β-adrenergic agents coupled to adenylate cyclase (Sharp 1979) these agents should be able to stimulate insulin gene transcription. Inhibition of adenylate cyclase, or stimulation of phosphodiesterase (e.g. by imidazole (Sams & Montague 1972; Ashcroft et al 1972b)), should abolish any stimulation of insulin gene transcription by such agents or glucose. Determination of the cellular targets for phosphorylation by the catalytic subunit of cAMP-dependent protein kinase would help in the identification of possible cAMP-mediated mechanisms in the translation and stabilization of preproinsulin mRNA and any change in affinity of the SRP-receptor for the SRP-preproinsulin ribosome complex.

Appendix

Gelmerge Program

This appendix contains the text of the Gelmerge program used to arithmetically average several sets of scan data from the laser densitometer. The program was written in UCSD-Pascal and because of its length has been written as several separate disk files. All the disk files should be stored on drive #5 and only the file #5:GELMERGE.TEXT needs to be compiled, the other files are automatically linked in by the compiler. The compiled program (GELMERGE.CODE) should be stored on the GELSCAN master disk for use.

Contents of Disk File #5:GELMERGE.TEXT

```
(*$C COPYRIGHT JON MARSHALL, LEICESTER UNIVERSITY. 1986*)
(*$S+*)
PROGRAM GELMERGE;

(*     TITLE:     GELMERGE
     VERSION:     1.1A
     AUTHOR:    JON MARSHALL, DEPT. OF BIOCHEMISTRY,
                      UNIVERSITY OF LEICESTER.
     WRITTEN:     JAN 1986
     MODIFIED: FROM V1.1 TO V1.1A (30/1/86)
     DESCRIPTION:

       ACCEPTS A LIST OF GELSCAN DATA FILE SPECIFICATIONS FROM
THE KEYBOARD AND MERGES THE CURVE DATA FROM THE FILES
TOGETHER. THE MERGED DATA IS WRITTEN TO A NEW DATA FILE FOR
FURTHER PROCESSING BY GELSCAN. THE PROGRAM INCORPORATES TRAPS
AND PROMPTS TO PREVENT THE MERGING OF INVALID DATA FILES. A
LIST OF THE MERGED FILES MAY BE OUTPUT TO A PRINTER IF ONE IS
ON LINE. *)

(* GLOBAL CONSTANTS *)

CONST
  COLON=':';  PERIOD='.'; HASH='#'; BLANK=' ';
  PRGTITLE='GELMERGE V1.1A';  MAXFILE=54;(* MAX. NO. OF MERGE
                                           FILES*)
  TOP=1000; DIM=5;  PMAX=50;  (* DATAFILE CONSTANTS *)
  DEFVOL='#5';  DEFEXT='DATA';  (* DEFAULTS *)
  RANGEDELIMETER=' TO ';
```

```
TYPE

  CURVE=ARRAY [0..TOP] OF INTEGER;
  DATE=PACKED RECORD MONTH:1..12;
                DAY:1..31;
                YEAR:0.99 END;

  DATALIST=RECORD
    NAME     :STRING[10];  (* NAME OF STORED DATA *)
    COMMENT  :STRING[40];  (* COMMENT LINE FOR STORED DATA *)
    DAY    :DATE;       (* DATE AS A PACKED RECORD*)
    ABR    :INTEGER;    (* ABSORBANCE RANGE SETTING OF
                                SCANNER*)
    BEG    :INTEGER;    (*VALUE OF FIRST POINT OF CURVE*)
    RES    :INTEGER;    (*(1,2,4) HIGH, MED OR LOW
                                RESOLUTION*)
    FFIT     :INTEGER;    (*(0...) NO. OF FIT PASSES *)
    FMAX     :INTEGER;    (*(-1..PMAX) NUMBER OF PEAKS*)
    AVPW2    :INTEGER;    (*CHANGE OF PEAK WIDTH WITH X *)
    AVPW1    :INTEGER;    (*AVERAGE PEAK WIDTH AT X=0 *)
    FPW    :ARRAY [0..PMAX] OF INTEGER;  (*PEAK WIDTH *)
    FPL    :ARRAY [0..PMAX] OF INTEGER;  (*PEAK LOCATION*)
    FPST     :ARRAY [0..PMAX] OF INTEGER;(*PEAK HEIGHT*)
    DTYP     :INTEGER;    (*TYPE OF CURVE, 1=MEAS., 2=CALC.,
                                3=BACKG *)
    BGNDSET  :INTEGER;    (* (0,1)...1=BACKGROUND SET *)
    C      :CURVE       (* STORED DATA *)
  END;

NEXT=^FILENTRY;

FILENTRY=RECORD
      NAME:STRING[23];  (* GELSCAN FILE NAME *)
      NEXTFILE:NEXT;    (* PTR TO NEXTFILESPEC IN LIST *)
      END;

CHARACTERS=SET OF CHAR;

VAR

  INFILE:FILE OF DATALIST;  (*INPUT & MERGED FILES *)
  LISTDEVICE:INTERACTIVE;
  MERGE:DATALIST;      (* RECORD FOR MERGED DATA *)
  NUMFILES,          (* NO. OF FILES ACTUALLY MERGED *)
  FILECOUNT:INTEGER;     (* NO. OF FILES IN MERGELIST *)
