STUDIES ON THE MODE OF ACTION OF CYCLIC AMP IN REGULATING THE RELEASE OF INSULIN FROM ISLETS OF LANGERHANS

A Thesis presented

by

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ABBREVIATIONS

AMP	: adenosine 5' - phosphate
ATP	: adenosine 5'- triphosphate
Cyclic AMP	: adenosine 3':5' cyclic phosphate
DEAE-cellulose	: diethylaminoethyl cellulose
DTT	: dithiothreitol
EDTA	: diaminoethanetetra-acetic acid
EGTA	: ethyleneglycol-bis-(-amino ethyl ether) N,N'-tetra acetic acid
Hepes	: N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IBMX	: isobutylmethylxanthine
NAD+	: nicotinamide-adenine dinucleotide
NADP	: nicotinamide-adenine dinucleotide phosphate
P_i	: orthophosphate
PGE	: $prostaglandin_E$
PMSF	: phenylmethane sulphonyl fluoride
RNA	: ribonucleic acid
TEMED	: N,N,N',N' - tetramethylethylenediamine
Tris	: 2-amino-2-hydroxymethyl propane-1,3-diol.

CHAPTER I

General Introduction

Brief Historical Survey

The presence of a cell form, not previously described within the pancreas gland, was noted by Paul Langerhans in 1869. He described these cell forms as being small irregular polygonal structures whose cytoplasm was brillant and free of any granules, their nuclei were distinct, round and of moderate size. They lay together, generally in considerable numbers, diffusely scattered in the parenchyma of the gland. In 1882, Kuhne and Lea demonstrated that these cells had a very rich glomerular blood supply while Doigel in 1893 showed that there was no connection between the cells and the duct system of the gland. In 1893 Laguesse found numerous groups of cells in human pancreas which he identified with those described by Langerhans, and named them islets of Langerhans. He suggested that the islets of Langerhans were the site of an internal secretion.

The connection between the islets of Langerhans and diabetes was demonstrated in 1893 by Hédon, who was able to prevent the occurrence of diabetes by grafting pancreatic tissue under the skin of depancreatised animals. Early attempts to extract the active hypoglycaemic factor from the pancreas were hampered by the presence of proteolytic enzymes. It was not until 1922 that Banting and Best using conditions of acid pH and low temperature were able to extract 'insulin' (the term proposed in 1909 by Jean de Mayer for the hypoglycaemic factor), which on injection into depancreatized dogs produced a dramatic lowering of the blood sugar levels, and a reduction of glycosuria. Further purification

enabled them to produce an insulin suitable for clinical use, but still relatively crude compared to the insulins at present in use.

Cellular Composition of Islets of Langerhans

Islets of Langerhans are composed of four cell types; the A and B cells which secrete glucagon and insulin respectively, a cell type which produces somatostatin (Luft et al., 1974; Dubois, 1975) and another cell type which produces pancreatic polypeptide (PP) (Lin and Chance, 1974; Larsson et al., 1975). The B cells are the most abundant cell type (78%) (Carpenter, 1966) while the A cells consist of 15-30% of the islet cell. It was assumed that the four cell types were present throughout all the pancreatic islets of most mammalian species. However, recent studies have shown the pancreas contains two distinct islet populations, differing each other by their cellular contents and topographical location (Orci et al., 1976). Using the technique of indirect immunofluoresence, it has been found, in islets of most species, that the B cells are arranged into a central mass surrounded by a rim of non B cells, i.e., glucagon, somatostatin and PP-containing cells. In the duodenal portion (head) of the pancreas, the non B cell rim is composed of a majority (20%) of PP-cells with only scarce glucagon cells (1.6%) and moderate numbers (5%) of somatostatin cells. In the splenic portion of the pancreas the non B cell rim is on the contrary rich in glucagon-containing cells (28%) with sparse PP-cells (2%) and moderate numbers of somatostatin cells (4%).

Diabetes Mellitus

After the discovery of insulin by Banting and Best (1922) it was believed that diabetes mellitus was a simple endocrine deficiency syndrome akin to Addison's Disease or myxoedma, which could be treated and controlled by insulin alone. However, this conception was soon dispelled with the discovery of other hormones such as Growth Hormone and steroids could worsen diabetes or even induce it.

During the past decade, concepts of diabetes mellitus have been in a state of evolution as new biologic characteristics of the disease have come to light. Most workers in the field now consider diabetes to be not one, but several diseases, possibly with differing causes and mechanisms of transmission. Thus the clinical entity diabetes appears to be an example of genetic heterogenity with carbohydrate intolerance as the cardinal feature.

Diabetes can be classified, by the age at which the disease was diagnosed, into two categories; Juvenile-onset and Maturity-onset. The former category are insulin dependent and generally have no or very few islets of Langerhans present in their pancreas. While in the latter category, the majority can control their disease on diet alone and have what appear to be normal islets of Langerhans which have impaired function for some unknown reason. The concept of a juvenile-onset and a maturity-onset form of the disease as a rigid categorisation is now under attack, for it is now increasingly apparent that age is a poor criterion upon which to classify diabetes. Clinical studies strongly suggest that the two major forms might reasonably be categorised as insulin-dependent and non-insulin-dependent diabetes.

The pathogenesis of diabetes mellitus is not yet fully understood (for review see Craighead, 1978). Diabetes is recognised clinically to represent several similar conditions having differing causes and pathogenetic mechanisms. It is apparent that heritable factors play a part in some cases, but it is uncertain whether or not genetic influences are invariably critical. Research evidence points to the role of external environment insults such as chemicals and viruses and constitutional factors such as nutrition or metabolic and psychic stresses, in the initiation and extent of B-cell injury of the disease, possibly in a genetically predisposed subject.

The Biochemical Mechanism of Insulin Secretion

An understanding of the biochemical mechanism of insulin secretion in the normal healthy islet of Langerhans is required (for reviews see Gerich et al., 1976; Lambert, 1976). In order to answer the questions, why do apparently healthy islets begin to malfunction and why does the disease occur in various degrees of severity?

In discussing insulin secretion one must first define what is meant by secretion. Secretion involves the movement of the secretory granule to the plasma membrane, this is called margination (see Diagram 1). This is followed by the fusion of the granule with the plasma membrane and the resulting release of insulin from the granule, this process is called exocytosis.

The cellular requirements for insulin secretion to occur are; adequate intracellular ATP and cyclic AMP levels, presence of extracellular Ca^{2+} ions, an intact microtubule system and a stimulus.

a) Glucose

Glucose is the primary physiological stimulus for insulin secretion. It is a primary stimulus because it can elicit a response on its own. A secondary stimulus such as Glucagon, the gut hormones or Prostaglandins can only stimulate secretion in the presence of glucose. Glucose-induced insulin secretion has a characteristic dose response curve (Diagram 2) which is modified in the presence of a secondary stimulator. The sigmoid curve indicates that glucose is having a co-operative effect on secretion, though the explanation of this in unclear. Glucose-induced insulin secretion also has a characteristic time course, which is biphasic (Curry et al.,

Diagram 1. Diagramatic representation of Insulin Secretion.

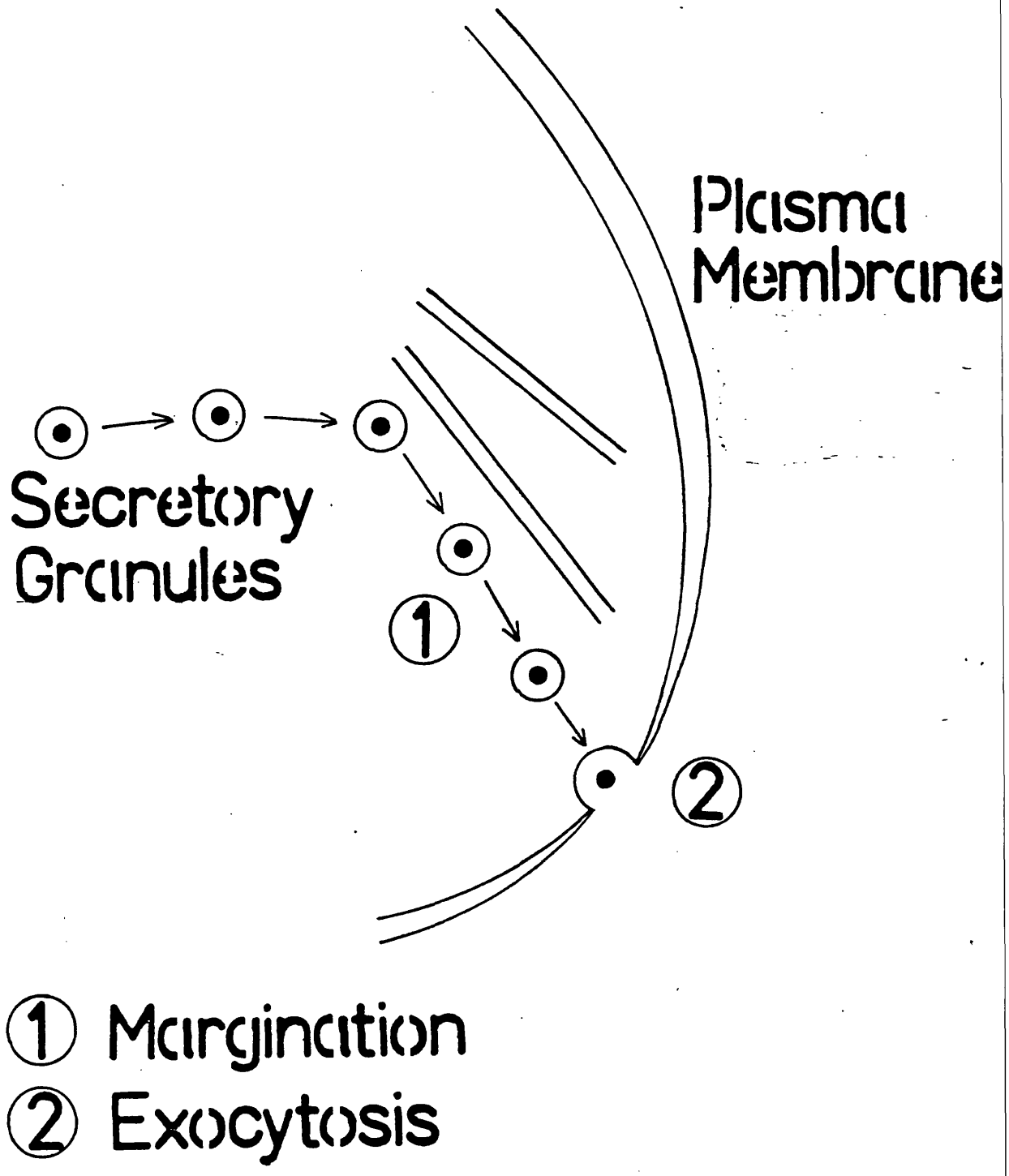
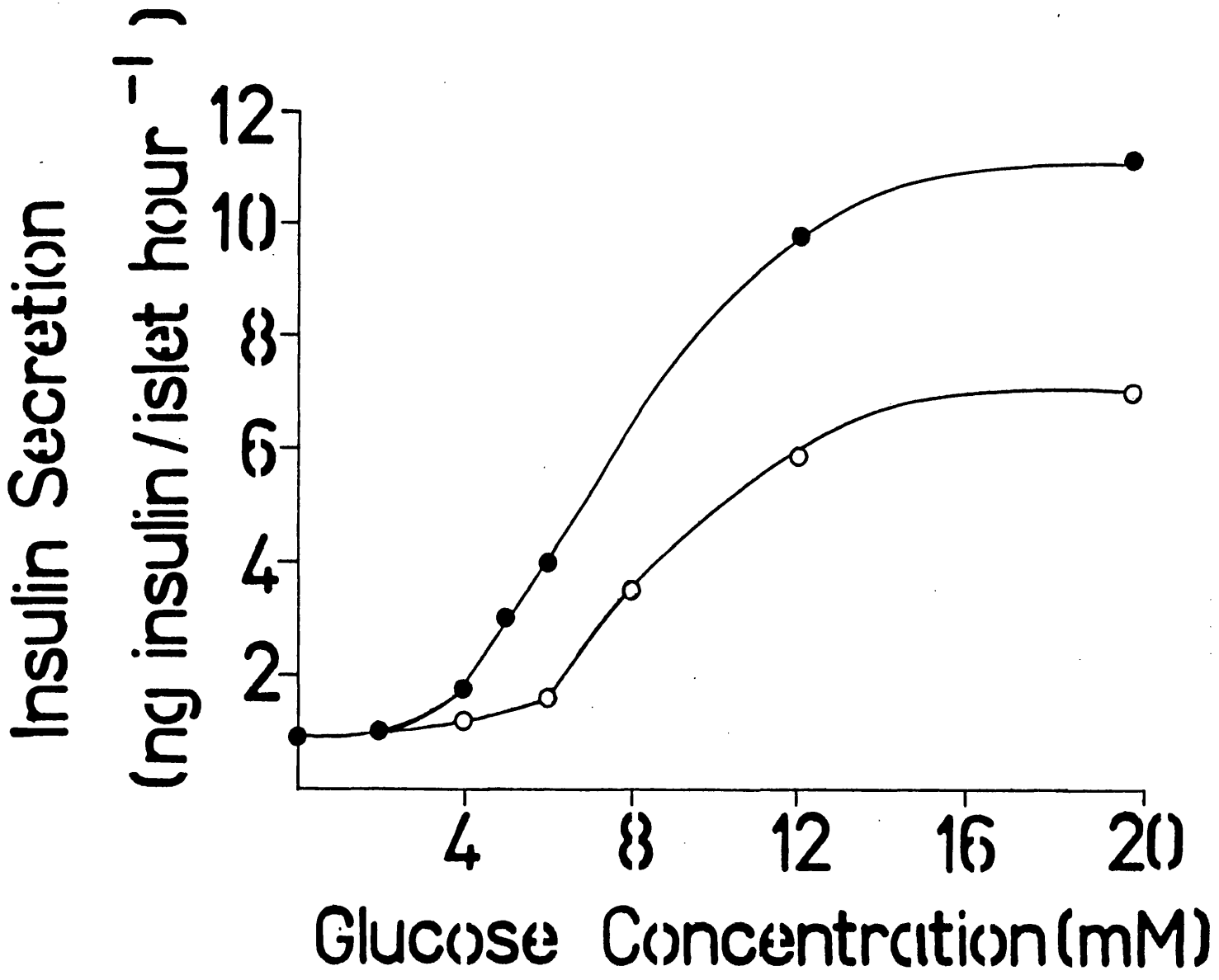


Diagram 2. Dose response curve for glucose-induced (O) and glucose plus a secondary stimulator -induced (●) insulin secretion. Taken from 'Cyclic 3',5'-nucleotides: Mechanism of Action'. Chapter 8. W. Montague (1977) p 134. (Eds. Cramer, H. and Schultz, J.) John Wiley and Sons.

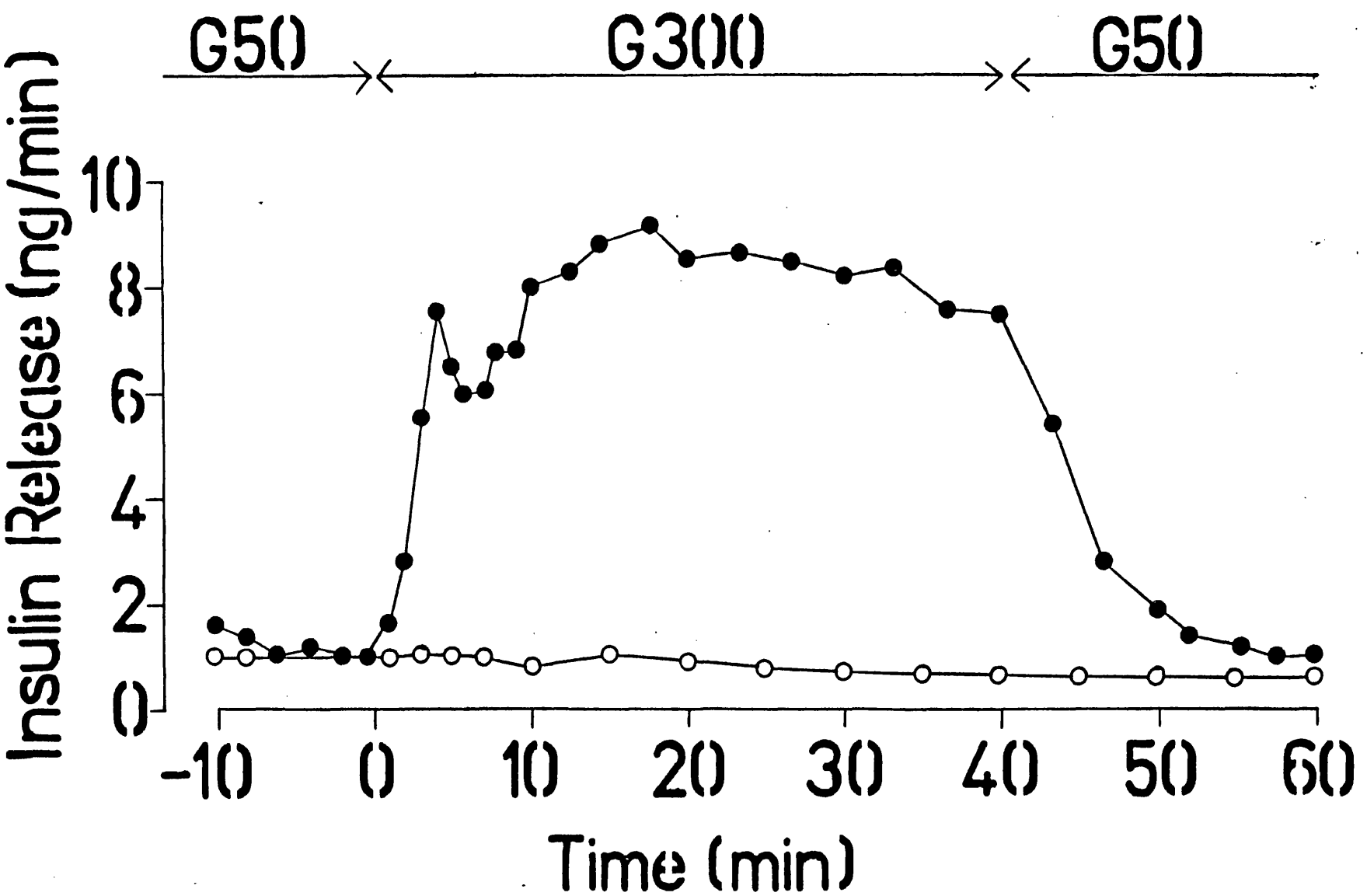

Insulin Secretion
(ng insulin/islet hour⁻¹)
12
10
8
6
4
2
4
8
12
16
20
Glucose Concentration (mM)

1968) (Diagram 3). There are several theories for the biphasic release of insulin, it may be due to a small labile pool of insulin-containing granules distinct from the majority of granules in that they are more susceptible to release, perhaps by their sensitivity to fusion with the plasma membranes or by close apposition to structures critical to the release process. (Lacy et al., 1968; Grodsky, 1972). Other possible explanations are; individual B cells may have differential sensitivities to glucose (Matthews and Dean, 1970); an initial burst of insulin release causing a transient feedback inhibition of subsequent release (Iversen and Miles, 1971; Pace et al., 1977); a concomitant release of somatostatin (Orci and Unger, 1975); a biphasic change in the concentration of an intracellular mediator of release (Randle and Hales, 1972); or a biphasic change in the concentration of cytosol Ca^{2+} ions. (Wollheim et al., 1978).