  MERGELIST:NEXT;      (* PTR TO HEAD OF MERGELIST *)
  PRINTER:BOOLEAN;       (* FLAG FOR PRINTER OUTPUT *)
  AGAIN,BELL,ESC:CHAR;     (* SPECIAL PRINT CHARS *)

(* PROCEDURE DECLARATIONS *)

FUNCTION STRTOINT (S:STRING):INTEGER;
FORWARD;

PROCEDURE TITLE;
FORWARD;

PROCEDURE QUIT;
FORWARD;
```

```
PROCEDURE INPUTSTRING (VAR LINE:STRING);
FORWARD;

PROCEDURE STRIPBLANKS (VAR ITEM:STRING);
FORWARD;

PROCEDURE HEADING;
FORWARD;

PROCEDURE CHECKFILESPEC (VAR ERRPOS:INTEGER;
                         VAR ERRMES:STRING;
                             FILESPEC:STRING);
FORWARD;

PROCEDURE PRINTFILEINFO(PRINTFILE:DATALIST);
FORWARD;

PROCEDURE PROMPTLINE (VAR REPLY:CHAR; OPTIONS:CHARACTERS);
FORWARD;

PROCEDURE PROCESSERROR (VAR ACTION:CHAR; ERROR:INTEGER;
                            NAME:STRING);
FORWARD;

SEGMENT PROCEDURE GETLIST (VAR BASE:NEXT);

(* PROMPTS FOR A LIST OF FILE ITEMS TO BE MERGED AND CHECKS
EACH  SYNTAX (& EXPANDS THEM IN THE CASE OF A FILE RANGE) AND
IF O.K.   ADDS THEM TO THE END OF A LINKED LIST OF FILENTRY
POINTED TO BY   BASE *)

VAR

  ITEM,
  LIST:STRING;       (* INPUT STRING AND SUB-PART *)
  LISTPLACE:INTEGER;  (* POSITION REACHED IN SCANNING LIST *)
  BACKLIST:NEXT;      (* PTR TO REAR OF MERGE FILE LIST *)

  PROCEDURE GETITEM (VAR PLACE:INTEGER;
                     VAR NEXTITEM:STRING;
                         LIST:STRING);

  (* GETS THE 'NEXTITEM' FROM 'LIST' STARTING FROM 'PLACE'
     STRIPBLANKS AND UPDATES 'PLACE' TO NEW POSITION. IF THE
'NEXTITEM'='' THEN NEXTITEM=BLANK *)

  CONST
    DELIMETER=',';

  VAR
    INDEX:INTEGER;    (* PLACE COUNTER *)
```

```
BEGIN;  (* GETITEM *)
INDEX:=PLACE-1;
REPEAT;
INDEX:=SUCC(INDEX);
UNTIL (INDEX=LENGTH(LIST)) OR (LIST[INDEX]=DELIMETER);
IF INDEX=PLACE THEN   (* NEXTITEM='' *)
  NEXTITEM:=BLANK
ELSE
  IF INDEX=LENGTH(LIST) THEN
    NEXTITEM:=COPY(LIST,PLACE,INDEX-PLACE);
IF INDEX<>LENGTH(LIST) THEN
  PLACE:=INDEX+1    (* INCR PAST DELIMETER *)
ELSE
  PLACE:=INDEX;   (* SET PLACE TO END OF LIST *)
END;    (* GETITEM *)

PROCEDURE PROCESSITEM(ITEM:STRING);

(* CHECKS THAT 'ITEM' IS OF CORRECT SYNTAX FOR A FILESPEC.
   AND ASKS FOR A CORRECTION IF NOT. ONCE A CORRECT SPEC. IS
   OBTAINED IT IS ADDED TO THE END OF THE MERGE FILE LIST *)

VAR
  ERROR:INTEGER;    (* POSITION OF ERROR IN ITEM,0 IF NONE*)
  MESSAGE:STRING; (* NATURE OF ERROR *)

  PROCEDURE ADDLIST (VAR FRONT,REAR:NEXT;ITEM:STRING);

  (* ADDS ITEM TO 'REAR' OF LINKED LIST POINTED TO BY
     'FRONT' AS LONG AS FILECOUNT<MAXFILE *)

  VAR
    ENTRY:NEXT;

  BEGIN;    (* ADDLIST *)
  IF FILECOUNT<=MAXFILE THEN (* STILL ROOM IN THE LIST *)
    BEGIN;
    NEW(ENTRY);     (* NEW FILENTRY *)
    ENTRY^.NAME:=ITEM;
    IF FRONT=NIL THEN   (* LIST EMPTY *)
      FRONT:=ENTRY
    ELSE
      REAR^.NEXTFILE:=ENTRY;
    REAR:=ENTRY;
    REAR^.NEXTFILE:=NIL;
    END         (* NEW FILENTRY *)
  ELSE
    WRITELN('FILE LIMIT EXCEEDED.');
  FILECOUNT:=SUCC(FILECOUNT);
  END;      (* ADDLIST *)

  PROCEDURE CORRECT (VAR ITEM:STRING);

  (* WRITES OUT 'ERROR' AND 'MESSAGE' AND REQUESTS FOR
     CORRECTED INPUT, PASSES IT BACK AS CALL TO
     PROCESSITEM *)
```

```
  VAR
    CORRECTION:STRING;
    START:INTEGER;     (* DUMMY FOR GETITEM CALL PARAM *)
    I:INTEGER;

  BEGIN;     (* CORRECT *)
  START:=1;
  WRITELN(ITEM);
  FOR I:=1 TO ERROR-1 DO
    WRITE(BLANK);
  WRITELN(MESSAGE);
  WRITELN('PLEASE RETYPE');
  INPUTSTRING(CORRECTION);
  IF CORRECTION=BLANK THEN
    BEGIN;     (* IGNORE WRONG ENTRY *)
    WRITELN('FILE DELETED FROM LIST.');
    EXIT(PROCESSITEM);
    END;       (* IGNORE *)
  STRIPBLANKS(CORRECTION);
  PROCESSITEM(CORRECTION):
  END;       (* CORRECT *)

PROCEDURE EXPANDITEM (ITEM:STRING);

(* EXPANDS A FILE RANGE SPEC. AND CHECKS BOTH THE SYNTAX OF
   THE FILE SPEC. AND THE RANGE PARAMETERS. ASKS FOR
   CORRECTION IF WRONG FOR THE RANGE SPEC. EACH NEW ITEM
   FROM EXPANSION IS PASSED TO PROCESSITEM *)

VAR
  DIGITS:CHARACTERS;
  I,
  POSITION,
  BOTTOMNUM,
  TOPNUM:INTEGER;
  FILESPEC,NUM,BOTTOMLIMIT,TOPLIMIT:STRING;

BEGIN;     (* EXPANDITEM *)
DIGITS:=['0'..'9'];
STRIPBLANKS(ITEM);
POSITION:=POS(RANGEDELIMETER,ITEM);
IF POSITION=1 THEN
  BEGIN;
  ERROR:=1;MESSAGE:='FILENAME EXPECTED.';
  CORRECT(ITEM);
  END
ELSE
  BEGIN;     (* EXTRACT & CHECK FILESPEC. *)
  FILESPEC:=COPY(ITEM,1,POSITION-1);
  STRIPBLANKS(FILESPEC);
  CHECKFILESPEC(ERROR,MESSAGE,FILESPEC);
  IF ERROR<>0 THEN
    CORRECT(FILESPEC)
  ELSE
    BEGIN;     (* CHK BOTTOM LIMIT *)
    BOTTOMLIMIT:=COPY(FILESPEC,LENGTH(FILESPEC)-1,2);
    IF NOT (BOTTOMLIMIT[1] IN DIGITS) AND
      NOT (BOTTOMLIMIT[2] IN DIGITS) THEN
      BEGIN;
      ERROR:=LENGTH(FILESPEC)-1;
```

```
  MESSAGE:='INVALID LOWER RANGE LIMIT.';
  CORRECT(ITEM);
  END
ELSE
  BEGIN;
  BOTTOMNUM:=STRTOINT(BOTTOMLIMIT);
  IF NOT(BOTTOMNUM IN [1..54] THEN
    BEGIN;
    ERROR:=LENGTH(FILESPEC)-1;
    MESSAGE:='INVALID LOWER RANGE LIMIT.';
    CORRECT(ITEM);
    END
  ELSE
    BEGIN;  (* CHK UPPER LIMIT *)
    IF (POSITION+LENGTH(RANGEDELIMETER)-1)=LENGTH(ITEM)
        THEN
      BEGIN;
      ERROR:=LENGTH(ITEM)+1;
      MESSAGE:='UPPER RANGE LIMIT EXPECTED.';
      CORRECT(ITEM);
      END
    ELSE
      BEGIN;
      TOPLIMIT:=
          COPY(ITEM,POSITION+LENGTH(RANGEDELIMETER),
  (LENGTH(ITEM)-(POSITION+LENGTH(RANGEDELIMETER)-1)));
      STRIPBLANKS(TOPLIMIT);
      I:=1;
      WHILE (TOPLIMIT[I] IN DIGITS) AND
                    (I<LENGTH(TOPLIMIT)) DO
        I:=SUCC(I);
      IF NOT (TOPLIMIT[I] IN DIGITS) THEN
        BEGIN;
        ERROR:=POSITION+LENGTH(RANGEDELIMETER)+I-1;
        MESSAGE:='ILLEGAL CHARACTER.';
        CORRECT(ITEM);
        END
      ELSE
        BEGIN;     (* CHK LIMITS *)
        TOPNUM:=STRTOINT(TOPLIMIT);
        IF NOT (TOPNUM IN [1..54]) THEN
          BEGIN;
          ERROR:=POSITION+LENGTH(RANGEDELIMETER);
          MESSAGE:='INVALID UPPER RANGE LIMIT.';
          CORRECT(ITEM);
          END
        ELSE
          BEGIN;     (* CHK RANGE *)
          IF BOTTOMNUM>=TOPNUM THEN
            BEGIN;
            ERROR:=LENGTH(FILESPEC)-1;
            MESSAGE:='UPPER LIMIT MUST EXCEED
                                LOWER.';
            CORRECT(ITEM);
            END
          ELSE
            BEGIN;  (*ALL O.K. TO EXPAND RANGE*)
            FILESPEC:=
              COPY(FILESPEC,1,LENGTH(FILESPEC)-2);
            FOR I:=BOTTOMNUM TO TOPNUM DO
```

```
                    BEGIN;
                    STR(I,NUM);
                    IF LENGTH(NUM)=1 THEN
                      NUM:=CONCAT('0',NUM);
                    ADDLIST(BASE,BACKLIST,
                          CONCAT(FILESPEC,NUM);
                    END
                  END
                END
              END
            END
          END
        END
      END
    END
  END;       (* EXPANDITEM *)

  BEGIN;     (* PROCESSITEM *)
  IF POS(RANGEDELIMETER,ITEM)<>0 THEN
    EXPANDITEM(ITEM)
  ELSE
    BEGIN;   (* PROCESS *)
    STRIPBLANKS(ITEM);
    CHECKFILESPEC(ERROR,MESSAGE,ITEM);
    IF ERROR<>0 THEN
      CORRECT(ITEM)
    ELSE
      ADDLIST(BASE,BACKLIST,ITEM)
    END    (* PROCESS *)
  END;       (* PROCESSITEM *)

BEGIN;       (* GETLIST *)
(* INITIALIZE *)
BACKLIST:=BASE;
WRITELN('LIST OF FILES(SEPARATED BY COMMAS)');
WRITELN('TO BE MERGED ?');
INPUTSTRING(LIST);
LISTPLACE:=1;    (* INITIALIZE FOR LIST SCANNING *)
WHILE LISTPLACE<LENGTH(LIST) DO
  BEGIN;     (* SCAN LIST *)
  GETITEM(LISTPLACE,ITEM,LIST);
  PROCESSITEM(ITEM);
  END;       (* SCAN LIST *)
END;         (* GETLIST *)

(*$I #5:GELMRGFILE.TEXT*)    (* PROCEDURE MERGEFILES *)

(*$I #5:GELWRTMRG.TEXT*)     (* PROCEDURE WRITEMERGEFILE *)

(* FORWARD DEFINITIONS *)

FUNCTION STRTOINT;
(* CONVERTS S TO AN INTEGER *)

VAR
  I,RESULT:INTEGER;
```

```
BEGIN;     (* STRTOINT *)
RESULT:=0;
FOR I:=1 TO LENGTH(S) DO
  RESULT:=RESULT*10+ORD(S[I])-ORD('0');
STRTOINT:=RESULT;
END;       (* STRTOINT *)