The mode of action of glucose on the B cell is still unclear. It is thought that either glucose itself interacts with a receptor (Regulator site mechanism) or a glucose metabolite interacts with the receptor (Substrate site mechanism, (Ashcroft et al., 1972)). Recent evidence suggests the latter may occur and phosphoenolpyruvate may be a possible intracellular trigger of insulin release (Sugden and Ashcroft, 1977).

The cellular position of the glucose receptor and how the glucose signal is translated into a response is unknown. The traditional second messengers, Ca^{2+} ions and cyclic AMP (for review see Rasmussen et al., 1972) have both been identified as playing a role in insulin secretion (Malaisse et al., 1967; Malaisse et al., 1973).

Diagram 3. Time course of insulin secretion by perifused rat islets in continuous presence of 50mg glucose/100ml (G50 O) and in response to stimulation with 300mg glucose/100ml (G300 ●) applied between min 0 and 40. (From Lambert, A.E., Henquin, J.C. and Malvaux, P. (1974) Endocrinology 95 1069-1077.)


G50
G300
G50
Insulin Release (ng/min)
0
2
4
6
8
10
-10
0
10
20
30
40
50
60
Time (min)

b) Calcium

It is well established that extracellular Ca^{2+} ions are an absolute requirment for insulin secretion (Grodsky and Bennet, 1966). Studies on Ca^{2+} fluxes in isolated islets of Langerhans have demonstrated that there is a striking parallelism between modifications in Ca^{2+} uptake by islet B cells and stimulation (Dean and Matthews, 1970). It is now thought that increased intracellular Ca^{2+} levels trigger the release mechanism (Malaisse et al., 1973). There are a number of theories of how this raised level of intracellular Ca^{2+} ions may be obtained in the B cell.

One of the theories is that it may be due to increased permeability of the plasma membrane to extracellular Ca^{2+} ions. The intracellular level of Ca^{2+} ions is much lower than the extracellular level. This concentration gradient is maintained by a Ca^{2+} ATPase. There is also an electrochemical gradient across the B cell extracellular plasma membrane, as the cytosolic side of the plasma membrane is negatively charged. It has been shown that a rapid depolarisation of the plasma membrane is observed when the B cell is stimulated by glucose, suggesting glucose inhibition of the Ca^{2+} ATPase activity.

Another theory is that raised intracellular levels of Ca^{2+} ions may be due to release from intracellular pools, such as mitochondria and secretory granules, which have a relatively high content of Ca^{2+} ions (Howell et al., 1975). Studies on mitochondrial fluxes of Ca^{2+} ions appear to indicate that this may be the case (Howell and Montague, 1975). The presence of an inactive pool of Ca^{2+}, such as bound calcium, should not be ignored, as its conversion to free active calcium would lead to an increase in intracellular

levels of Ca^{2+} ions.

There are several theories on how raised intracellular levels of Ca^{2+} ions could initiate the processes of margination and exocytosis.

One possibility is the presence of Ca^{2+}-activated contractile proteins in the islet B cell, similar to the actin/myosin proteins found in muscle. The process of margination involves the vectorial transport of the secretory granule to the plasma membrane. An increase in intracellular levels of Ca^{2+} ions would cause the contraction of such proteins thereby providing the means by which the secretory granules could marginate.

The second possibility is that the raised levels of intracellular Ca^{2+} ions could promote microtubule formation (Montague and Howell, 1976), by catalysing the polymerisation of tubulin. Alternatively Ca^{2+} ions may play a role in regulating the interaction between secretory granules and microtubules, an interaction which would be necessary to ensure the vectorial transport of granules to the plasma membrane of the B cell.

The third possibility is that Ca^{2+} ions may play a role in the fusion of the granule with the plasma membrane. This could be brought about by either the neutralisation of the electrical potential barrier which exists between the secretory granule and the plasma membrane or by calcium stimulation of Phospholipase C activity (which converts plasma membrane phospholipids to diacylglycerides and glycerol) thereby making the plasma membrane more fusagenic.

The identification of a calcium binding protein, Calmodulin (for reviews see Klee et al., 1980; Means and Dedman,

1980), which activates cyclic nucleotide phosphodiesterase (Cheung, 1970) has suggested the possibility that Ca^{2+} ions may regulate cellular mechanisms by altering intracellular nucleotide concentrations. Calmodulin has been identified in rat islets (Sugden et al., 1979a) and Syrian hamster insulinoma cells (Schubart et al., 1980). However, as cyclic AMP acts as a potentiator rather than a direct trigger for glucose-induced insulin release, it is thought likely that an alternative or additional role of calmodulin regulation of intracellular cyclic nucleotide levels occurs in islets. Such roles as the regulation of microtubule assembly (Marcum et al., 1978) and Ca^{2+} ions transport (Katz and Remtulla, 1978) have been proposed (Sugden et al., 1979a). Finally it has been demonstrated that calmodulin mediates Ca^{2+}-dependent protein phosphorylation in membranes from various tissues. (Schulman and Greengard, 1978).

c) Cyclic AMP

Cyclic AMP has been demonstrated to be the second messenger for the secondary stimulators of insulin secretion such as Glucagon, the gut hormones and the Prostaglandins (Montague and Howell, 1973). These secondary stimulators raise intracellular levels of cyclic AMP by activating adenyl cyclase. Adrenaline has been shown to inhibit insulin secretion by inhibiting adenyl cyclase.

The role of cyclic AMP can be summarised in the following way:-

1) SHORT TERM: mediating the action of hormones in modulating insulin secretion.

2) LONG TERM: mediating the action of hormones during pregnancy where there are maintained high levels

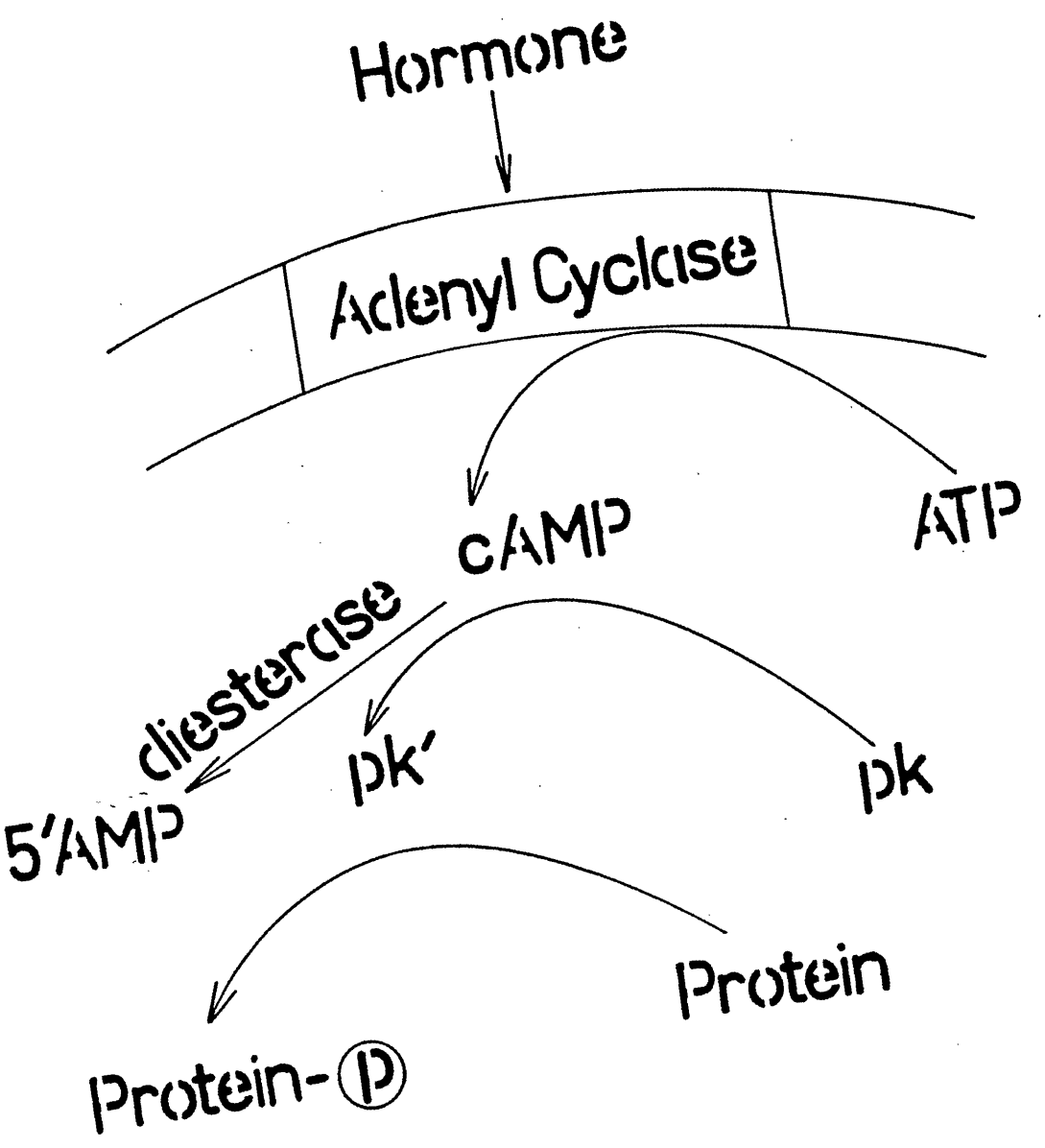
of hormones. In pregnancy there is an increased sensitivity to glucose which has been shown to be due to an increased activity in adenyl cyclase.

3) LONG TERM: starvation and excessive carbohydrate intake. In starvation there is a decreased sensitivity to glucose.

How does cyclic AMP act? Diagram 4 outlines the classic cyclic AMP 'pathway' which has been found to be true in all of the cases where cyclic AMP is the second messenger. Adenyl cyclase, phosphodiesterase and cyclic AMP stimulated protein kinase activities have all been identified in the islets of Langerhans (Montague and Howell, 1972; Sams and Montague 1972). The substrate of the cyclic AMP-dependent protein kinase has not yet been identified, and this is the aim of this study. The identification of this substrate would be a big step in elucidating the biochemical mechanism of the modulators of insulin secretion and maybe throw some light on the action of glucose on the B cell.

There are several postulates as to the nature of the substrate of the cyclic AMP-dependent protein kinase. One idea is that there may be a cyclic AMP mediated mitochondrial permeability change caused by phosphorylation of a membrane protein and leading to the efflux of Ca^{2+} ions from the mitochondria thus raising cy tosolic Ca^{2+} ion levels, triggering the release mechanism. It is important to note that several cyclic AMP mediated permeability changes have been observed in other tissues, e.g., the action of ADH (Anti-diuretic Hormone) on the toad bladder has been demonstrated to be mediated by cyclic AMP and increased phos-

Diagram 4. The mode of action of cyclic AMP.



phorylation of a membrane protein has been correlated with increased fluxes of Na^+ ions across the membrane, the transport of water molecules following that of Na^+ ions. (DeLorenzo et al., 1973).

Synaptic transmission is also mediated by cyclic AMP and phosphorylation of a membrane protein has also been observed, but unfortunately correlations between this phosphorylation and the magnitude of the action potential is difficult to prove due to the time course of the action potential being of the order of a few milliseconds.

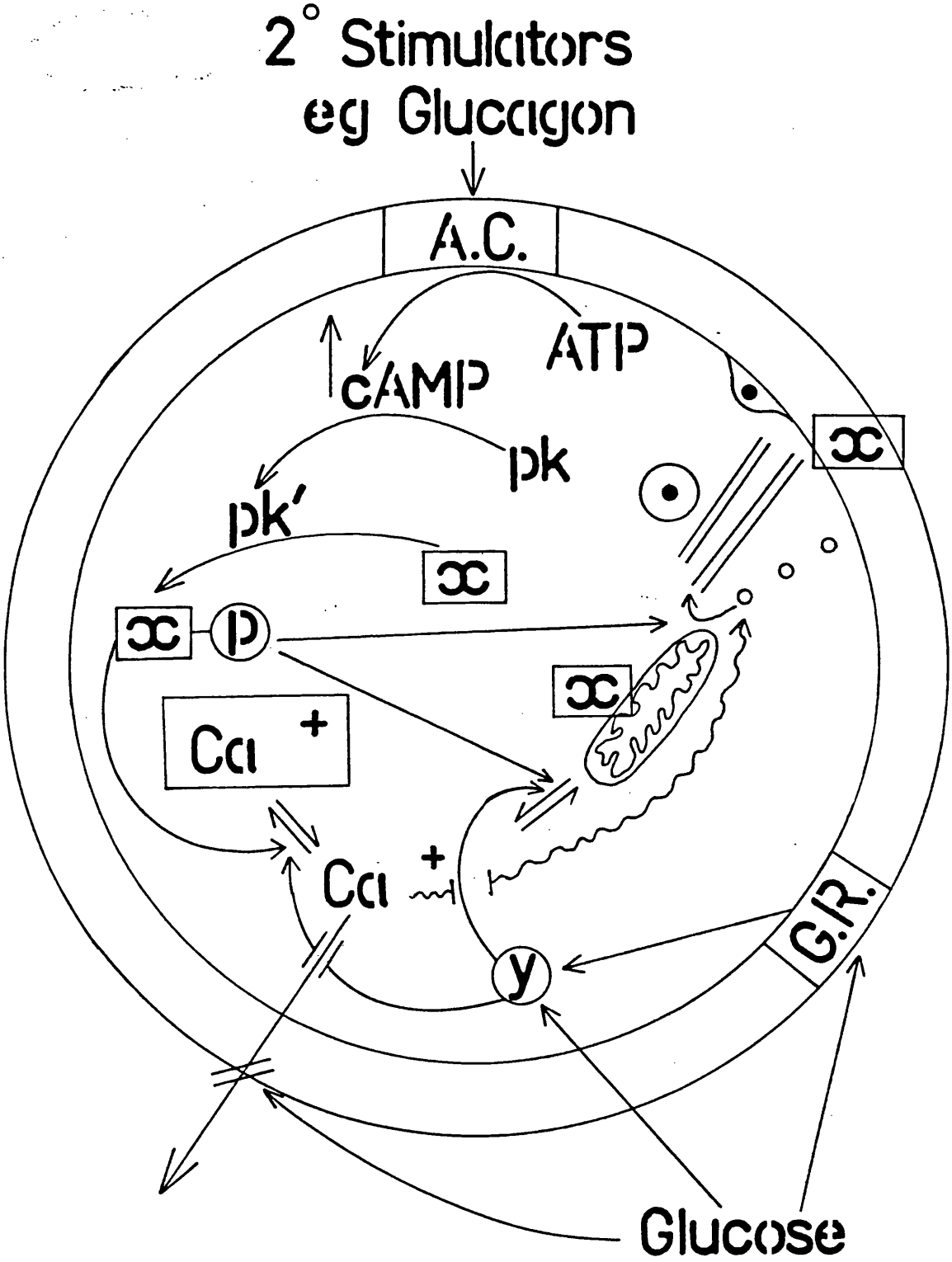
A closer analogy with the islet system is that of cardiac relaxation in which cyclic AMP mediated phosphorylation has been correlated with increased transmembrane movement of Ca^{2+} ions and stimulated Ca^{2+} ATPase activity. (Katz et al., 1975). This substrate of the cyclic AMP dependent protein kinase has now been tentatively named as Phospholambian by Katz.

Other roles for cyclic AMP in insulin secretion have been suggested and these should not be neglected. One such role is the cyclic AMP mediated phosphorylation of a microtubule protein or a protein closely associated with the microtubules. Evidence seems to suggest that phosphorylation promotes the polymerisation of tubulin and microtubular protein phosphorylation has been demonstrated in brain. (Sheterline, 1977).