PROCEDURE QUIT;

VAR
  ANSWER:CHAR;

BEGIN;     (* QUIT *)
WRITE('QUIT (Y/N)?');
PROMPTLINE(ANSWER,['Y','N']);
IF ANSWER='Y' THEN
  BEGIN;
  TITLE;
  WRITELN('              RUN ABORTED....');
  EXIT(PROGRAM);
  END;
END;       (* QUIT *)

PROCEDURE INPUTSTRING;
(* READS 'LINE' FROM THE KEYBOARD AND CHECKS IF FIRST CHAR. IS
<ESC> IN WHICH CASE THE OPTION TO EXIT THE PROGRAM IS GIVEN *)

BEGIN;       (* INPUTSTRING *)
WRITE(BELL,'>');
READLN(LINE);
IF LINE<>'' THEN
  BEGIN;
  IF LINE[1]=ESC THEN
    BEGIN;QUIT;INPUTSTRING(LINE);END;
  END
ELSE
  LINE:=BLANK
END;         (* INPUTSTRING *)

PROCEDURE STRIPBLANKS;

(* STRIPS LEADING AND TRAILING BLANKS FROM 'ITEM'. IF 'ITEM'
   IS ALL BLANKS THEN ITEM=' ' ON EXIT *)

VAR
  FRONT,BACK:INTEGER;

BEGIN;       (* STRIPBLANKS *)
FRONT:=1;
WHILE (ITEM[FRONT]=BLANK) AND (FRONT<LENGTH(ITEM)) DO
  FRONT:=SUCC(FRONT);
IF FRONT=LENGTH(ITEM) THEN    (* ITEM ALL BLANKS *)
  ITEM:=BLANK
ELSE
  BEGIN;     (* FIND BACK CHAR *)
  BACK:=LENGTH(ITEM);
  WHILE ITEM[BACK]=BLANK DO
    BACK:=PRED(BACK);
  ITEM:=COPY(ITEM,FRONT,(BACK-FRONT)+1);
  END;       (* FIND BACK CHAR *)
```

```
END;        (* STRIPBLANKS *)

PROCEDURE HEADING;

(* WRITES PROGRAM HEADING TO THE SCREEN *)

BEGIN;    (* HEADING *)
WRITELN('         COPYRIGHT JON MARSHALL 1986');
WRITELN;WRITELN;
WRITELN('  THIS PROGRAM WILL MERGE TOGETHER THE');
WRITELN('CURVES OF A LIST OF GELSCAN DATAFILES');
WRITELN('INTO A NEW DATAFILE SUITABLE FOR FURTHER');
WRITELN('PROCESSING BY THE GELSCAN PROGRAM.');
WRITELN;
END;      (* HEADING *)

PROCEDURE TITLE;

(* WRITES PRGTITLE TO SCREEN *)

CONST
  HEIGHT=10;    (* POS OF TITLE DOWN SCREEN *)

VAR
  I:INTEGER;

BEGIN;      (* TITLE *)
PAGE(OUTPUT);
GOTOXY(TRUNC(40-LENGTH(PRGTITLE))/2),HEIGHT);
WRITELN(PRGTITLE);
GOTOXY(TRUNC(40-LENGTH(PRGTITLE))/2),HEIGHT+1);
FOR I:=1 TO LENGTH(PRGTITLE) DO
  WRITE('-');
WRITELN;WRITELN;
END;        (* TITLE *)

(*$I #5:GELCHKFL.TEXT*)       (* PROCEDURE CHECKFILESPEC *)

PROCEDURE PRINTFILEINFO;

(* PRINTS DETAILS OF 'PRINTFILE' TO 'LISTDEVICE' *)

  PROCEDURE CONVDATE(TDAY:DATE);

  VAR
    MONTHN:STRING[3];
    DY,YR :STRING[2];

  BEGIN;  (* CONVDATE *)
  WITH TDAY DO
    BEGIN;
    STR(MONTH,MONTHN);
    STR(DAY,DY);
    STR(YEAR,YR);
    END;
  WRITE(LISTDEVICE,DY,'-',MONTHN,'-',YR);
  END;    (* CONVDATE *)
```

```
BEGIN;    (* PRINTFILEINFO *)
WITH PRINTFILE DO
  BEGIN;
  WRITE(LISTDEVICE,'NAME:',NAME:10,'   ');
  WRITE(LISTDEVICE,'DATE:');CONVDATE(DAY);
  WRITE(LISTDEVICE);
  WRITE(LISTDEVICE,'COMMENT:);
  WRITELN(LISTDEVICE,COMMENT);
  WRITE(LISTDEVICE,'RESOLUTION:');
  CASE RES OF
    1:WRITE(LISTDEVICE,'HIGH);
    2:WRITE(LISTDEVICE,' MED');
    3:WRITE(LISTDEVICE,' LOW');
  END;  (* CASE *)
  WRITE(LISTDEVICE,'       TYPE:');
  CASE DTYP OF
    1:WRITE(LISTDEVICE,'MEASURED');
    2:WRITE(LISTDEVICE,'CALCULATED');
    3:WRITE(LISTDEVICE,'BACKGROUND');
  END;  (* CASE *)
  WRITELN(LISTDEVICE);
  WRITE(LISTDEVICE,'ABSORBANCE:',ABR/10:4:1);
  WRITELN(LISTDEVICE,'     START OF CURVE:',(BEG DIV 40):4);
  END;  (* WITH *)
WRITELN(LISTDEVICE);
END;        (* PRINTFILEINFO *)

PROCEDURE PROMPTLINE;

BEGIN;    (* PROMPTLINE *)
IF 'I' IN OPTIONS THEN
  WRITELN('TO INCLUDE FILE.....I');
IF BLANK IN OPTIONS THEN
  WRITELN('TO CONTINUE...<SPACE>');
IF 'S' IN OPTIONS THEN
  WRITELN('TO SKIP FILE........S');
IF 'T' IN OPTIONS THEN
  WRITELN('TO TRY AGAIN........T');
IF ESC IN OPTIONS THEN
  WRITELN('TO QUIT.........<ESC>');
IF 'D' IN OPTIONS THEN
  WRITELN('TO CHANGE DATE......D');
IF 'C' IN OPTIONS THEN
  WRITELN('TO CHANGE COMMENT...C');
WRITE(BELL);
REPEAT;READ(KEYBOARD,REPLY);
UNTIL REPLY IN OPTIONS;WRITELN(REPLY);
END;        (* PROMPTLINE *)

PROCEDURE PROCESSERROR;

(* DISPLAYS ERROR MESSAGE FOR 'ERROR' & A PROMPTLINE FOR
  POSSIBLE ACTION AND RETURNS THE RESPONSE AS 'ACTION' *)

VAR
  ERRORMESS:STRING;
  CHOICE:CHARACTERS;
```

```
BEGIN;      (* PROCESSERRROR *)
CASE ERROR OF
  0:BEGIN;
    ACTION:=BLANK;
    EXIT(PROCESSERROR);
    END;
 2,3:BEGIN;
    ERRORMES:='BAD DEVICE.';
    CHOICE:=[ESC,BLANK];
  5:BEGIN;
    ERRORMESS:='DEVICE NO LONGER ON LINE.';
    CHOICE:=[ESC,BLANK];
    END;
  6:BEGIN;
    ERRORMESS:='FILE NO LONGER IN DIRECTORY.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  7:BEGIN;
    ERRORMESS:='ILLEGAL FILENAME.';
    CHOICE:=[ESC,BLANK];
    END;
  8:BEGIN;
    ERRORMESS:='NO ROOM ON DISC';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  9:BEGIN;
    ERRORMESS:='VOLUME NOT ON LINE.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  10:BEGIN;
    ERRORMESS:='NO SUCH FILE.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  11:BEGIN;
    ERRORMESS:='DUPLICATE FILENAME.';
    CHOICE:=[ESC,BLANK];
    END;
  12:BEGIN;
    CLOSE(INFILE);
    ERRORMESS:='FILE ALREADY OPEN.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  13:BEGIN;
    ERRORMESS:='FILE CLOSED.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  16:BEGIN;
    ERRORMESS:='DISC WRITE PROTECTED.';
    CHOICE:=[ESC,BLANK,'T'];
    END;
  64:BEGIN;
    ERRORMESS:='BAD DATA OR ADDRESS ON DISC.';
    CHOICE:=[ESC,BLANK];
    END;
END;  (* CASE *)
WRITELN(NAME);
WRITELN(ERRORMESS);WRITELN;
PROMPTLINE(ACTION,CHOICE);
END;      (* PROCESSERROR *)
```

```
(* GELMERGE *)

BEGIN;     (* MAIN *)
(* INITIALIZE *)
BELL:=CHR(7);ESC:=CHR(27);
TITLE;
HEADING;
REPEAT
  MERGELIST:=NIL;FILECOUNT:=0;
  GETLIST(MERGELIST);
  WRITELN;
  MERGEFILES(MERGE,MERGELIST,NUMFILES);
  IF NUMFILES>0 THEN
    WRITEMERGEFILE(MERGE);
  WRITELN;WRITELN;
  WRITE('MERGE MORE FILES (Y/N)?');
  PROMPTLINE(AGAIN,['Y','N']);
  PAGE(OUTPUT);
UNTIL AGAIN='N';
TITLE;
WRITELN;WRITELN('                    BYE...');
END.       (* MAIN *)
```

Contents of Disk File #5:GELWRTMRG.TEXT

```
SEGMENT PROCEDURE WRITEMERGEFILE(MERGED:DATALIST);

VAR
  FILESPEC,FILENAME:STRING;
  FILERROR:INTEGER;

  PROCEDURE GETNAME(VAR NAME:STRING);

  VAR
    I,ERRORPOSITION:INTEGER;
    ERRORMESSAGE:STRING;

  BEGIN;     (* GETNAME *)
  WRITELN('NAME FOR MERGED FILE?');
  INPUTSTRING(NAME);
  CHECKFILESPEC(ERRORPOSITION,ERRORMESSAGE,NAME);
  IF ERRORPOSITION<>0 THEN
    BEGIN;  (* CORRECT *)
    FOR I:=1 TO ERRORPOSITION DO
      WRITE(BLANK);
    WRITELN('^');WRITELN(ERRORMESSAGE);
    GETNAME(NAME);
    END;     (* CORRECT *)
  END;       (* GETNAME *)

PROCEDURE CHANGE;

VAR
  RESPONSE:CHAR;
  COMMENT,DATE:STRING;
  DY,MNTH,YR:INTEGER;
```

```
  PROCEDURE CHANGEDATE(VAR DAY,MONTH,YEAR:INTEGER);

  VAR
    PLACE:INTEGER;
    DATESTRING:STRING;
    ERROR:BOOLEAN;

    PROCEDURE NEXTNUM(VAR NUMBER:INTEGER);

    VAR
      START:INTEGER;

    BEGIN;     (* NEXTNUM *)
    START:=PLACE;
    WHILE (DATESTRING[PLACE] IN ['0'..'9']) AND
                  (PLACE<LENGTH(DATESTRING)) DO
      PLACE:=SUCC(PLACE);
    IF NOT(DATESTRING[PLACE] IN ['0'..'9','-']) OR
(PLACE=START)
                                    THEN
      ERROR:=TRUE
    ELSE
      IF (PLACE=LENGTH(DATESTRING) THEN
        BEGIN;
        IF DATESTRING[PLACE]='-' THEN
          ERROR:=TRUE
        ELSE
        NUMBER:=STRTOINT(COPY(DATESTRING,START,PLACE-START+1))
        END
      ELSE
        BEGIN;
        NUMBER:=STRTOINT(COPY(DATESTRING,START,PLACE-START));
        PLACE:=SUCC(PLACE);
        END;
    END;       (* NEXTNUM *)

  BEGIN;     (* CHANGEDATE *)
  WRITE('DATE ?');INPUTSTRING(DATESTRING);
  PLACE:=1;ERROR:=FALSE;
  NEXTNUM(DAY);
  IF ERROR OR NOT(DAY IN [1..31]) THEN
    CHANGEDATE(DY,MNTH,YR)
  ELSE
    BEGIN;
    NEXTNUM(MONTH);
    IF ERROR OR NOT(MONTH IN [1..12]) THEN
      CHANGEDATE(DY,MNTH,YR)
    ELSE
      BEGIN;
      NEXTNUM(YEAR);
      IF ERROR OR NOT(YEAR IN [1..99]) THEN
        CHANGEDATE(DY,MNTH,YR);
      END;
    END;
  END;       (* CHANGEDATE *)
```

```
BEGIN;     (* CHANGE *)
CLOSE(LISTDEVICE);
REWRITE(LISTDEVICE,'CONSOLE:');
PAGE(OUTPUT);
WRITELN('                MERGED FILE');