Another role for cyclic AMP could be to increase phosphorylation of proteins in the secretory granule and/or plasma membrane. This perhaps could control the rate of membrane fusion in exocytosis.

Diagram 5 summarises some of these events. The

Diagram 5. A schematic representation of the Biochemical Mechanism of Insulin Secretion. 'x' represents a protein whose phosphorylation is mediated by cyclic AMP. 'y' represents the glucose intracellular receptor or 'trigger' of glucose-induced insulin secretion.


2° Stimulators
eg Glucagon
A.C.
ATP
cAMP
pk
pk'
Ca +
Ca +
G.R.
Glucose

organelles of protein synthesis have been ommitted as secretion involves the release of insulin already present in the secretory graules of the cytosol, not with de novo synthesis.

As has been mentioned this study's main objective was originally to identify the substrate of cyclic AMP-dependent protein kinase. But first of all it was decided to investigate the cyclic AMP-dependent protein kinase itself, the reasons for this being twofold.

Firstly, although cyclic AMP-dependent protein kinase was identified in islets of Langerhans in the early 70's by several workers. (Montague & Howell, 1972; Dods and Burdowski, 1973; Müller and Sharp, 1974). There have been several reports of cyclic AMP-dependent kinase isozymes in several tissues. (Corbin and Keeley, 1977; Uno et al., 1976; Knight and Skala, 1977). Secondly, preliminary work on substrate phosphorylation in the islets of Langerhans (Montague and Howell, 1976) has shown that several proteins are phosphorylated under conditions which raise intracellular cyclic AMP levels and difficulties have been encountered in identifying them individually. The explanation for this last observation could be:-

a) There are several substrates for one cyclic AMP-dependent protein kinase, this would not be an unusual situation as in assaying for these kinases a variety of substrates can be used, e.g., histone (the most common one used), protamine and casein. Though the majority of cyclic AMP-dependent kinases do have a higher affinity for histone than for the others.

b) There are cyclic AMP-dependent protein kinase

isozymes present in the islets of Langerhans.

Cyclic AMP-dependent protein kinase isozymes Type I and Type II have been recently identified in rat islets of Langerhans (Sugden et al., 1979; Oliver and Kemp, 1980) although the subcellular distribution of the different molecular forms was not determined. The first aim of this study was to investigate the subcellular distribution of cyclic AMP-dependent protein kinase isozymes in guinea-pig islets of Langerhans.

Cyclic AMP-dependent Protein Kinase Isozymes

Cyclic AMP-dependent protein kinases have been shown to consist of two regulatory subunits and two catalytic subunits. Cyclic AMP activates the protein kinase through the formation of a regulatory subunit(R)-cyclic AMP-catalytic subunit(C) complex. (Tsuzuki and Kiger, 1978; Builder et al., 1979), as shown in Equation 1.

$$R_2C_2 + 4cAMP \rightleftharpoons R_2 \cdot 4cAMP \cdot C_2 \rightleftharpoons R_2 \cdot 4cAMP + 2C \qquad (1)$$

Although the subsequent dissociation of the ternary complex produces active catalytic subunit, it has not yet been proven whether the ternary complex is active and, in consequence, if dissociation is absolutely necessary. Table 1.1 summarises the differences in properties of the cyclic AMP-dependent protein kinase Type I and Type II isozymes. The first major difference, the concentration of salt at which the isozyme is eluted from DEAE-cellulose chromatography columns, is exploited in separating the isozymes. The second property, the relative amounts in particular fractions is a generalisation as there are quite a few exceptions, such as bovine liver (Uno et al., 1976), due to the broad species and tissue specificity of the isozyme types. The third property, the dissociation rate in the presence of histone (0.75 mg/ml)

TABLE 1.1 - Differences in Properties of Cyclic AMP-dependent Kinase Isozymes I and II

Taken from Corbin, J. D. and Keely, S. L. (1977) J. Biol. Chem., 252, p914.

Property	Isozyme	
	Type I	Type II
DEAE-cellulose elution	<0.1M NaCl	>0.1M NaCl
Relative amounts in particulate fractions	Low	High
Rate of dissociation into subunits by Histone (0.75 mg/ml) or high salt (0.5M)	Rapid	Slow
Rate of reassociation of subunits in dilute (10mM) buffer	Slow	Rapid
Rate of autophosphorylation	Slow or nil	Rapid
Approximate molecular weight	151,000	183,000
ATP binding affinity	High	Low

and NaCl (0.5M) is the main criteria by which the Type I and Type II isozymes are distinguished. As one would expect the re-association rate of the two isozymes is the inverse of their dissociation rate. The differences in the rate of auto-phosphorylation and ATP binding affinity of the two isozymes is discussed in detail in Chapter VI (General Discussion). The catalytic subunits of the isozymes are very nearly identical (Hofmann et al., 1975), hence the difference in properties of the Type I and Type II isozymes is a reflection of a difference in regulatory subunits.

CHAPTER II

Methods of Study

Introduction

This chapter contains a detailed description of the methods used in this thesis. It has been divided into two main sections. The first deals with the methods used to characterise islet cyclic AMP-dependent protein kinases. The second contains the procedures used to measure islet cell endogenous protein phosphorylation and identif y the substrates of islet cyclic AMP-dependent protein kinases.

Animals

The Guinea-Pigsused in the isolation of islets of Langerhans were all of a Duncan-Hartley strain bought from the School of Agriculture, University of Nottingham, Sutton Bodington. All animals were housed in the University of Leicester, Biological Sciences Animal House until used. They were fed ad lib and weighed 800-1000 g at slaughter.

Reagents

Carrier free ^{32}P and (^{3}H)-cyclic AMP were purchased from the Radiochemical Centre, Amersham,Bucks.

Collagenase, N-2-hydroxyethylpiperazine-N^{1}-2-ethanesulphonic acid (Hepes), Sodium β-glycerophosphate, Tris base, Ethyleneglycol-bis-(-amino ethyl ether) N, N^{1}-tetra acetic acid (EGTA), Sodium fluoride, Theophylline, Activated charcoal, Histone, Protamine sulphate, Casein hydrolysate, Dithiothreitol (DTT), Phenylmethanesulphonyl fluoride (PMSF), Dowex^{R}-1 Cl^{-} (1 x 8-200), Polyoxyethylene 20 cetyl ether (Brij 58) and Rabbit Muscle Type I and Type II cyclic AMP-dependent protein kinases were all purchased from Sigma

(London) Co. Ltd., Kingston-upon-Thames, Surrey. Isobutyl-1-methylxanthine (IBMX) and Benzamidine hydrochloride hydrate were purchased from Aldrich Chemical Co. Ltd., Gillingham, Dorset.

Collagenase, 3-phosphoglycerate kinase, Glycerophosphate dehydrogenase, Triosephosphate isomerase, Glyceraldehyde-3-phosphate dehydrogenase, Lactate dehydrogenase, pyruvate, adenosine diphosphate (ADP), adenosine triphosphate (ATP), Nicotinamide-adenine dinucleotide (NAD) and molecular weight calibration proteins:- Ribonucleic acid (RNA) polymerase, Bovine serum albumin, Ovalbumin and Chymotrypsinogen were purchased from Boehringer Co. (U.K.) Ltd., Lewes, Sussex.

Acrylamide, Bis-Acrylamide, Ammonium persulphate and N, N, N^1, N^1-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad, Watford, Herts.

Coomassie Blue R-250, Nonidet-P40 and Triton X-100 were purchased from B.D.H. Ltd., Poole, Dorset.

Diethylaminoethyl (DEAE)-cellulose, Phosphocellulose (P-81) paper and glass fibre membranes GF/C (2.1 cm diameter) were purchased from Whatman, Maidstone, Kent.

Sartorius cellulose nitrate membrane filters (0.45 μm pore size) were purchased from V. A. Howe and Co. Ltd., London, SW6.

All inorganic salts used in buffers and incubation media, and all other reagents, were of analar grade or of the purest available commercially.

Methods Used in the Characterisation of Islet Cyclic AMP-dependent Protein Kinases

Introduction

In these studies guinea-pig islets of Langerhans were used, as the yield of islets obtained from guinea-pigs using the collagenase digestion method was higher (300-800) than that obtained from rats (100-300).

Isolation of Islets of Langerhans

Principle

Islets of Langerhans were isolated by collagenase digestion. In this method pancreatic acinar tissue which surrounded the islets of Langerhans was preferentially broken down by collagenase, thereby releasing islets of Langerhans free of acinar tissue.

With the introduction of this method it was first thought that the use of collagenase for isolating islets of Langerhans might alter the structural intergrity of the islet cells and thus make them unsuitable for the study of insulin release. Ultrastructural and secretion studies have demonstrated that the collagenase isolated islets have a normal ultrastructure with no evidence of cellular disruption (Lacy and Kostianovsky, 1967; Lacy et al., 1968). They behave similarly to islets in other pancreatic preparations and the secretory responses observed are very similar to those obtained *in vivo* with microdissected islets.

Incubation Medium

The incubation medium used in the isolation of islets of Langerhans was a bicarbonate buffered salt solution (Gey and Gey, 1936), containing 4 mM Glusose and brought to pH 7.4 by

gassing with O_2: CO_2 (95:5) at 37°C immediately before use.

Procedure

Islets of Langerhans were prepared by the collagenase digestion of guinea-pig pancreatic tissue (Howell and Taylor, 1968). For each preparation one guinea-pig was killed by cervical dislocature. The splenic portion of the pancreas was rapidly removed and transferred to a 250 ml conical flask containing prewarmed (37°C) incubation medium. The pancreas was treated in the following way: using a syringe fitted with a fine needle (25 G, 5/8 gauge) 15 ml of medium was injected subperitoneally into the tissue. This resulted in gross distention of the organ, enabling blood vessels and excess fat to be dissected away. The distended pancreas was then transferred to a beaker and cut into small pieces with fine scissors. The chopped pancreas was poured into a 10 ml disposable centrifuge tube, more medium added and centrifuged (setting 8, 1 second) in a MSE Bench centrifuge. This allowed the separation of small pieces of adipose tissue to float to the surface, leaving the pancreatic tissue which was pelleted at the bottom of the tube. The supernatant was discarded and the pellet of pancreatic tissue was transferred to a 25 ml conical flask containing 12 mg Collagenase (Boehringer), 2 mg Collagenase (Sigma) and sufficient medium to give a ratio 1:1 of tissue:medium. The flask was then shaken at 37°C using a Griffin and George flask shaker (set at 5/8 full speed). The digestion was monitored by taking aliquots at various time intervals and transferring them to a black bottomed petri dish for examination under a disecting microscope (15 times magnification). When free islets (white opaque spheres) were observed (approximately 10 min), the

digestion was terminated by transferring the contents of the flask to a 10 ml disposable centrifuge tube and adding excess medium. The tube was centrifuged (MSE Bench centrifuge, setting 8 for 1 second), the supernatant discarded and the pellet resuspended in 10 ml of medium. An aliquot of the resultant suspension was transferred to a black bottomed petri dish and diluted with excess medium for examination under a dissecting microscope. Islets were visualised, collected in finely drawn Pasteur pipettes and transferred to a 10 ml disposable centrifuge tube containing 5 ml medium. When all the intact islets had been collected from this sample, further aliquots of the original suspension were treated in similar fashion.

The islets, collected in the centrifuge tube (usually 300-800) were incubated at 37^{o}C for 30 min. This preincubation period was to allow fading of possible insulin release, induced by the isolation procedure. At the end of the preincubation, the medium containing islets was poured into a black bottomed petri dish. The islets were visualised using a dissecting microscope, collected by finely drawn Pasteur pipettes and transferred to a 10 ml disposable centrifuge tube placed on ice, containing incubation medium. The tube was centrifuged (MSE Bench centrifuge, setting 8 for 1 second) in order to pellet the islets. The resultant supernatant was discarded and an appropriate volume of homogenisation buffer was added.

Preparation of Islet Subcellular Fractions

Isolated islets were manually homogenised in an appropriate buffer (500-800 islets/ml) using a glass homogeniser (12 turns).

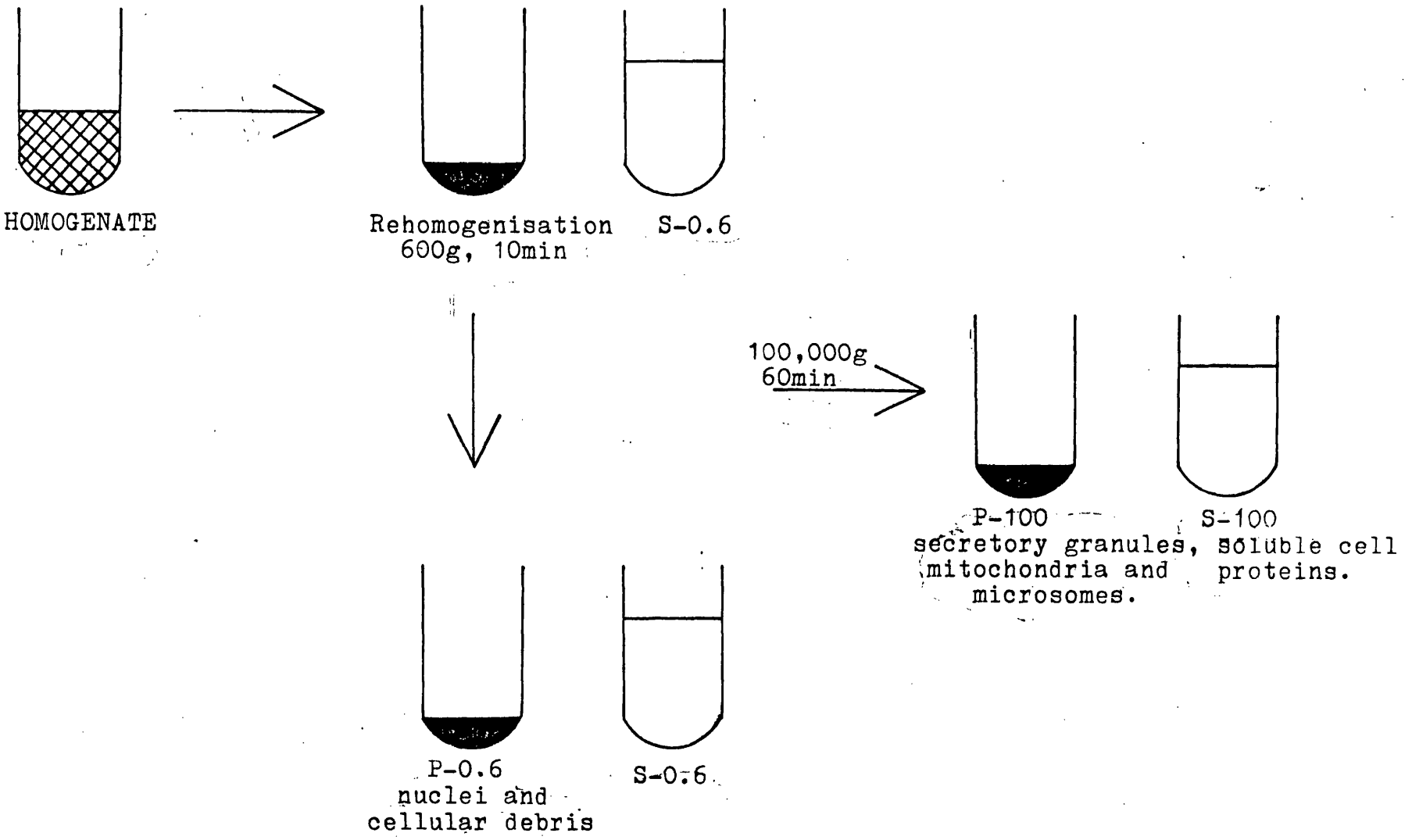
Subcellular fractions were prepared by differential centrifugation as shown in Figure 2.1. The homogenate was poured into a 3 ml centrifuge tube and centrifuged at 600 g for 10 min (2,000 r.p.m., MSE Bench centrifuge). The resultant supernatant was decanted into another 3 ml centrifuge tube and the pellet was rehomogenised in buffer (1000-1600 islets/ ml) and recentrifuged at 600 g for 10 min. The resultant supernatant was pooled with that previously obtained and called the S-06 fraction, this contained the cytosol, microsomes, mitrochondria and secretory granules. The pellet was called the P-0.6 fraction and contained the nuclei and cell debris.

The S-0.6 fraction was centrifuged at 100,000 g for 60 min (MSE 65 centrifuge, 3 x 3 ml rotor, 45,000 r.p.m.), the resultant supernatant contained the soluble cell proteins and was called the S-100 fraction. The pellet containing microsomes, mitochondria and secretory granules was called the P-100 fraction.

The P-0.6 and P-100 subcellular fractions were re-homogenised in buffer containing 0.5% Nonidet-P40 (a non-ionic detergent), in order to solublise the membrane bound proteins.

In experiments where protein kinase activity, ^{32}P production and (γ-^{32}P)-ATP hydrolysis were being measured, the subcellular fractions were diluted with homogenisation buffer to give the following concentrations; Homogenate (106-125 islets/ml), P-0.6 (318-375 islets/ml), S-0.6 (48 - 75 islets/ml), P-100 (53-225 islets/ml) and S-100 (48-125 islets/ml).

Figure 2.1. Diagramatic scheme of the Preparation of Islet Subcellular Fractions by Differential Centrifugation.


HOMOGENATE
Rehomogenisation
600g, 10min
S-0.6
100,000g
60min
P-100
secretory granules,
mitochondria and
microsomes.
S-100
soluble cell
proteins.
P-0.6
nuclei and
cellular debris
S-0.6

Preparation of Islet Sonicates

Guinea-pig islets of Langerhans were isolated and treated ultrasonically using a MSE 150 Watt Ultrasonic disinte grator (three bursts at five seconds each) in 10 mM Hepes/NaOH pH 7.5, containing 1 mM Diaminoethanetetra-acetic acid (E.D.T.A.) and 0.2 mM Phenylmethanesulphonyl fluoride (P.M.S.F.) at 4°C. The ratio of islets to buffer was 500-800 islets per ml.

Diethylaminoethyl (DEAE) Cellulose Chromatography

Preswollen DEAE cellulose was added to 10 mM Hepes/NaOH, pH 7.5, containing 1 mM EDTA (Equilibration buffer) in the ratio of 15 ml per gram. In the initial experiments the resultant slurry was poured into a chromatograpy column (0.9 cm x 5.0 cm) and packed by washing with the Equilibration buffer. This was found to be too large a column for the amount of islet tissue applied, and in later experiments better elution profiles were obtained using a smaller column (0.5 cm x 3.0 cm).

Using the larger column (0.9 cm x 5.0 cm) the sample was applied, washed with 20 ml of Equilibration buffer, and then eluted with a linear gradient (total volume 150 ml) of 0-500 mM NaCl in Equilibration buffer. 3 ml fractions were collected using a flow rate of 3 ml per min.

With the smaller column (0.5 cm x 3.0 cm), the sample was applied and washed with 5 ml of Equilibration buffer and then eluted with a linear gradient (total volume 20 ml) of 0-500 mM NaCl in Equilibration buffer. 0.5 ml fractions were collected using a flow rate 0.5 ml per min. Any alternative procedures will be discussed in Chapter III.

NaCl Gradient Preparation

Initially linear NaCl gradients were obtained by using two beakers of the same cross-sectional areas. The size of the beakers was determined by the volume of the gradient, e.g., for a gradient volume of 100 ml, 100 ml beakers were used. Equilibration buffer (the volume being half of the total gradient volume) was placed in one beaker which was magnetically stirred and connected to the other beaker (which contained half the total gradient volume of 500 mM NaCl in Equilibration buffer) by a glass U-tube or plastic tubing. The beaker containing the lower concentration buffer was then connected to the chromatography column by plastic tubing.

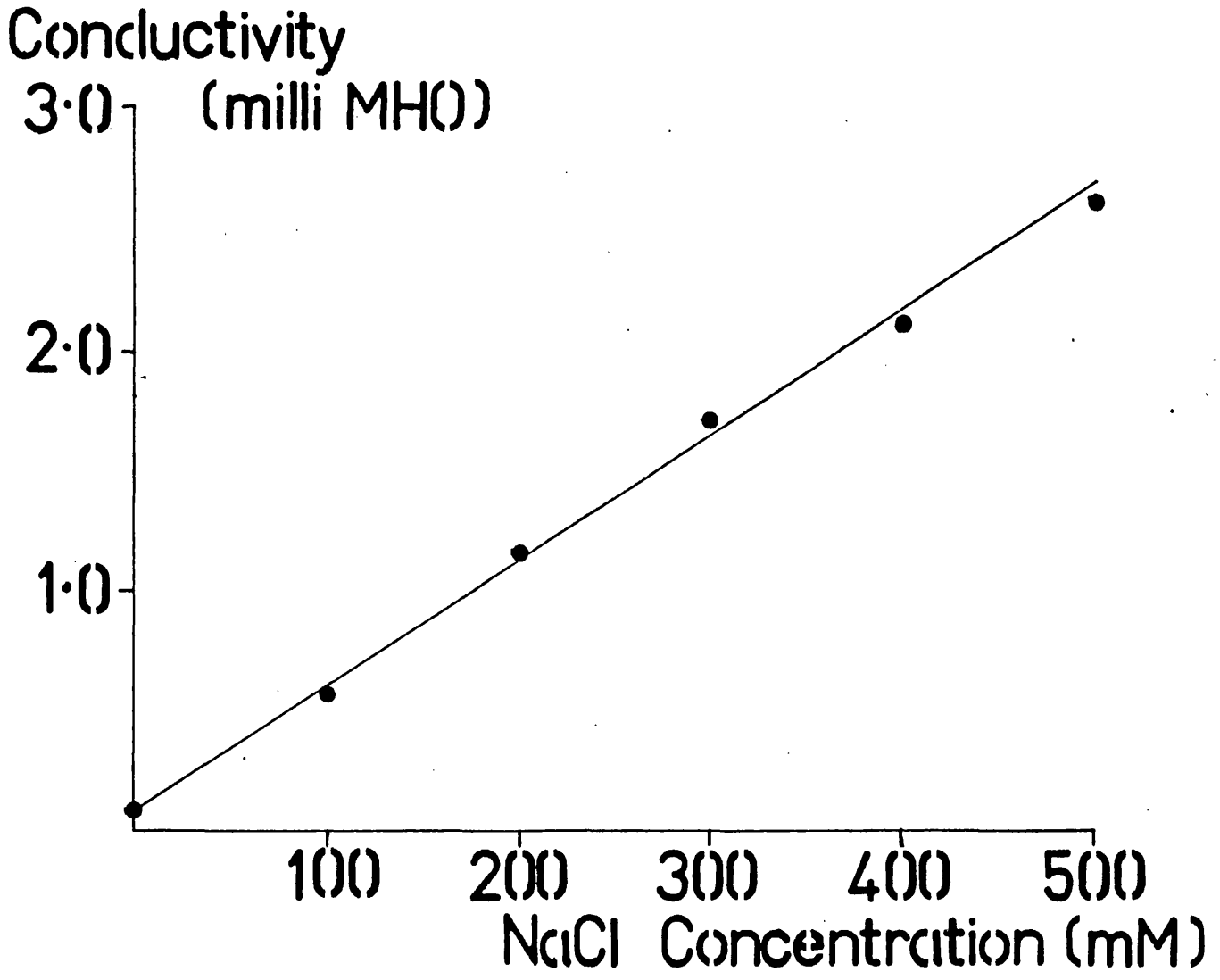
In later experiments gradient formers were used. For large gradient volumes (greater than 20 ml) a gradient former made in the laboratory workshops was used, while for gradient volumes of 20 ml, a MSE gradient former (Cat. No. 36658) was used.

For both methods, linear NaCl gradients (as determined by conductivity readings) were easily and reliably obtained.

Measurement

To determine the NaCl concentration of the chromatography column eluant, conductivity measurements of alternate fractions were made, using a conductivity Type CDM/2 Radiometer and a CDC 114 electrode (Radiometer, Copenhagen). 0.1 ml of the eluant fraction was diluted to 2 ml with Equilibration buffer and the conductivity was measured. To convert the conductivity reading to a value of NaCl concentration, the conductivity of standard NaCl solutions prepared and diluted 1:20 in Equilibration buffer was measured. A calibration

Figure 2.2. Conductivity Meter Calibration Curve. Standard NaCl solutions were diluted 20 fold and their conductivity (●) was measured using a Radiometer CDM2 Conductivity meter with a CDC114 electrode.


Conductivity
(milli MHO)
3·0
2·0
1·0
100
200
300
400
500
NaCl Concentration (mM)

curve was then constructed. Figure 2.2 shows a typical calibration curve.

Protein Estimation

E_{280} measurements of the DEAE-cellulose chromatography column eluants using a Pye Unicam SP500 spectrophotometer were sometimes found to be insensitive, particularly when measuring eluant fractions of islet tissue.

Lowry Method of Protein Determination

Initially the Lowry (1951) method of protein determination was used. 0.1 ml samples were pipetted in duplicate into glass flat bottomed (10 mm x 50 mm) tubes. 1 ml of a freshly prepared solution containing 100 ml of 2% $NaCO_3$ in 0.1M NaOH, 1 ml of 1% $CuSO_4$ and 1 ml of 2% Sodium potassium tartrate was added to each tube and the contents of the tubes were mixed. After 10 min 0.1 ml of Folins Reagent was added, and the contents of the tube mixed. The tubes were incubated at room temperature for 45 min. 0.5 ml of distilled water was pipetted into each tube and the absorbance of the tube contents was read at 750 nm, using a Pye Unicam SP6-500 spectrophotometer. Standard solutions of Bovine serum albumin with concentrations 400, 200, 100, 50 and 25 μg per ml were assayed in duplicate, in order to construct a calibration curve.

When the protein concentration of the DEAE-cellulose chromatography column eluant fractions was determined, using the Lowry Method, it was observed that the fractions preceeding the NaCl gradient fractions gave a lower absorbance reading at 750 nm than the Equilibration buffer-reagent blank samples. In order to examine more closely what was happening to the Equilibration buffer as it passed through the DEAE-cellulose chromatography column, a column with a

NaCl gradient was run without the application of a tissue sample. The E_{280} and protein concentration (Lowry Method) of the fractions collected was determined. As shown in Figure 2.3, a gradual rise in the Lowry A_{750} was observed in the eluant fractions preceeding the NaCl gradient, which increased into a large peak on applying the NaCl gradient. The Lowry A_{750} then leveled off to the "normal blank" of the buffer at 50 mM NaCl (approx.), this "normal blank" Lowry A_{750} being higher than that observed in the eluant fractions preceeding the NaCl gradient. No peak of E_{280} was observed, suggesting that the cause of interference was not due to protein being eluted. It was thought that the large peak of Lowry A_{750} may have been due to a contaminant of the DEAE-cellulose which was eluted by low NaCl concentrations. The experiment was repeated, using DEAE-cellulose which had been pre-cycled with acid (0.2M HCl) and alkali (0.2M NaOH) washings prior to use. The rationale being that if there was a contaminant present in the DEAE-cellulose which could be eluted by low concentrations of NaCl, precycling of the DEAE-cellulose would remove such a contaminant. The absorbance at 750 nm and the protein content of the eluant fractions was determined by the Lowry Method and by a modified Lowry described by Peterson (1977) which was designed to measure protein concentration in the presence of interferring buffers.

Figure 2.4, shows the low value of Lowry Absorbance at 750 nm obtained in the eluant fractions preceeding the NaCl gradient and the peak of Lowry Absorbance at 750 nm at low concentrations of NaCl were still present when the DEAE-cellulose had been pre-cycled. The A_{750} measurement of the

Figure 2.3. Protein Measurement of the Collected Fractions from a DEAE-cellulose Chromatography Column.
Sample buffer containing no tissue sample was applied to a DEAE-cellulose chromatography column (0.9 cm x 5.0 cm) and eluted with a linear NaCl gradient (△). The protein content of the collected fractions was measured using the Lowry method (◯) and E_{280} (∩) measurement.

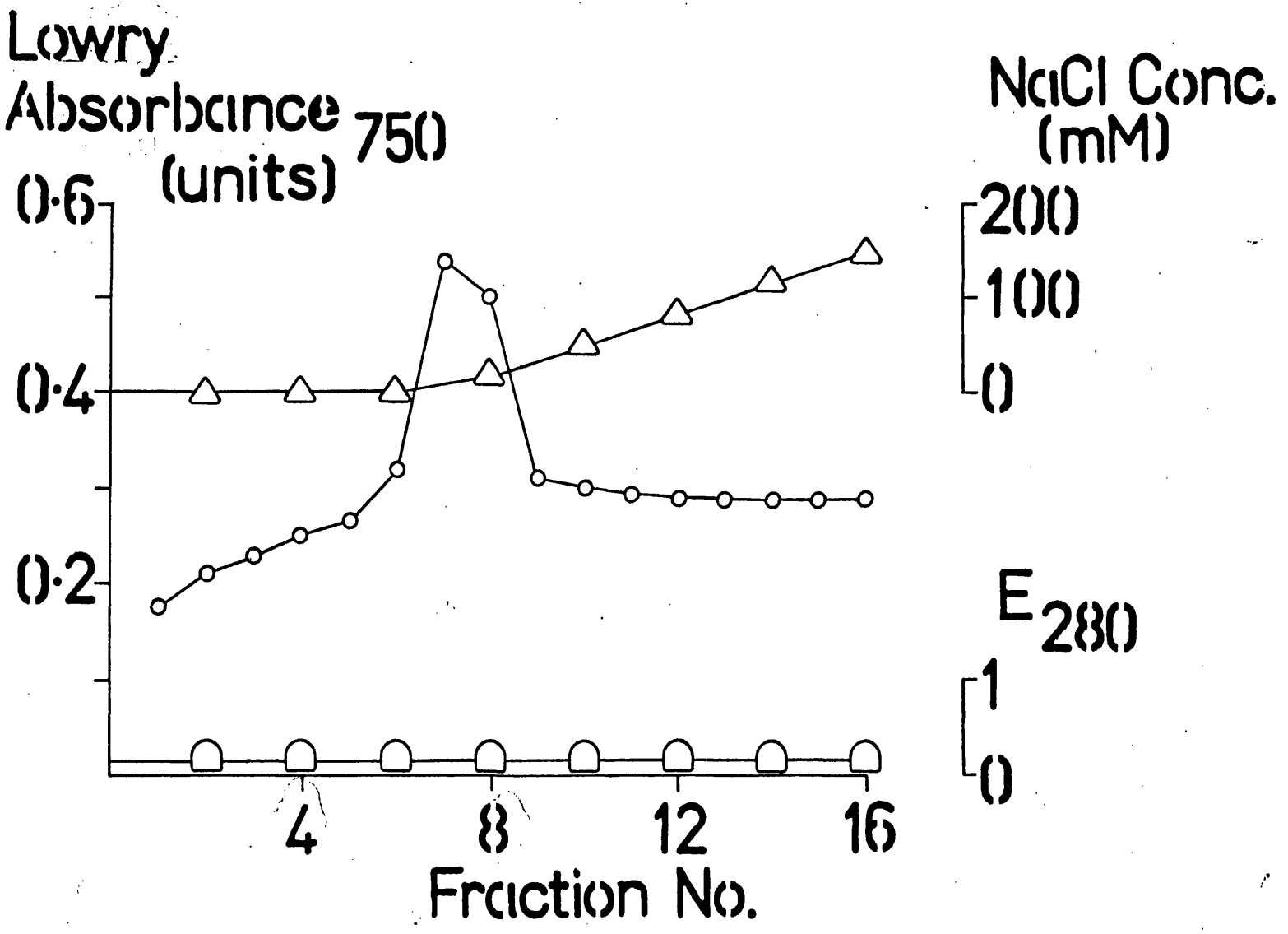
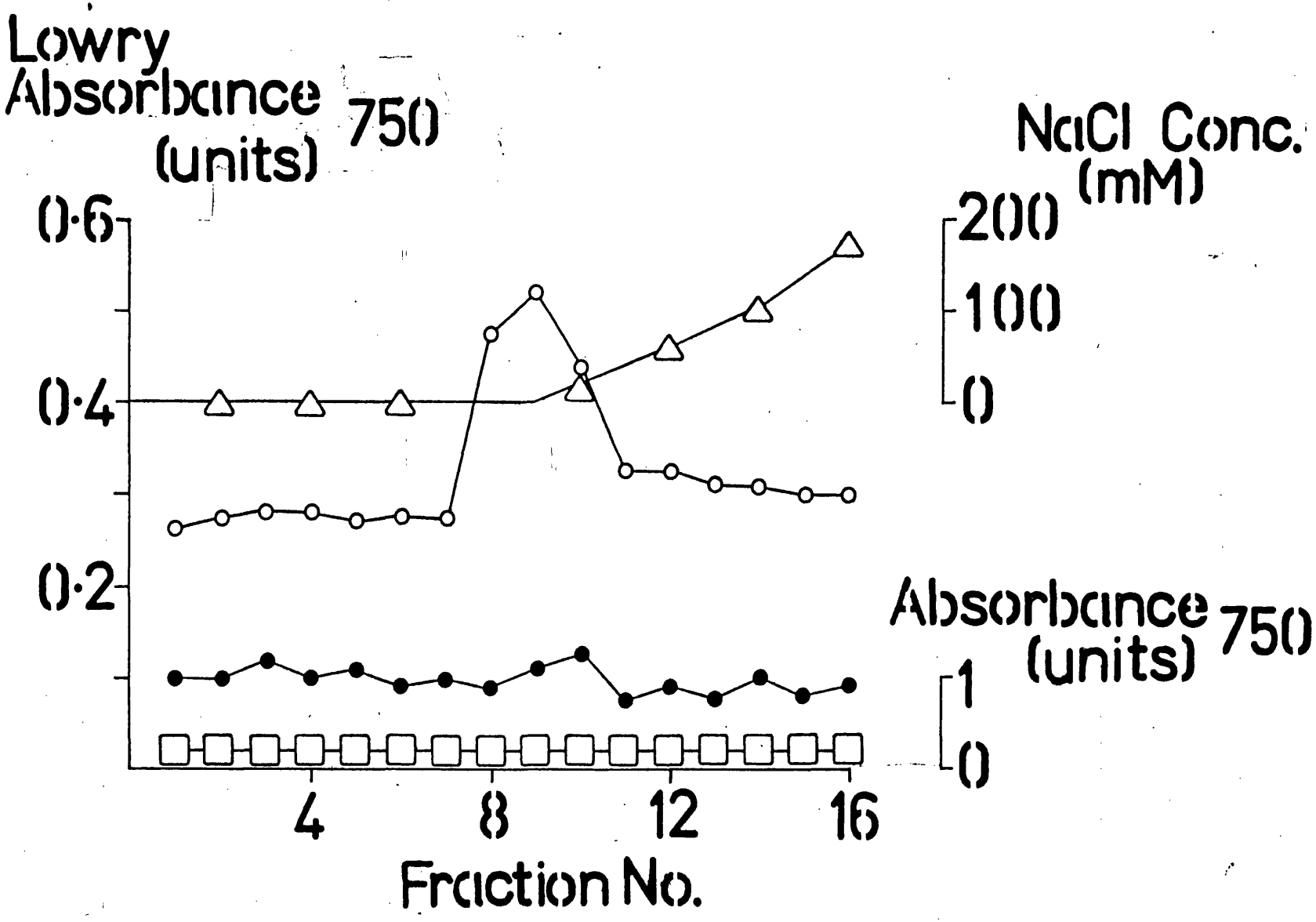

Lowry
Absorbance 750
(units)
0·6
0·4
0·2
NaCl Conc.
(mM)
200
100
0
E 280
1
0
4
8
12
16
Fraction No.