WRITELN('                -----------');
WRITELN;
PRINTFILEINFO(MERGED);
PROMPTLINE(RESPONSE,[BLANK,ESC,'D','C']);
IF RESPONSE=ESC THEN
  BEGIN;QUIT;CHANGE;END;
IF RESPONSE='C' THEN
  BEGIN;     (* COMMENT *)
  WRITELN('COMMENT ?');
  INPUTSTRING(COMMENT);
  IF LENGTH(COMMENT)>40 THEN
    MERGED.COMMENT:=COPY(COMMENT,1,40)
  ELSE
    MERGED.COMMENT:=COMMENT;
  CHANGE;
  END;       (* COMMENT *)
IF RESPONSE='D' THEN
  BEGIN;     (* DATE *)
  CHANGEDATE(DY,MNTH,YR);
  WITH MERGED.DAY DO
    BEGIN;DAY:=DY;MONTH:=MNTH;YEAR:=YR;END;
  CHANGE;
  END;       (* DATE *)
END;       (* CHANGE *)

PROCEDURE WRITEFILE;

VAR
  I:INTEGER;
  RESPONSE:CHAR;
  OPTIONS:CHARACTERS;

BEGIN;     (* WRITEFILE *)
(*$I-*)RESET(INFILE,FILESPEC);(*$I+*)
FILERROR:=IORESULT;
IF FILERROR=0 THEN
  BEGIN;   (* FILE ALREADY EXISTS *)
  WRITELN(FILESPEC,' ALREADY EXISTS.');
  CLOSE(INFILE);
  PROMPTLINE(RESPONSE,[ESC,'T',BLANK]);
  IF RESPONSE='T' THEN
    WRITEFILE
  ELSE
    WRITEMERGEFILE(MERGED);
  END
ELSE
  BEGIN;     (* FILE DOESN'T EXIST, CREATE NEW FILE *)
  (*$I-*)REWRITE(INFILE,FILESPEC);(*$I+*)
  FILERROR:=IORESULT;
  PROCESSERROR(RESPONSE,FILERROR,FILESPEC);
  IF FILERROR<>0 THEN
```

```
    BEGIN;     (* FILERROR *)
    IF RESPONSE=ESC THEN
      BEGIN;QUIT;WRITEFILE;END;
    IF RESPONSE='T' THEN
      WRITEFILE;
    IF RESPONSE=BLANK THEN
      WRITEMERGEFILE(MERGED);
    END      (* FILERROR *)
  ELSE
    BEGIN;     (* NO FILERROR *)
    (* SET FIELDS TO DEFAULT BEFORE WRITING *)
    WITH MERGED DO
      BEGIN;
      FFIT:=0;     (* 0 FIT PASSES *)
      FMAX:=-1; (* 0 PEAKS FITTED *)
      BGNDSET:=0; (* NO BACKGROUND SET *)
      DTYP:=1;     (* 'MEASURED' DATA *)
      END;
    INFILE^:=MERGED;
    PUT(INFILE);
    CLOSE(INFILE,LOCK);
    CLOSE(LISTDEVICE);
    IF PRINTER THEN
      RESET(LISTDEVICE,'PRINTER:')
    ELSE
      RESET(LISTDEVICE,'CONSOLE:');
    WRITELN(LISTDEVICE);WRITELN(LISTDEVICE);
    WRITELN(LISTDEVICE,NUMFILES:2,' FILES MERGED INTO :-');
    WRITELN(LISTDEVICE,'-----------------------');
    PRINTFILEINFO(MERGED);
    END;     (* NO FILERROR *)
  END;
END;         (* WRITEFILE *)

BEGIN;         (* WRITEMERGEFILE *)
GETNAME(FILESPEC);
IF POS(COLON,FILESPEC)=0 THEN
  FILENAME:=FILESPEC
ELSE
  FILENAME:=COPY(FILESPEC,POS(COLON,FILESPEC)+1,
              LENGTH(FILESPEC)-POS(COLON,FILESPEC));
IF POS(COLON,FILESPEC)=0 THEN
  FILESPEC:=CONCAT(DEFVOL,COLON,FILESPEC,PERIOD,DEFEXT)
ELSE
  FILESPEC:=CONCAT(FILESPEC,PERIOD,DEFEXT);
MERGED.NAME:=FILENAME;
CHANGE;
WRITEFILE;
CLOSE(LISTDEVICE);
END;           (* WRITEMERGEFILE *)
```

Contents of Disk File #5:GELMRGFILE.TEXT

```
SEGMENT PROCEDURE MERGEFILES(VAR MERGE:DATALIST;BASE:NEXT;
                             VAR FILENUMBER:INTEGER);

(* MERGES THE FILES IN THE LINKED LIST POINTED TO BY 'BASE'
   INTO 'MERGE'. ONLY THOSE FILES CONTAINING DATA COMPATIBLE
   WITH THE FIRST FILE IN THE LIST ARE ALLOED TO BE MERGED. IF
   DATA ARE DIFFERENT BUT NOT TOTALLY INCOMPATIBLE THE USER IS
   REQUESTED TO CONFIRM MERGING. ALL I/O ERRORS ARE TRAPPED
   AND DEALT WITH. THE NUMBER OF FILES ACTUALLY MERGED IS
   PASSED BACK AS 'FILENUMBER'. *)

CONST
  LONGLEN=7;  (* LONG INTEGER LENGTH FOR MERGING FILES, MUST
                 BE LONG ENOUGH TO AVOID OVERFLOW FOR
                 'MAXFILE' NUMBER OF FILES. *)

VAR
  ACCUMULATOR:ARRAY [0..TOP] OF INTEGER[LONGLEN];
  ENTRY:NEXT;
  RESPONSE:CHAR;
  FILENAME:STRING;    (* NEXT FILE IN LIST *)
  I,FILERROR:INTEGER;

  PROCEDURE PROCESSFILE(TITLE:STRING);

  (* OPENS FILE 'TITLE' AND IF NO I/O ERROR AND IF DATA ARE
     COMPATIBLE THE FILES CURVE DATA IS MERGED INTO
     'ACCUMULATOR'. *)

  VAR
    I:INTEGER;

    PROCEDURE ERRORACTION(VAR ACTION:CHAR);

    BEGIN;    (* ERRORACTION *)
    IF ACTION=BLANK THEN
      BEGIN;
      WRITELN(ENTRY^.NAME,' SKIPPED.');WRITELN;
      EXIT(PROCESSFILE);
      END;
    IF ACTION='T' THEN
      PROCESSFILE(TITLE);
    IF ACTION=ESC THEN
      BEGIN;QUIT;PROCESSERROR(ACTION,FILERROR,ENTRY^.NAME);
      END;
    END;      (* ERRORACTION *)

  BEGIN;    (* PROCESSFILE *)
  (*$I-*) (* I/O ERROR CHECKING OFF *)
  RESET(INFILE,TITLE);
  (*$I+*) (* I/O ERROR CHECKING ON *)
  FILERROR:=IORESULT;
  PROCESSERROR(RESPONSE,FILERROR,ENTRY^.NAME);
  IF FILERROR=0 THEN
```

```
    BEGIN;  (* PROCESS *)
    GET(INFILE);
    IF FILENUMBER=0 THEN
      MERGE:=INFILE^;
    IF MERGE.RES<>INFILE^.RES THEN
      BEGIN;
      WRITELN(ENTRY^.NAME);
      WRITELN('GEL RESOLUTION INCOMPATIBLE.');
      PROMPTLINE(REPONSE,[ESC,BLANK]);
      ERRORACTION(RESPONSE);
      END
    ELSE
      BEGIN;
      IF MERGE.ABR<>INFILE^.ABR THEN
        BEGIN;
        WRITELN(ENTRY^.NAME);
        WRITELN('ABSORBANCE RANGE INCOMPATIBLE.');
        PROMPTLINE(REPONSE,[ESC,BLANK,'I']);
        ERRORACTION(RESPONSE);
        END;
      IF MERGE.BEG<>INFILE^.BEG THEN
        BEGIN;
        WRITELN(ENTRY^.NAME);
        WRITELN('CURVE START POINT DIFFERENT.');
        PROMPTLINE(REPONSE,[ESC,BLANK,'I']);
        ERRORACTION(RESPONSE);
        END;
      IF MERGE.DTYP<>INFILE^.DTYP THEN
        BEGIN;
        WRITELN(ENTRY^.NAME);
        WRITELN('CURVE DATA TYPE DIFFERENT.');
        PROMPTLINE(REPONSE,[ESC,BLANK,'I']);
        ERRORACTION(RESPONSE);
        END;
      (* O.K. TO ADD FILE *)
      FOR I:=0 TO TOP DO
        ACCUMULATOR[I]:=ACCUMULATOR[I]+INFILE^.C[I];
      FILENUMBER:=SUCC(FILENUMBER);
      WRITELN(LISTDEVICE,FILE:',FILENUMBER:3);
      WRITELN(LISTDEVICE,'--------');
      PRINTFILEINFO(INFILE^);
      END
    ELSE
      ERRORACTION(RESPONSE);
    (*$I-*)CLOSE(INFILE);(*$I+*)
    END;          (* PROCESSFILE *)

BEGIN;        (* MERGEFILES *)
FOR I:=0 TO TOP DO     (* INIT. ARRAYS *)
  BEGIN;
  ACCUMULATOR[I]:=0;
  END;
FILENUMBER:=0;
(*$I-*)RESET(LISTDEVICE,'PRINTER:');(*$I+*)
IF IORESULT IN [0,12] THEN     (* PRINTER: ON LINE *)
  BEGIN;     (* PRINTER OPTION *)
  WRITE('LIST OF FILES TO PRINTER (Y/N)?');
  PROMPTLINE(RESPONSE,['Y','N']);
```

```
  IF RESPONSE='N' THEN
    BEGIN;
    CLOSE(LISTDEVICE);
    (*$I-*)RESET(LISTDEVICE,'CONSOLE:');(*$I+*)
    PRINTER:=FALSE;
    END
  ELSE
    BEGIN;
    PRINTER:=TRUE;
    WRITELN(LISTDEVICE,PRGTITLE);
    FOR I:=1 TO LENGTH(PRGTITLE) DO
      WRITE(LISTDEVICE,'-');
    WRITELN(LISTDEVICE);WRITELN(LISTDEVICE);
    END;
  END
ELSE
  BEGIN;
  (*$I-*)RESET(LISTDEVICE,'CONSOLE:');(*$I+*)
  PRINTER:=FALSE;
  END;
WRITELN;
ENTRY:=BASE;
WHILE ENTRY<>NIL DO
  BEGIN;     (* SCAN LIST *)
  FILENAME:=ENTRY^.NAME;
  IF POS(COLON,FILENAME)=0 THEN
    FILENAME:=CONCAT(DEFVOL,COLON,FILENAME);
  FILENAME:=CONCAT(FILENAME,PERIOD,DEFEXT);
  PROCESSFILE(FILENAME);
  ENTRY:=ENTRY^.NEXTFILE;
  END;       (* SCAN LIST *)
IF FILENUMBER=0 THEN
  BEGIN;
  WRITELN;WRITELN(BELL,'NO FILES MERGED.');
  EXIT(MERGEFILES);
  END;
IF FILENUMBER>1 THEN
  FOR I:=0 TO TOP DO
    MERGE.C[I]:=TRUNC(ACCUMULATOR[I] DIV FILENUMBER)
ELSE
  FOR I:=0 TO TOP DO
    MERGE.C[I]:=TRUNC(ACCUMULATOR[I]);
END;          (* MERGEFILES *)
```

Contents of Disk File #5:GELCHKFL.TEXT

```
PROCEDURE CHECKFILESPEC;

(* CHECKS THAT FILESPEC IS CORRECT SYNTAX FOR A GELSCAN
   DATAFILE, IF AN ERROR IS PRESENT ITS LOCATION AND NATURE
   ARE RETURNED IN ERRPOS AND ERRMES. IF NO ERROR THEN
   ERRPOS=0 AND ERRMES='' *)

CONST
  MAXVOLEN=7;    (* MAXIMUM VOLUME NAME LENGTH *)
  MAXNAMELEN=10;  (* MAXIMUM NAME LENGTH *)
```

```
VAR
  LETTERS,
  DIGITS,
  SPECIALCHARS,
  VALIDLETTERS:CHARACTERS;
  VOLUMESPEC,
  NAMESPEC:STRING;
  PLACE,COLONPOS:INTEGER;

  PROCEDURE SETERROR (POSITION:INTEGER;MESSAGE:STRING);

  BEGIN;     (* SETERROR *)
  ERRPOS:=POSITION;ERRMES:=MESSAGE;
  END;       (* SETERROR *)

  PROCEDURE CHECKVOLUME (VOLUME:STRING);

  VAR
    I,NUMBER:INTEGER;

  BEGIN;       (* CHECKVOLUME *)
  IF VOLUME[1]=HASH THEN
    BEGIN;  (* CHECK VOLUME NUMBER *)
    IF LENGTH(VOLUME)=1 THEN
      SETERROR(2,'VOLUME NUMBER EXPECTED.')