Figure 2.4. Protein Measurement of the Collected Fractions from a precycled DEAE-cellulose Chromatography Column. Sample buffer containing no tissue sample was applied to a DEAE-cellulose chromatography column (0.9cm x 5.0cm) which had been precycled, as described in the text, with acid and alkali washes. The column was eluted with a linear NaCl gradient (△) and the absorbance at 750nm (□) of the collected fractions was measured along with the protein concentration using the Lowry (O) and Peterson (●) method.


Lowry
Absorbance
(units) 750
0·6
0·4
0·2
NaCl Conc.
(mM)
200
100
0
Absorbance
(units) 750
1
0
4
8
12
16
Fraction No.

eluant fractions demonstrated that the eluant did not contain varying amounts of A_{750} absorbing chemicals. The Peterson A_{750} showed no change in the eluant fractions. From these results it was inferred that the interference was due to the Hepes buffer and not due to the DEAE-cellulose. A likely mechanism for the interference, was that the Hepes itself was being adsorbed onto the DEAE-cellulose, and eluted at low concentrations of NaCl. This would explain the lower "blank" values obtained in the eluant fractions preceeding the NaCl gradient, the peak of Lowry A_{750} at low NaCl concentrations being an eluted 'peak' of Hepes buffer.

These experiments suggested that the Lowry Method of protein determination was unsuitable for eluant fractions of DEAE-cellulose chromatography using Hepes buffer. Thus when large quantities of islet tissue were used E_{280} measurements were obtained and when this was insufficient, the protein content of the eluant fractions was determined using the Peterson method.

Peterson Protein Determination

Reagents

Reagent A: This consisted of equal volumes of:-

a) Copper-Tartrate-Carbonate (C.T.C.) Solution. This was prepared by adding equal volumes of 20% Na_2CO_3 and a solution containing 0.2% $CuSO_4$ and 0.2% Potassium Tartrate. The 20% Na_2CO_3 solution was added slowly while magnetically stirring the 0.2% $CuSO_4$, 0.2% Potassium Tartrate solution. This solution was stable for 2 months at 20°C.

b) 0.8M NaOH.

c) 10% Sodium dodec yl sulphate (S.D.S.).

d) H_2O.

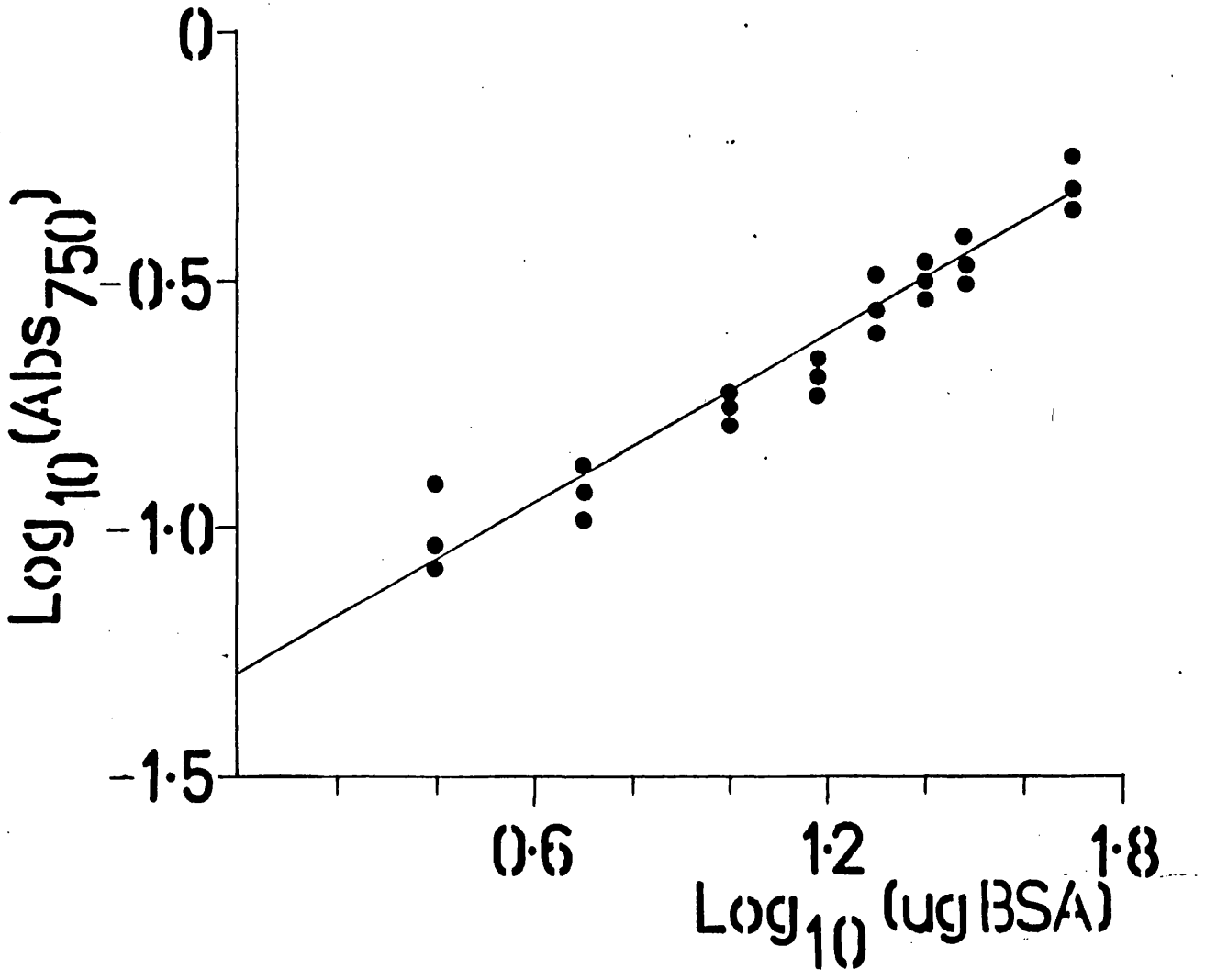
This solution could be kept for 2 weeks at room temperature. The presence of a small amount of dark precipitate or white flocculent precipitate (S.D.S.) did not affect colour development if the solution was shaken well before use.

Reagent B: Folin-Ceocalteu phenol reagent stored at 4^oC in an amber bottle.

Procedure

0.1 ml samples were pipetted in triplicate into small plastic test tubes (10 mm x 75mm) and brought to a total volume of 1 ml with distilled water. 0.1 ml of 0.15% Sodium deoxycholate was added, mixed and allowed to stand at room temperature for 10 min. 0.1 ml of 72% Trichloroacetic acid was added and mixed. The samples were centrifuged at 2,000 r.p.m. (MSE Mistral 6L, 192 x 3 ml swing-out rotor) for 30 min. The supernatant was carefully discarded and the protein pellet resuspended in 1 ml of distilled water. 1 ml of Reagent A was added, the contents of the tube mixed and allowed to stand at room temperature for 10 min. 0.1 ml of Reagent B was added and the contents of the tube mixed immediately and left to stand for 30 min at room temperature. The absorbance of the tubes was then measured at 750 nm using a Pye Unicam SP6-500 Spectrophotometer. The absorbance was converted to protein concentration by construction of a calibration curve, plotting $\log_{10} Abs_{750}$ against $\log_{10}$ µg of protein standard. Protein standards were Bovine serum albumin solutions of concentrations 500, 300, 250, 150, 100, 50 and 25 µg/ml. These were assayed in triplicate alongside the samples. Figure 2.5 shows a typical standard curve obtained using the Peterson assay.

Figure 2.5. Peterson Standard Calibration Curve. Standard solutions of Bovine serum albumin were measured in triplicate for their protein concentration (●) using the Peterson method described in detail in the text.


0
-0·5
-1·0
-1·5
Log10 (Abs750)
0·6
1·2
1·8
Log10 (ug BSA)

The usefulness of the Peterson method in determining protein concentrations in the presence of interfering agents was further examined, using Nonidet-P40 (a non-ionic detergent which interfers with the Lowry assay). The results presented in Table 2.1 demonstrated that the presence of increasing amounts of Nonidet-P40 did not affect the gradient or intercept of the standard curve obtained.

TABLE 2.1 - The Effect of Nonidet-P40 on the Peterson Standard Curve

% Nonidet-P40	Gradient of Standard Curve	Intercept of Standard Curve
-	0.56 (± 0.03)	- 1.27 (± 0.03) (8)
0.001	0.54 (± 0.03)	- 1.26 (± 0.03) (8)
0.01	0.56 (± 0.02)	- 1.30 (± 0.02) (8)
0.02	0.46 (± 0.04)	- 1.22 (± 0.05) (8)
0.05	0.54 (± 0.02)	- 1.28 (± 0.02) (8)

The gradient (± standard deviations) and the intercept (± standard deviations) were obtained using a Least Square Analysis computer programme. The number of observations is shown in parenthesis.

Protein Kinase Activity

Principle of Assay

This assay used the ability of the protein kinase enzyme to catalyse the transfer of the γ-phosphate group of ATP to an artificial protein substrate, such as histone, protamine and casein. By using (γ -^{32}P)-ATP the transfer of the γ-phosphate group could be monitored, after separating the protein bound ^{32}P from free ^{32}P and (γ -^{32}P)-ATP. This method gave a measure of ^{32}P incorporation into endogenous islet cell protein as well as exogenous protein substrate. As the concentration (2 mg/ml) of exogenous protein substrate used was in excess of the islet protein concentration (50-100 µg/ml) it was thought and in later experiments confirmed, that the activity due to endogenous phosphorylation would be negligible compared to that measured in the presence of an exogenous protein substrate.

Preparation of (γ -^{32}P)-ATP

Initially the method used, was that described by Glynn and Chappell (1964). This method makes use of the exchange reaction between inorganic phosphate and the terminal phosphate group of ATP that occurs in the presence of phosphoglycerate kinase (reaction 1), glyceraldehyde 3-phosphate dehydrogenase (reaction 2) and suitable substrates.

(Reaction 1)

3-Phosphoglycerate + ATP* $\rightleftharpoons$ 1*,3 diphosphoglycerate + ADP

(Reaction 2)

1*,3 diphosphoglycerate + glyceraldehyde 3-phosphate dehydrogenase $\rightleftharpoons$ 3-phosphoglycerolenzyme + P_i*

The asterick denotes the presence of ^{32}P.

The equilibrium position of reaction (1) is far to the left, so that ADP will be present only in catalytic amounts.

Safety

The preparation of (γ-^{32}P)-ATP was carried out in a fume hood, well protected by lead shielding. Two pairs of disposable gloves were worn and when contaminated the outer gloves were removed and placed into a suitably protected disposable bag. β-radiation was monitored by a bench β-counter throughout the experiment. Contaminated glassware was rinsed under a tap in a sealed sink unit until the radiation level was acceptable, care was taken to prevent splashing. Acceptably contaminated glassware was placed in a bucket containing 1% (approx.) DECON^{R} 90 solution and soaked overnight, the glassware was then rinsed with tap water in a sealed sink unit. The process of DECON^{R} soaking and rinsing of glassware was repeated until no radiation was detected. The glassware was then washed and dried in the normal manner. Solid non-glass waste was placed in a disposable bag shielded by lead and kept there until the radiation was at an acceptable level, then was disposed of as normal solid waste.

Experimental Procedure

Two syringe needles (guage 25G, 5/8) were inserted into the rubber stopper of the vial (still in its original lead pot) containing 10 mCi ^{32}P in 1 ml of 1M HCl. The extra syringe needle was inserted to prevent pressure build up on addition of the reagents to the vial. 1 ml of distilled water, 1 ml of 0.1M NaOH, 0.5 ml of 1M Tris/HCl, pH 8.0 and 1 ml of H_2O containing 12 mg $MgCl_2.6H_2O$ and 2.4 mg Cysteine free base were slowly added to the vial separately, using 1 ml disposable syringes. The pH of the vial contents were

checked, to see if it was approximately pH 8.0, by spotting a small drop onto pH paper. If the drop was acidic, more 0.1M NaOH had to be added, but in all the experiments performed none had an acidic pH at this stage. 1 ml of distilled water containing 36 mg ATP (disodium salt) and 5 mg 3-Phosphoglycerate (tricyclohexyl) and then 0.2 ml of Glycero-3-phosphate dehydrogenase/Phosphoglycerate kinase enzyme mix where slowly added separately using 1 ml disposable syringes. The contents of the vial were mixed by gentle agitation and incubated at 26^{o}C for 60 min. The reaction was terminated by adding the contents of the vial to a 500 ml round bottomed flask containing 35 ml of ethanol, using a Pasteur pipette. The vial was washed with 1 ml of water and this was added to the flask. The resultant cloudy ethanolic solution was cleared by the addition of water in 1 ml aliquots (approximately 7 ml was required). The round bottomed flask was connected to a Gallenkamp Thin Film Evaporator (Model EU-100) and the contents evaporated to approximately 8 ml. The evaporated solution was applied to a Dowex^{R}-1 (1 x 8-200 Chloride form) column (0.5 cm x 3.0 cm), which had been previously prepared in the following way:- Dowex^{R} was added to an excess of 1M HCl, the resultant slurry was poured into a column where it was packed to a height of 3 cm, the column was then washed with distilled water until the pH of the washings was neutral. The sample eluant was collected. The column was then washed with 10 ml of 0.02M HCl containing 11 mg NH_4Cl, the eluant which contained AMP, ADP and P_i was collected. The column was then washed with 10 ml water and the eluant containing NH_4^+ ions was collected. Finally, the column was washed with 0.25M HCl to remove the

ATP, 14 ml of the eluant was collected. The 0.25M HCl (final) eluant collected was neutralised to pH 7.0 by the addition of 1M Tris in 0.5 ml aliquots. It was found that between 4.0 and 4.5 ml was required to neutralise this fraction. 10 µl aliquots of the four eluant fractions were counted for ^{32}P using Čérenkov radiation (Clausen, 1968) (Packard Tri-Carb liquid-Scintillation spectrometer). The ATP concentration in the eluant fractions were determined by measuring the extinction at 259 nm of a 200 fold diluted aliquot, using a quartz cuvette reserved for radioactive solutions. The molar extinction coefficient was taken to be 15.4×10^3 at pH 7.0. Every precaution to prevent spillage of the radioactive solutions was taken. In the event of spillage the contaminated area was wiped with a neat solution of DECON^R-90 and then thorougly rinsed with water using a paper towel. This was repeated until no contamination could be detected with a bench β-monitor. The paper towels were disposed of using the procedure described in the Safety section.

TABLE 2.2 - Preparation of (γ-^{32}P)-ATP Using the Glynn and Chappell Method (1964)

Experiment	I	II	III
Sample Wash (mCi)	0.70	1.98	0.78
0.02M HCl Wash (mCi)	0.10	0.34	0.06
H_2O Wash (mCi)	0.01	0.03	0.02
0.25M HCl Wash (mCi)	1.70	3.91	5.40
% Recovery	68	62	86
[(ATP)] (mM)	2.60	1.77	1.30
Specific Activity (γ-^{32}P)-ATP (mCi/m mole)	67	135	229

Table 2.2 shows that even with a 86% recovery of total radioactivity, (γ-^{32}P)-ATP with a specific activity of 229 mCi per m mole was obtained. Although this value of specific activity was sufficient for use in the protein kinase assays, a higher specific activity was particularly necessary for experiments in which the endogenous phosphorylation of islet cell proteins was being measured. Using (γ-^{32}P)-ATP of higher specific activity had the additional advantage that it could be used for a longer period of time, the two week half life of ^{32}P meant that after six weeks, the low specific (γ-^{32}P)-ATP was unusable.

To prepare higher specific activity (γ-^{32}P)-ATP by the Glynn and Chappell method required larger amounts of $^{32}P_i$, e.g., 100 mCi $^{32}P_i$ was required to prepare (γ-^{32}P)-ATP of a specific activity of 1.52 Ci per m mole. Such large amounts of radioactivity were far in excess of the quota allowed to the department by the National Radiation Protection Board and also very expensive. Hence another method (Johnson and Walseth, 1979) was used which claimed to pro-

duce high specific activity (γ-^{32}P)-ATP. This method was designed in order to prepare (α-^{32}P)-ATP, the first stage of the procedure being the preparation of high specific activity (γ-^{32}P)-ATP which was required for the subsequent stages.

Principle of the Johnson and Walseth Preparation of (γ-^{32}P)-ATP

This method was a modification of that described by Schendell and Wells (1973), in which the substrate level phosphorylation of ADP occurs during the enzymatic conversion of L-α-glycerophosphate to 3-phosphoglycerate (Figure 2.6). The modificiations of the Schendell and Wells procedure were:

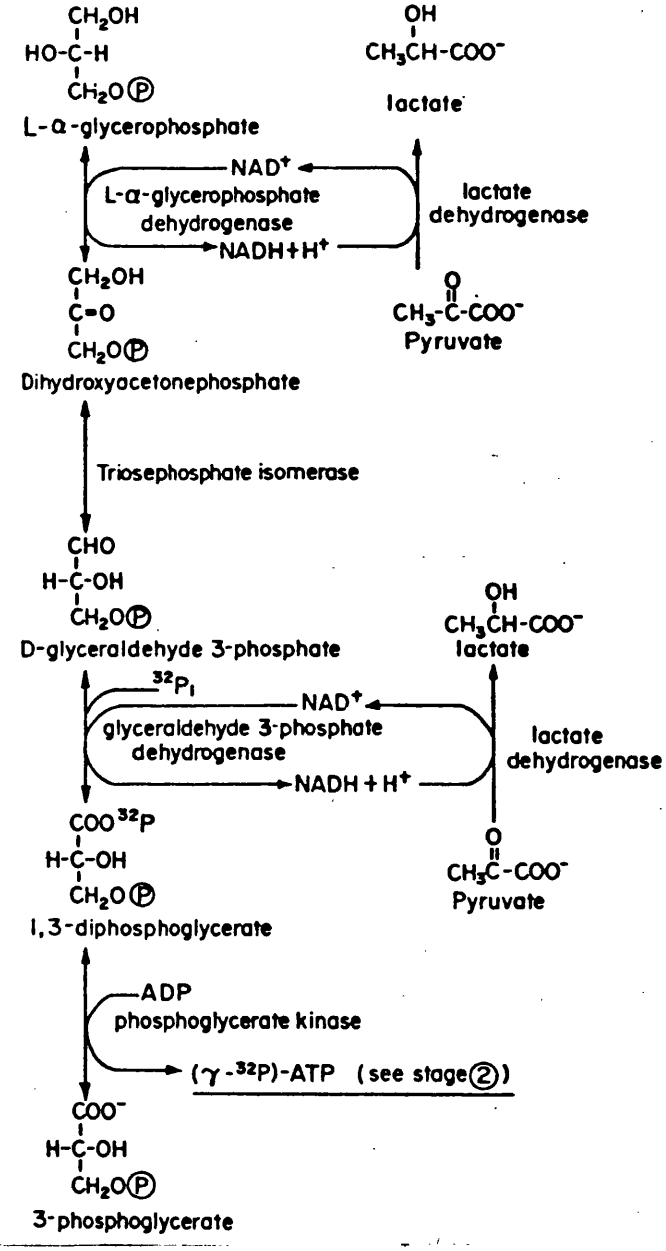
a) the use of lactate dehydrogenase and pyruvate to maintain NAD^+ concentrations instead of the oxidising dyes phenazine methosulphate and thioazoyl blue;

b) the use of L-α-glycerolphosphate as primary substrate and the enzymes necessary to convert it to D-glyceraldehyde-3-phosphate. The use of L-α-glycerophosphate prevented the hydrolysis of the diethylacetal of glyceraldehyde-3-phosphate before beginning the reaction and also resulted in a smaller amount of unlabelled P_i (as contaminant) being introduced into the reaction thereby decreasing the specific activity of the resulting (γ-^{32}P)-ATP.

Reagents

All of the enzymes were obtained as suspensions in 3.2M $(NH_4)_2SO_4$ from Boehringer/Mannheim, where indicated enzymes were diluted with 3.2M $(NH_4)_2SO_4$ to produce volumes that could be easily and accurately pipetted. 1M Tris was used to neutralise the 1M HCl in which the $^{32}P_i$ was contained. The original method used 500 mM Tris/HCl pH 9.0, because the $^{32}P_i$ used, was obtained in HCl free water.

Figure 2.6. The Reaction Sequence for the Synthesis of (γ—^{32}P)-ATP using the method described by Johnson and Walseth (1979).
Taken from Johnson,R.A. and Walseth,T.F. (1979) Adv. Cyclic Nucleotide Res. 10 p. 138.


CH2OH
HO-C-H
CH2O(P)
L-α-glycerophosphate
NAD+
L-α-glycerophosphate dehydrogenase
NADH+H+
CH2OH
C=O
CH2O(P)
Dihydroxyacetonephosphate
Triosephosphate isomerase
CHO
H-C-OH
CH2O(P)
D-glyceraldehyde 3-phosphate
32P1
NAD+
glyceraldehyde 3-phosphate dehydrogenase
NADH + H+
COO 32P
H-C-OH
CH2O(P)
1,3-diphosphoglycerate
ADP
phosphoglycerate kinase
(γ-32P)-ATP (see stage②)
COO-
H-C-OH
CH2O(P)
3-phosphoglycerate
OH
CH3CH-COO-
lactate
lactate dehydrogenase
CH3-C-COO-
Pyruvate
OH
CH3CH-COO-
lactate
lactate dehydrogenase
CH3C-COO-
Pyruvate

Method

A 120 µl aliquot of the stock mixture of enzymes (see Table 2.3) was centrifuged at 16,000 g for 15 mins (3 x 3 ml rotor, MSE 65 Centrifuge, 13,000 r.p.m.). The supernatant fraction was discarded and the pellet resuspended in 120 µl of 50 mM Tris/HCl pH 9.0. The enzyme-DTT mix was prepared by adding 28 µl of 1M Tris, 140 µl of 10 mM DTT and 92 µl of distilled water to 20 µl of the resuspended enzyme pellet, this gave a total volume of 280 µl.

Two syringe needles (guage 25G, 5/8) were inserted into the rubber cap of the vial (still in its lead pot) containing 1 ml of 10 mCi $^{32}P_i$ in 1M HCl. 800 µl of the reagent mixture (Table 2.4 was added to the vial followed by 200 µl of the enzyme-DTT mixture, using 1 ml disposable syringes. The vial was gently shaken and the reaction allowed to proceed at room temperature for 30 min. The enzymes were inactivated by immersing the reaction vessel for 10 min in water which had been previously brought to the boil. The reaction mixture was then applied to a Dowex^{R}-1 (1 x 8-200 Chloride form) column (0.5 cm x 3.0 cm) to separate (γ-^{32}P)-ATP from the rest of the reaction mixture. The reaction vessel was washed twice with 2 ml of distilled water and once with 2 ml of 0.02M HCl and the washings were applied to the column. The column was then eluted in the same manner previously described in the Glynn and Chappell method. In some preparations 10 ml of the final eluant of 0.25M HCl was collected, this was known to contain at least 60% of the total ATP, but being a smaller volume required less (approx. 2.5 ml) 1M Tris base to neutralise to pH 7.0. An estimate of the minimum specific activity of the prepared (γ-^{32}P)-ATP was calculated, as the ATP concen-

TABLE 2.3 - Enzymes for the Preparation of (γ -^{32}P)-ATP Using the Johnson and Walseth (1979) Method

Enzyme	Dilution for Stock	Stock Mixture		Final Conc. in Reaction Mix
		(µl)	(mg/ml)	(U/ml)
Glycerophosphate Dehydrogenase (Rabbit muscle, 170 U/mg, 2 mg/ml)	--	35	0.48	0.58
Triosephosphate Isomerase (Rabbit muscle, 5,000 U/mg, 2 mg/ml)	x 50	50	0.014	0.50
Glyceraldehyde-3-P Dehydrogenase (Rabbit muscle, 80 U/mg, 10 mg/ml)	--	20	1.40	0.80
3 - Phosphoglycerate Kinase (Yeast, 450 U/mg, 10 mg/ml)	x 10	20	0.14	0.44
Lactate Dehydrogenase (Rabbit muscle, 550 U/mg, 5 mg/ml)	--	20	0.70	2.75

The indicated volumes were mixed and stored at 4°C as stock mixture

(total volume 145 µl)

TABLE 2.4 - Reagents for the Preparation of (γ-^{32}P)-ATP Using the Johnson and Walseth (1979) Method

Reagent	Stock Concentration (mM)	Vol. Used (µl)	Final Conc. in Reaction Mix (mM)
Tris	1000	375	100
$MgCl_2$	300	100	12
DTT	100	150	6
L-α-Gycerolphosphate	2.4	125	0.12
β NAD^+	10	125	0.5
ADP	2	62.5	0.05
Pyruvate	40	62.5	1

All reagents were stored as frozen stock solutions except ADP and pyruvate, which were prepared freshly for each preparation. The indicated volumes were mixed prior to use (total volume 1 ml).

tration of the (γ-^{32}P)-ATP solution obtained was too dilute to safely measure its extinction or 259 nm. Thus it was assumed that a 100% conversion of ADP to (γ-^{32}P)-ATP occurred (Walseth and Johnson, 1979) and that a 100% recovery of (γ-^{32}P)-ATP was obtained. (In the case were only 10 ml of the final eluant was collected, it was assumed that a 60% recovery of the prepared (γ-^{32}P)-ATP was obtained).

Table 2.5 shows the prepared (γ-^{32}P)-ATP had an approximate 600 fold higher specific activity than that prepared by the Glynn and Chappell method. The reason why the Johnson and Walseth method produced a higher specific activity (γ-^{32}P)-ATP than the Glynn and Chappell method was due to experimental design. The latter method relied on the production of 1,3 diphosphoglycerate and ADP from ATP and 3-phosphoglycerate an equilibrium reaction that favours the formation of ATP and 3-phosphoglycerate. The 1-phosphate group of the 1,3 diphosphoglycerate was then exchanged for $^{32}P_i$ in another equilibrium reaction. The now labelled *1,3 diphosphoglycerate exchanged its phosphate group to ADP to produce (γ-^{32}P)-ATP and 3-phosphoglycerate. Hence dilution of the (γ-^{32}P)-ATP occurred due to the addition of ATP to initiate the reaction sequence and due to liberation of unlabelled phosphate during the equilibration reaction. The Johnson and Walseth method also used the conversion of labelled *1,3-diphosphoglycerate and ADP to produce 3-phosphoglycerate and (γ-^{32}P)-ADP, but the labelled *1,3-diphosphoglycerate was produced by the enzymic conversion of L-α-glycerophosphate. This conversion required several enzymes of which two were NAD^+ dependent, this meant that the whole reaction sequence could be driven to the formation

TABLE 2.5 - Preparation of (γ-^{32}P)-ATP Using the Method of Johnson and Walseth (1979)

Experiment	I*	II*	III*	IV	V
Fraction 1 (mCi)	<0.001	<0.001	<0.001	0.01	<0.001
Fraction 2 (mCi)	1.78	2.15	6.90	0.79	1.00
Fraction 3 (mCi)	0.17	0.10	0.18	0.26	0.18
Fraction 4 (mCi)	7.40	3.57	2.80	6.25	5.35
[ATP] (μM)	5.6	5.9	5.9	4.8	5.0
Specific Activity (Ci/mmole)	73	38	28	104	89
% Recovery	79	61	28	86	82
Volume (ml)	18	17	17	12	12

* Denotes preparations in which the stored stock enzymes were diluted with distilled water and not 3.2M $(NH_4)_2SO_4$. The decreasing yield obtained in these preparations demonstrated that the reagent enzymes were losing activity. Hence in following experiments the reagent enzymes were diluted when required in 3.2M $(NH_4)_2SO_4$ and this prevented the loss of enzymic activity.

of (γ-^{32}P)-ATP and 3'-phosphoglycerate by maintaining NAD^+ levels, using pyruvate and lactate dehydrogenase. Hence no dilution of (γ-^{32}P)-ATP and $^{32}P_i$ occurred.

Protein Kinase Activity

Assay Buffer

100 mM Sodium β-glycerophosphate brought to pH 6.0 by the addition of conc. HCl, containing 40 mM NaF, 0.6 mM E.G.T.A., 0.2 mM E.D.T.A., 20 mM Magnesium acetate, 1 mM Isobutylmethylxanthine (I.B.M.X.), 0.2 mM (γ-^{32}P)-ATP (40-100 cpm/p mole) and 4 mg/ml exogenous protein substrate. NaF was present in the assay buffer as an inhibitor of protein phosphatase (Maeno and Greengard, 1972) and Mg^{2+} ions were used as the cofactor. Histone (Calf Thymus IIa), Protamine (Salmon) or Hydrolysed casein prepared by the method of Reimann et al. (1971) were used as exogenous protein substrates.

Incubation Procedure

Equal volumes (50-200 µl) of sample and protein kinase assay buffer were mixed and incubated for 10-20 min at 37°C in disposable tubes (8 mm x 65 mm). Cyclic AMP dependency was measured in the absence and presence of 1 µM cyclic AMP.

Two methods for terminating the reaction and separating the proteins of the reaction mixture from (γ-^{32}P)-ATP and ^{32}P were tried. The principle of the first method was to TCA precipitate the proteins in the reaction tube, a protein carrier was added to ensure a large enough precipitate was obtained. The proteins were pelleted by centrifugation, ^{32}P contamination was removed by dissolving the pellet in 2M NaOH, reprecipitating it in 10% TCA and then by further washings and centrifugations.

Procedure

2 ml of 10% TCA were added to the tubes to terminate the reaction and two drops of carrier protein (B.S.A. (10 mg/ml) were added, using a Pasteur pipette. The tubes were centrifuged at 2,000 rpm (MSE 6L Mistral, 192 x 3 ml swing-out rotor) for 15 min. The supernatant was removed and disposed of as aqueous radioactive waste, and the protein pellet redissolved by the addition of 2 drops of 2M NaOH. 2 ml of 10% TCA was then added and the tubes re-centrifuged, the supernatant was discarded, and the pellet was then washed with 2 ml 10% TCA and recentrifuged. The resultant supernatant was discarded and the pellet dissolved in 0.2 ml 2M NaOH. The reaction tubes were then placed inside disposable insert vials which in turn were placed inside glass counting vials. The ^{32}P content of the vials was determined by Cerenkov counting. This method coped adequately with the large number of samples but the reproducibility of the duplicates was found to be poor.

The second method was based on that described by Glass et al. (1978) and used the principle of selective adsorption of protein in acid conditions onto phosphocellulose paper (Whatmans P-81).

Procedure

The reaction was terminated by the addition of an equal volume of 60% Acetic acid, this gave a final concentration of 30% Acetic acid. The terminated reaction solutions could then be stored at $4^{o}C$ for up to 18 hr if required.

50 µl aliquots of the terminated reaction solutions were placed onto Whatmans P81 paper (2 cm x 2 cm) and

immediately placed into 15% Acetic acid which was magnetically stirred. To prevent disinte gration of the P81 papers, they were separated from the magnetic follower with a wire mesh. The volume of 15% Acetic acid was determined from the number of samples, 500 ml was sufficient for 100 samples. The P-81 papers were washed twice (15 min each) with 15% Acetic acid and then washed in Acetone for 5 min. The papers were dried in an oven, then placed individually into disposable insert vials and 4 ml of water was added. The insert vials were capped and placed inside glass counting vials for determination of ^{32}P content using Čerenkov counting. (Clausen (1968).

This method was found to be very reliable and could cope with several samples in one assay.

Calculation of Protein Kinase Activity

A unit of protein kinase activity was arbitrarily defined as the incorporation of one pico mole of ^{32}P per min per mg of exogenous substrate.

Preparation of Hydrolysed Casein

This preparation was based on the method described by Reimann et al. (1971). Casein (100 mg/ml) was suspended in distilled water and heated at 100°C for 10 min, while maintaining the pH at 9.5 using 2M NaOH. The solution was cooled and adjusted to a final protein concentration of 40 mg/ml at pH 6.0, using concentrated HCl.

Preparation of Walsh Protein

Introduction

The protein kinase assay described measured both cyclic AMP-dependent and cyclic AMP-independent activities. When measuring the protein kinase activities of the DEAE-cellulose column fractions, peaks of activities which increased

in the presence of cyclic AMP could be taken as being solely due to cyclic AMP-dependent protein kinase activity. But when the protein kinase activities of the subcellular fractions prepared by differential centrifugation were determined, the difference between the activity in the presence and absence of cyclic AMP could not be taken as the total activity due to cyclic AMP-dependent protein kinases, as some of the activity measured in the absence of cyclic AMP may have been due to dissociated catalytic subunits of the cyclic AMP-dependent protein kinase holoenzyme. For this reason the cyclic AMP-dependent protein kinase activity of the subcellular fractions was determined indirectly by measuring the activity in the presence and absence of cyclic AMP and Walsh protein, a specific inhibitor of the catalytic subunit of the cyclic AMP-dependent protein kinase enzyme (Ashby, C. D. and Walsh, D. A., 1972). The difference between the protein kinase activity in the presence of cyclic AMP and in the presence of cyclic AMP plus Walsh protein was assumed to be due solely to cyclic AMP-dependent protein kinases.

Procedure

The preparation of Walsh protein was based on the method described by Ashby and Walsh, (1972). The hind leg muscle of a freshly killed rabbit was removed and immediately stored at -20^{o} prior to use. The muscle (20 g) was thawed, minced with scissors and then homogenised at 4^{o}C in 60 ml of 4 mM EDTA, pH 7.0, using a Ato-Mix blendor. The homogenate was centrifuged at 7,000 g (8 x 50 ml angle rotor, 7,000 rpm) for 30 min at 0^{o}C and the resultant supernatant was decanted through glass wool. The supernatant was placed in a water bath (90^{o} - 95^{o}C) for 5 min and then chilled. T.C.A. was added to the super-

natant to give a final concentration of 15%. The resultant suspension was centrifuged at 27,000 g (8 x 50 ml angle rotor, 17,000 rpm) for 10 min. The supernatant was then dialysed against 5 mM Sodium phosphate, 1 mM EDTA, pH 7.0 for 18 hours at 4°C. The pH of the dialysed solution was adjusted to pH 5.0 using 15% Acetic Acid. The dialysed solution was applied to a DEAE-cellulose chromatography column (0.9 cm x 3.0 cm) which had been previously equilibrated with 5 mM Sodium acetate, 1 mM EDTA, pH 5.0, the column was then washed with 3 ml of the same buffer. The Walsh protein was eluted with 250 mM Sodium acetate, 1 mM EDTA, pH 5.0 and 0.5 ml fractions were collected. The first 0.5 ml fraction was discarded and the following 3 ml which contained the Walsh protein was pooled, and stored in 0.5 ml aliquots at -20°C. The activity of the Walsh protein preparation was determined by measuring the inhibition of protein kinase activity in the presence of cyclic AMP of an islet S-0.6 fraction. A unit of activity was arbitrarily defined as the inhibition of 1 picomole of ^{32}P transferred per min.