    ELSE
      BEGIN
      VALIDLETTERS:=DIGITS;I:=1;
      REPEAT
      I:=SUCC(I);
      UNTIL NOT(VOLUME[I] IN VALIDLETTERS) OR
                          (I=LENGTH(VOLUME));
      IF NOT(VOLUME[I] IN VALIDLETTERS) THEN
        SETERROR(I,'ILLEGAL CHARACTER.')
      ELSE
        BEGIN
        NUMBER:=STRTOINT(COPY(VOLUME,2,LENGTH(VOLUME)-1));
        IF NOT(NUMBER IN [4,5,9..12] THEN
          SETERROR(2,'INVALID VOLUME NUMBER.');
        END
      END
    END   (* CHECK VOLUME NUMBER *)
  ELSE
    BEGIN   (* CHECK VOLUME NAME *)
    VALIDLETTERS:=LETTERS+DIGITS;
    I:=0;
    REPEAT
    I:=SUCC(I);
    UNTIL NOT(VOLUME[I] IN VALIDLETTERS) OR
(I=LENGTH(VOLUME));
    IF NOT(VOLUME[I] IN VALIDLETTERS) THEN
      SETERROR(I,'ILLEGAL CHARACTER.')
    ELSE
      IF I>MAXVOLEN THEN
        SETERROR(MAXVOLEN+1,'VOLUME NAME TOO LONG.');
    END;
  END;       (* CHECK VOLUME *)
```

```
  PROCEDURE CHKNAME(NAME:STRING);

  VAR
    I:INTEGER;

  BEGIN;       (* CHKNAME *)
  VALIDLETTERS:=LETTERS+DIGITS+SPECIALCHARS;
  I:=0;
  REPEAT;I:=SUCC(I);
  UNTIL NOT(NAME[I] IN VALIDLETTERS) OR (I=LENGTH(NAME));
  IF NOT(NAME[I] IN VALIDLETTERS) THEN
    SETERROR(PLACE+I,'ILLEGAL CHARACTER.')
  ELSE
    IF I>MAXNAMELEN THEN
      SETERROR(PLACE+MAXNAMELEN+1,'FILENAME TOO LONG.');
  END;         (* CHKNAME *)

BEGIN;     (* CHECKFILESPEC *)
(* INITIALIZE *)
LETTERS:=['A'..'Z'];DIGITS:=['0'..'9'];
SPECIALCHARS:=['&','/','-','(',')','+'];
ERRPOS:=0;ERRMES:='';PLACE:=0;
COLONPOS:=POS(COLON,FILESPEC);
IF COLONPOS<>0 THEN
  BEGIN;
  PLACE:=COLONPOS;
  IF COLONPOS=1 THEN
    SETERROR(1,'VOLUME SPECIFICATION EXPECTED.');
  VOLUMESPEC:=COPY(FILESPEC,1,COLONPOS-1);
  CHECKVOLUME(VOLUMESPEC);
  END;
(* CHECK NAME *)
IF COLONPOS=LENGTH(FILESPEC) THEN
  SETERROR(COLONPOS+1,'FILENAME EXPECTED.');
NAMESPEC:=COPY(FILESPEC,COLONPOS+1,LENGTH(FILESPEC)-COLONPOS);
CHKNAME(NAMESPEC);
END;       (* CHECKFILESPEC *)
```

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Regulation of Insulin Gene Expression in Rat Islets of Langerhans

PhD. Thesis

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Abstract

The rate of glucose stimulated in vitro (pro)insulin synthesis in islets of Langerhans isolated from fed male rats was determined. Increases in the glucose concentration of the incubation medium, over the physiological range (2 to 20 mM), stimulated the rates of both (pro)insulin and total protein synthesis during a two hour incubation. This stimulation was preferential for (pro)insulin synthesis and had a sigmoidal dose response curve with a concentration threshold of between 2.5 mM and 5.0 mM and a maximal rate at 20 mM glucose. Inhibition of islet RNA synthesis by actinomycin D depressed the rate of total protein synthesis within 30 minutes of the application of a 20 mM glucose stimulus. A specific inhibition of (pro)insulin synthesis by actinomycin D, that occured 60 minutes after the application of a 20 mM glucose stimulus, was thought to reflect the inhibition of glucose stimulated preproinsulin mRNA synthesis.

The effect of glucose on islet preproinsulin mRNA content during in vitro incubations was also determined. To achieve this a dot blot hybridization assay for preproinsulin mRNA was developed which used a ^{32}P-labeled cloned human insulin gene as the hybridization probe. The assay proved to be sensitive enough to detect preproinsulin mRNA in as few as fifty islets. Incubation of islets, for 2 hours, at 2.5 mM glucose had no effect on islet preproinsulin mRNA content but incubation at 20 mM glucose increased islet preproinsulin mRNA content. Actinomycin D had no effect on this pattern of glucose stimulation and it is suggested that the degradation of preproinsulin mRNA is in some way dependent on RNA synthesis and that its inhibition by glucose plays an important role in the regulation of islet preproinsulin mRNA content.