Measurement of (γ-^{32}P)-ATP Hydrolysis and ^{32}P Production

(γ-^{32}P)-ATP hydrolysis and ^{32}P production by islet preparations was measured using the reaction conditions described in the procedure for the determination of protein kinase activity, in order to monitor any substantial decrease in (γ-^{32}P)-ATP concentration.

Procedure

(γ-^{32}P)-ATP hydrolysis and ^{32}P production was measured using the method described by Cooper et al, (1974). The reaction conditions and assay buffer used were the same as that described in the protein kinase assay. The reaction was terminated by aliquoting 20µl of the reaction mixture into a disposable test tube (10 mm x 75 mm) containing 500µl of Activated Charcoal (10 mg/ml) in 1M HCl. The test tube was centrifuged at 2,500 rpm (MSE Mistral 6L, 192 x 3 ml swing-out rotor) for 15 min. A 50µl aliquot of the supernatant was removed and placed into a plastic insert vial contained inside a glass counting vial. 4 ml distilled water was added and the ^{32}P content was determined using Čerenkov counting in a Packard Tri-carb Scintillation spectrometer. The remaining supernatant was discarded as aqueous radioactive waste and the resultant charcoal pellet was counted for (γ-^{32}P)-ATP content in a Packard Tri-carb liquid scintillation spectrometer.

Calculation of (γ-^{32}P)-ATP Hydrolysis and ^{32}P Production Activity

A unit of (γ^{32}P)-ATP hydrolysis activity was arbitrarily defined as the hydrolysis of 1 picomole of (γ^{32}P)-ATP per min. A unit of ^{32}P production activity was arbitrarily defined as the production of 1 picomole of ^{32}P per min.

^{32}P Scintillation Counting

Optimisation of Liquid-Scintillation Spectrometer Procedure

10 µl of protein kinase assay buffer containing (γ-^{32}P)-ATP was pipetted into a glass scintillation vial, the vial was placed in an oven (60°C) until the liquid had evaporated. 4 ml scintillant (2,5 diphenlyoxazole:toluene, 4:100 w/v) was added. The vial was placed in a Packard Tri-Carb liquid scintillation spectrometer and counted for ^{32}P using a window setting of 50 to 10,000 and varying percentage gains. (Figure 2.7). A blank value was determined by counting 4 ml of scintillant. The optimum percentage gain was found to be 2.0%. A counting efficiency of 90% was assumed for ^{32}P in scintillant and the quantity of radioactivity was calculated.

Cerenkov Counting (Clausen, 1968)

10 µl of protein assay buffer containing (γ-^{32}P)-ATP was pipetted onto P81 phosphocellulose paper (2 cm x 2 cm), placed inside a glass counting vial and then in an oven (60°C) until dry. 4 ml water was then added and the vial counted for ^{32}P in a Packard Tri-Carb scintillation spectrometer, using a window setting of 35-10,000. The percentage gain was varied as shown in Figure 2.8 and the optimum was found to be 50%. By comparing the mean count rate obtained with scintillant with that using Cerenkov counting, the counting efficiency of Cerenkov counting was calculated to be 53%.

In all experiments where the specific activity of (γ-^{32}P)-ATP was required, an aliquot (10 µl) of protein kinase assay buffer containing 0.2 mM (γ-^{32}P)-ATP was

Figure 2.7. Calibration of Packard Tri-Carb Liquid Scintillation Spectrometer for ^{32}P Radiation.
The variation of ^{32}P (●) and background (O) mean count rate with percentage gain was determined using a 'window setting' of 50 - 10,000.

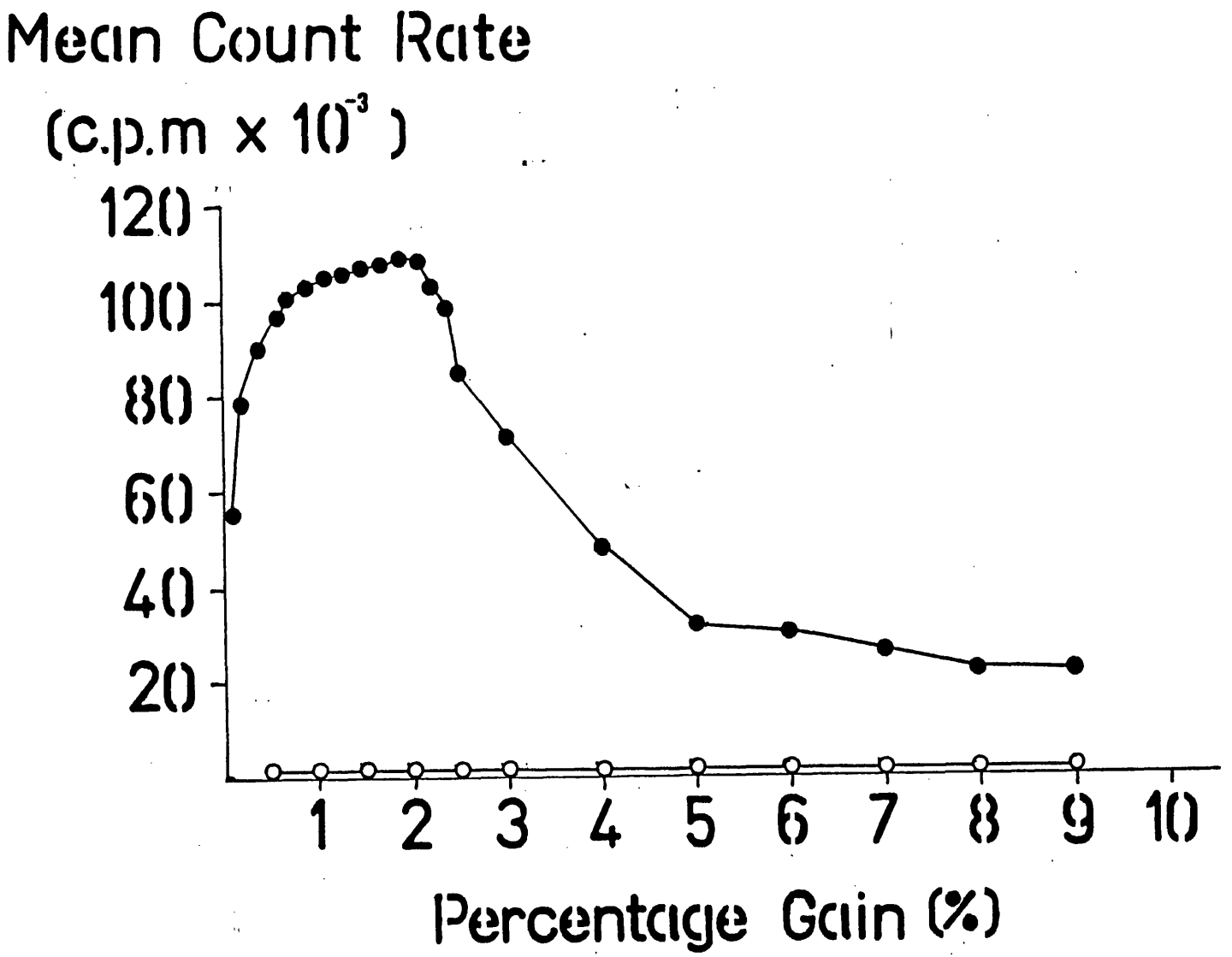
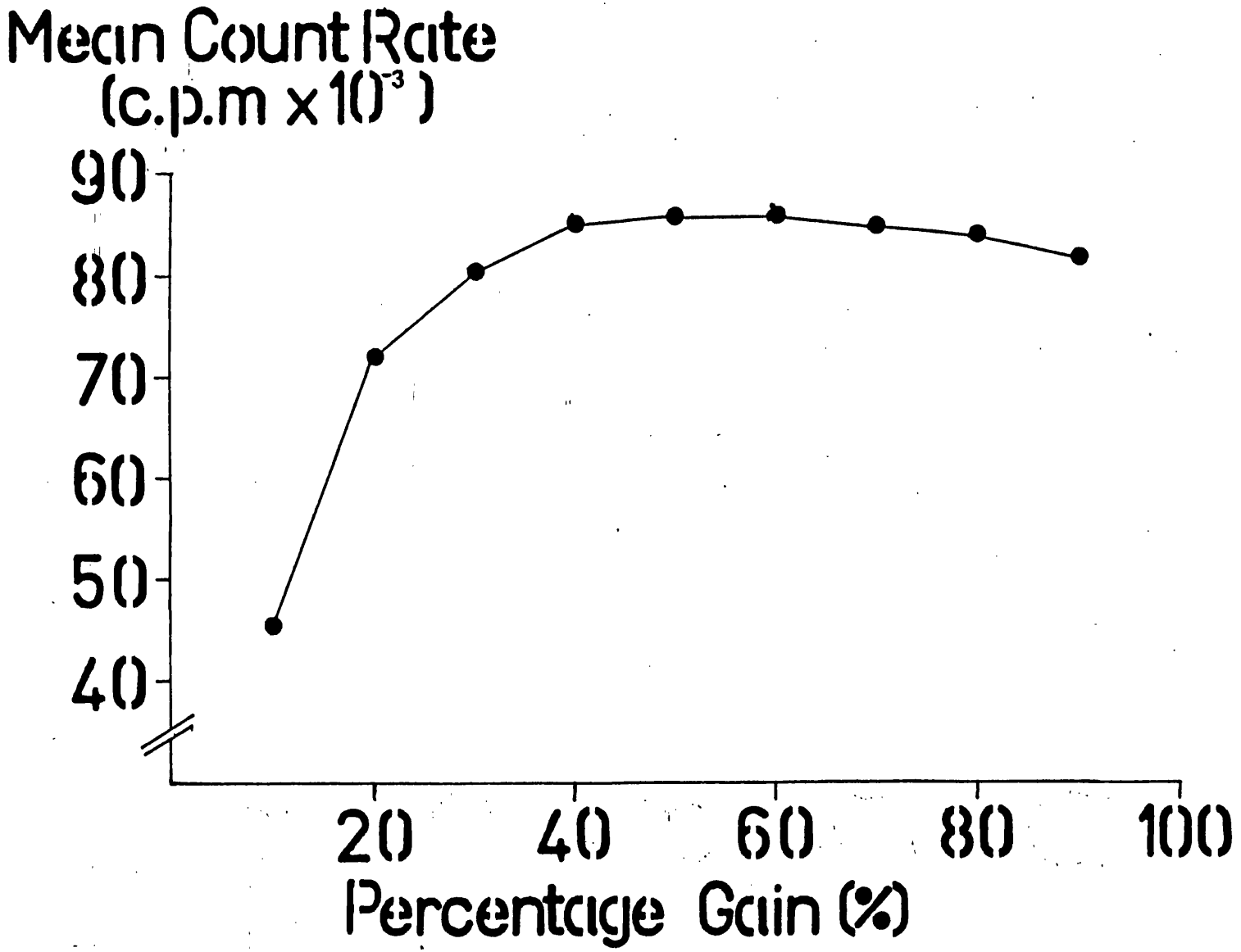

Mean Count Rate
(c.p.m x 10⁻³)
120
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10
Percentage Gain (%)

Figure 2.8. Calibration of Packard Tri-Carb Liquid Scintillation Spectrometer for Cerenkov Radiation. The variation of Cerenkov radiation (●) with percentage gain was determined using a 'window setting' of 35 - 10,000.


Mean Count Rate
(c.p.m x 10⁻³)
90
80
70
60
50
40
20
40
60
80
100
Percentage Gain (%)

counted in the same manner alongside the samples. This was to eliminate any discrepancies in counting efficiency due to machine variation.

(^{3}H)-Cyclic AMP Binding Assay

Principle

This assay used the ability of the receptor subunit of the cyclic AMP-dependent protein kinase holenzyme to bind (^{3}H)-cyclic AMP. The protein bound (^{3}H)-cyclic AMP was then separated from the free (^{3}H)-cyclic AMP by filtration, using either sartorius membrane filters or glass fibre filters.

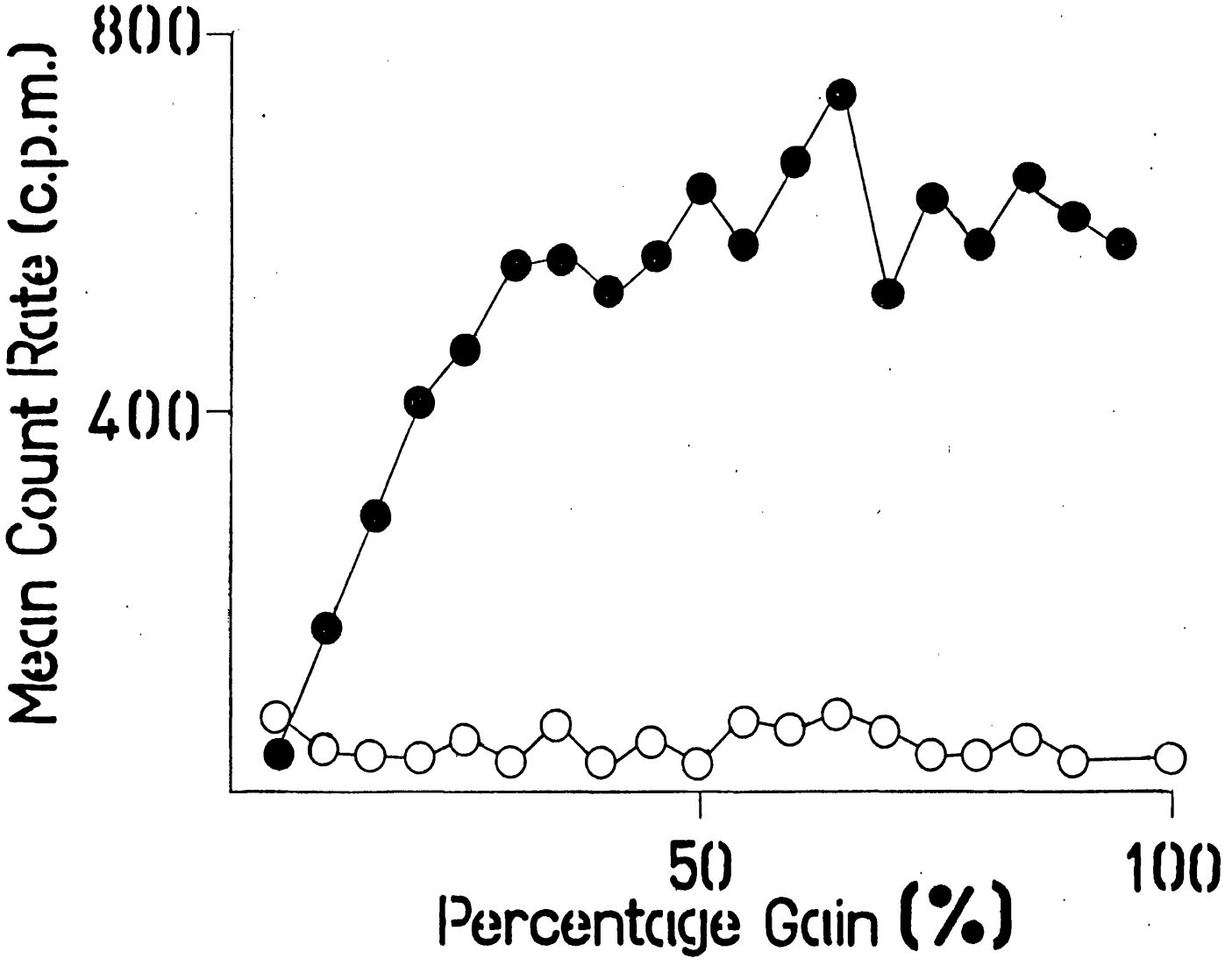
The method used was based on that described by Døskeland and Ueland (1977) and Montague and Howell (1972).

Procedure

100 µl-200 µl aliquots of DEAE-cellulose chromatography eluant fractions were pipetted into disposable tubes (10 mm x 75 mm) containing 300 µl of (^{3}H)-cyclic AMP binding assay buffer:- 50 mM Hepes/NaOH, pH 7.4, containing 5 mM $MgCl_2$, 0.5 mM I.B.M.X. and 0.1 µM (^{3}H)-cyclic AMP (specific activity 5.2-10.6 Ci/m mole). The tubes were incubated at 4^{o}C for 1 hour. Samples were assayed for (^{3}H)-cyclic AMP binding activity in the absence and presence of Histone (0.2 mg/ml) and NaCl (800 mM).

200 µl aliquots were taken from the incubation tube and placed in disposable test tubes (14 mm x 100 mm) containing 2 ml of 0.1 mM cyclic AMP in ice cold 80% saturated $(NH_4)_2SO_4$. The cyclic AMP was present to prevent binding of unlabelled ligand during precipitation (Døskeland et al., 1977). Initially filtration on sartorius membrane filters (0.45 µm pore size) was used to separate the bound (^{3}H)-cyclic AMP from the unbound, but the $(NH_4)_2SO_4$ solution proved very

Figure 2.9. Calibration of Packard Tri-Carb Liquid Scintillation Spectrometer for ^{3}H Radiation.
The variation of ^{3}H (●) and background (O) mean count rate with percentage gain was determined using a 'window setting' of 50 - 10,000.


800
400
Mean Count Rate (c.p.m.)
50
100
Percentage Gain (%)

difficult to filter because of its high viscosity. By replacing the membrane filters with glass fibre filters (Whatman GF/C) easier filtration was obtained with similar results. The filters were placed in disposal insert vials and 4 ml scintillant (toluene: 2,5 diphenyloxazole (1000:4 v/w)) was added. The vials were capped and placed in glass scintillation vials and the ^{3}H content was determined using a Packard Tri-Carb liquid-scintillation spectrometer.

(^{3}H) Scintillation Counting

An 0.1 ml aliquot of (^{3}H)-cyclic AMP (specific activity 5.2 Ci/m mole) binding assay buffer was pipetted onto a fibre glas filter (Whatman's GF/C), which was placed inside a disposable insert vial (10 mm x 50 mm). 4 ml scintillant was added and the vial was capped and placed into a glass scintillation vial. The optimum mean count rate for (^{3}H) was determined by varying the percentage gain of the Packard Tri-Carb liquid-scintillation spectrometer while maintaining the 'window-setting' at 50-10,000. Figure 2.9 shows the optimum mean count rate was obtained with a percentage gain of 65%. The background mean count rate was obtained by counting 4 ml scintillant.

Disc Native Polyacrylamide Gel Electrophoresis (P.A.G.E.)

The procedure used was based on the method described by Davis (1964).

Solutions

A) 36.6 g Tris base, 48 ml 1M HCl, 0.25 ml N,N,N^{1},N^{1}-tetramethylene diamine (TEMED) and water to 100 ml (pH 8.9).

B) 5.98 g Tris base, 48 ml 1M HCl, 0.5 ml TEMED and water to 100 ml (pH 6.7).

C) 28.0 g Acrylamide, 0.74 g Bis acrylamide and water to 100 ml.

D) 10.0 g Acrylamide, 2.5 g Bis acrylamide and water to 100 ml.

E) 4 mg Riboflavin in 100 ml water.

F) 20% Sucrose.

G) 0.14% Ammonium persulphate freshly prepared before use.

Stock Electrophoresis Buffer:- 6.0 g Tris base, 28.8 g Glycine and water to 1 litre (pH 8.3). This was diluted 100 ml to a litre before use.

Procedure

The running gel was prepared by adding 1.5 ml solution A, 3.0 ml solution C, 1.5 ml distilled water and 6 ml solution G to a 25 ml conical flask. This gave a final running gel concentration of 375 mM Tris/HCl, pH 8.9, containing 7% Acrylamide, 0.2% Bis-Acrylamide, 0.07% Ammonium persulphate and 0.03% TEMED. When larger volumes of running gel were required the volumes of the solutions were proportionately increased. The contents of the flask were mixed by gentle shaking and poured into glass tubes (7 mm x 85 mm), sealed at the bottom with Nescofilm^{R}, by means of a finely drawn Pasteur pipette, to a height of 70 mm. A layer of water was carefully added dropwise over the gel solution. The gel was allowed to polymerise for 30 min. The water layer was carefully removed and the surface of the gel carefully blotted dry with tissue. The stacking gel was prepared by adding 1.0 ml solution B, 2.0 ml solution D, 1.0 ml solution E and 4.0 ml solution F to a 25 ml conical flask. This gave a final stacking gel concentration of 62.5 mM Tris/HCl, pH 6.7, containing 20% Sucrose, 2.5% Acrylamide, 0.6% Bis-

Acrylamide, 0.06% TEMED and 0.5 mg/ml Riboflavin. The contents of the flask were mixed by gentle shaking and the top of the running gel was washed briefly with portions of the stacking gel solution. The stacking gel was added to the top of the running gel by means of a finely drawn Pasteur pipette to a height of 5 mm. A layer of water was carefully added over the stacking gel. The gel was allowed to polymerise by placing the tubes close to a lamp for 15 min. Polymerisation was indicated by the bleaching of the riboflavin and a slight opacity of the gel. The water layer was carefully removed and the surface of the gel carefully blotted dry with tissue.

The Nescofilm^{R} was carefully removed, the tubes placed in the disc gel electrophoresis cell (Bio-Rad Model No. 150A) with the stacking-gel uppermost and the lower end submerged in electrophoresis buffer in the anode compartment. The anode compartment was magnetically stirred to prevent the formation of bubbles below the tubes. For each gel, 0.1 ml sample was added to 0.1 ml solution F and 10 µl 0.1% Bromophenol blue, and mixed thoroughly. The sample-sucrose mixture was added to the space above the gel using a Pasteur pipette and electrophoresis buffer was carefully layered over the sample so as to fill completely the remaining space in the tubes. The upper (cathode) chamber was then filled with electrophoresis buffer ensuring the tubes were submerged and the electrode was in contact with the buffer. Electrophoresis was run at 4 mA per tube until the tracker dye had migrated a distance of 50 mm. Gels were removed from the tubes and sectioned into 2 mm segments using a Mickle gel slicer or used intact for protein staining. These

gels were called native gels.

Protein Staining

Protein Staining Solution:- 125 mg Coomassie Blue R250 dissolved in 405 ml Methanol and 520 ml water. Glacial Acetic acid was added in the ratio 93:7 just before use.

Destaining Solution:- Same as staining solution with the omission of Coomassie Blue R250.

Procedure

The intact gels were placed in glass tubes (1.2 cm x 16 cm) containing 10 ml of protein staining solution. The tubes were incubated at room temperature for 18 hours. The gels were destained by replacing the protein staining solution with destaining solution, this was replaced with further fresh destaining solution every hour until the background stain was removed and protein bands were clearly usable.

Determination of Protein Kinase Activity on Native Disc Polyacrylamide Gels

2 mm gel segments were placed in disposable test tubes (8 mm x 40 mm) and 0.2 ml of protein kinase assay buffer (see page 41) was added to the tubes. The tubes were incubated at 30°C for 30 min. The reaction was terminated with the addition of 2 ml 10% TCA and the gel segments were removed. Two drops of B.S.A. solution (1 mg/ml) were added to each tube, using a Pasteur pipette, and the tubes were centrifuged at 2,000 rpm for 20 min using a MSE Mistral 6L. The excess T.C.A. was removed and the precipitated proteins dissolved in two drops of 2M NaOH, reprecipitated with the addition of 2 ml 10% TCA and centrifuged at 2,000 rpm for 20 min. The TCA washing of the precipitated proteins was then repeated. 0.2 ml NaOH was added to the tubes which were then placed inside plastic insert vials, contained in

glass scintillation vials. The ^{32}P content was determined in a Packard Tri-Carb liquid-scintillation counter using Cerenkov radiation.

Attempts to determine (^{3}H)-cyclic AMP binding activity on native polyacrylamide gels proved unsuccessful.

Disc Sodium Dodecyl Sulphate (S.D.S.) Polyacrylamide Gel Electrophoresis (P.A.G.E.)

The method used was based on that described by Laemmli, (1970).

Solutions

A) 29.2% Acrylamide.
0.8% BIS (N.N^{1} methylene bis acrylamide).

B) 1M Tris/HCl, pH 8.8.

C) 20% Sodium dodecyl sulphate (S.D.S.).

D) 10% Ammonium Persulphate freshly prepared before use.

E) TEMED (N,N,N^{1},N^{1}-tetramethylene diamine).

F) 1M Tris/HCl, pH 6.8.

Procedure

Running gels were prepared by the addition of 11.3 ml solution A, 18.6 ml solution B, 19.85 ml distilled water and 0.25 ml solution C to a 250 ml Buchner flask. This gave a final concentration of 7% Acrylamide, 0.2% Bisacrylamide, 0.1% S.D.S. in 370 mM Tris/HCl, pH 8.8. The contents of the Buchner flask were degassed using a laboratory vacuum line. 100 µl of solution D and 30 µl of solution E was then added to the flask to give a final concentration of 0.032% Ammonium persulphate and 0.06% TEMED. The flask was gently swirled to mix the contents and immediately poured into tubes (0.5 cm x 13 cm), using a Pasteur pipette, sealed at the bottom with Nescofilm^{R}. The gel solution was overlayed with a few drops of distilled water and left for 30 min to

polymerise.

Stacking gels were prepared by the addition of 2.66 ml of solution A, 2.5 ml of solution F, 14.72 ml of distilled water and 0.1 ml solution C to a 100 ml Buchner flask, this gave a final concentration of 4.0% Acrylamide, 0.1% Bis-acrylamide, 0.1% S.D.S. in 125 mM Tris/HCl, pH 6.8. The contents of the flask were degassed for 5 min. The running gel overlay was removed and the tops of the gels were rinsed with distilled water and blotted dry with tissue. The flask was removed from the vacuum line and 100 µl of solution D and 30 µl of solution E was added to give a final concentration of 0.05% Ammonium persulphate and 0.15% TEMED. The flask was gently swirled to mix the contents, which were then immediately poured on top of the running gels. The stacking gels were overlayed with a few drops of distilled water and left for 60 min to polymerise. When the gels had polymerised the water overlay was removed and the gels rinsed with distilled water and blotted dry with tissue. 10 µl of 0.1% Bromophenol blue solution was added to the prepared electrophoresis samples and 100 µl (30-40 µg protein) of the sample was applied individually to the disc tubes. Electrophoresis was performed at 4 mA per tube using a magnetic stirrer in the anode compartment and the water cooling system of the disc electrophoresis kit (Bio-Rad Model 150A). When the tracker dye had migrated a standard distance of 7 cm the gels were removed from the tubes using a 20 ml disposable syringe filled with distilled water. Gels were either stained for protein as previously described or used for the determination of (^{3}H)-cyclic AMP binding activity.

Sample Preparation for Sodium Dodecyl Sulphate (S.D.S.) Polyacrylamide Gel Electrophoresis (P.A.G.E.)

30 µl of 20% S.D.S. was added to the sample (100-200 µl) followed immediately by the addition of 100 µl of Electrophoresis sample buffer:- 0.1M Tris/HCl, pH 6.8, containing 15% glycerol and 2 mM Phenylmethanesulphonyl fluoride (P.M.S.F.). The contents of the sample vials were mixed, and immediately placed in a boiling water bath for 20 min after capping the vials. The sample vials were removed from the boiling water bath, cooled and 30 µl 1M Dithiothreitol (D.T.T.) was added to the vials. The contents of the vial were mixed and the vials were replaced in the boiling water bath for a further 10 min. The samples after cooling were either applied to the S.D.S. polyacrylamide gels or were stored at -20°C until used.

Determination of (^{3}H)-cyclic AMP Binding on Disc S.D.S. Polyacrylamide Gels

The washing method used to remove the S.D.S. was based on that described by Knight and Skala (1977) and the assay method used was based on that described by Montague and Howell (1972).

Procedure

The intact S.D.S. polyacrylamide disc gels were placed in glass test tubes (1.2 cm x 10.6 cm) containing 10 ml ice cold 250 mM Tris/HCl, pH 7.0 and incubated for 30 min at 4°C with two changes of buffer. The buffer was then replaced with 10 ml of ice cold 50 mM Tris/HCl, pH 7.0 and incubated for a further 10 min at 4°C.

The buffer was then replaced with 10 ml (^{3}H)-cyclic AMP binding assay buffer:- 50 mM Hepes/NaOH, pH 7.4 containing 5 mM $MgCl_2$, 5 mM Theophylline and 0.1 µM (^{3}H)-cyclic AMP

(specific activity 5 Ci/m mole). The tubes were then incubated at 4°C for 3 hours. Gels were assayed for (^{3}H)-cyclic AMP binding in the absence and presence of Histone (0.2 mg/ml) and NaCl (800 mM). The gels still in the glass test tubes were then washed under running tap water for 18 hours and then sectioned into 2 mm segments using a Mickle gel slicer. The gel segments were placed in flat bottomed glass test tubes (10 mm x 50 mm) and 0.2 ml of 30% H_2O_2 (v/v) was added, the tubes were then lightly capped and placed in capped glass scintilliation vials. The vials were incubated at 60°C for 18 hours. 2 ml of scintillant (toluene-Triton X-100 - 2,5, diphenyloxazole (140:60:1 v/v/w)) was added and the ^{3}H content was determined using a Packard Tri-Carb liquid-scintillation spectrometer.

Attempts to measure protein kinase activity on S.D.S. disc polyacrylamide gels proved unsuccessful. This was thought to be either due to the inefficiency of the washing procedure to remove sufficient S.D.S. to allow renaturation of the cyclic AMP-dependent protein kinase catalytic subunit or due to S.D.S. treatment of the catalytic subunits being irreversible.

Methods Used to Measure Islet Cell Endogenous Phosphorylation and Identify the Substrates of Islet Cyclic AMP-dependent Protein Kinases

Introduction

Endogenous phosphorylation was determined by measuring ^{32}P incorporation into islet cell proteins from (γ-^{32}P)-ATP, using a cell free system.

Subcellular Distribution of Islet Cell Endogenous Protein Phosphorylation

Guinea-pig islets of Langerhans were isolated and homogenised (500-800 islets/ml) in Buffer B (see Appendix). Subcellular fractions were prepared as previously described by differential centrifugation. Endogenous phosphorylation of subcellular fractions was determined in the absence and presence of 1 μM cAMP, using the method described in the procedure for the determination of "Protein Kinase Activity", with the omission of the exogenous protein substrate. Islet proteins were separated from the reaction mixture using the method described by Glass et al. (1978).

A unit of endogenous phosphorylation activity was arbitrarily defined as the incorporation of 1 femtomole of ^{32}P per min.

Identification of the Substrates of Islet Cyclic AMP-dependent Protein Kinases

Introduction

In these studies, S-0.6 fractions prepared by differential centrifugation from an islet homogenate were used, as the study was particularly designed to investigate the secretory process and not mechanisms involved in de novo synthesis. Changes in the phosphorylation state of non-nuclear protein(s) in response to agents which are known to

affect insulin secretion, would indicate that these proteins might play an important role in the secretory processes of granule migration and emiocytosis.

Two methods were used in this study, in the first method (A), S-0.6 fractions were prepared and incubated in Buffer A (see Appendix). The second method (B) followed a protocol described by Rudolph, S. A. and Kreuger, B. K. (1979) for investigating endogenous phosphorylation in various tissues. The differences between the two methods will be discussed in Chapter V.

The phosphorylated non-nuclear proteins were analysed by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (S.D.S. P.A.G.E.) and autoradiography after incubation with (γ-^{32}P)-ATP.

Procedure A

Guinea-pig islets of Langerhans were prepared and homogenised (500-800 islets/ml) in Buffer A (see Appendix). The S-0.6 fraction was prepared by centrifugation (600 g, 10 min) and aliquots (100 µl) were pipetted into glass screw top vials (9mm x 30 mm) for Sodium Dodecyl Sulphate (S.D.S.) Polyacrylamide Gel Electrophoresis (P.A.G.E.). The reaction was initiated by the addition of an equal volume of the above mentioned buffer containing 0.2 mM (γ-^{32}P)-ATP and other test substances. The incubation was terminated by the addition of 30 µl of 20% Sodium Dodecyl Sulphate (S.D.S.) for sample preparation for S.D.S. P.A.G.E. (see Methods used in the Characterisation of islet cyclic AMP-dependent protein kinases).

Procedure B

This method was based on that described by Rudolph. S. A.

and Krueger, B. K. (1979), the basic difference between this method and that previously described was that the reaction was conducted at pH 7.0 and a ten-fold lower concentration of ATP was used. This meant that the specific activity of the (γ-^{32}P)-ATP used was increased ten-fold. As a high ATPase activity had been detected in the islet extracts it was thought that a ten-fold decrease in ATP concentration would result in its rapid breakdown, for this reason the reaction time was reduced to 15 secs, 1 min and 5 min.

The Rudolph and Krueger method suggested the use of Hepes as the sample preparation buffer but it was found that on the addition of S.D.S. to the sample a white precipitate was formed which made it very difficult to apply aliquots accurately to the S.D.S. polyacrylamide electrophoresis gels. By substituting sodium β glycerophosphate for Hepes this problem was resolved , even after storing the samples at -20°C a clear solution was obtained after warming to room temperature. The disadvantage mentioned by Rudolph and Krueger of using Sodium β glycerophosphate buffer was that dilution of the specific activity of the (γ-^{32}P)-ATP would occur. The advantage of obtaining better samples for S.D.S. polyacrylamide gel electrophoresis was thought to outweigh the disadvantage of isotope dilution. In practise it was found that similar results were obtained using either the Hepes or the Sodium β-glycerophosphate buffer.

Procedure

Guinea-pig islets were prepared and homogenised in 100 mM Sodium β glycerophosphate, pH 7.0 containing 40 mM NaF, 0.6 mM EGTA, 1 mM IBMX, 20 mM Benzamidine, 2 mM DTT and 250 mM Sucrose to give a final protein concentration of

1-2 mg/ml (approx. 300-500 islets/ml). The S-0.6 fraction was prepared by centrifugation. 100 µl aliquots were placed in screw top glass sample vials (9 mm x 30 mm) placed in ice. To these 20 µl 100 mM $MgCi_2$, test substances and distilled water were added to give a final volume of 180 µl. The reaction was initiated by the addition of 20 µl of 10 µM (γ-^{32}P)-ATP (specific activity 10-20 Ci/m mole). The reaction was terminated either by the addition of 30 µl of 20% S.D.S. for sample preparation for S.D.S. P.A.G.E. or by the addition of 2 ml of 10% TCA for the determination of S-0.6 endogenous protein phosphorylation.

Determination of S-0.6 Endogenous Protein Phosphorylation

2 ml of 10% TCA was added to the tubes followed by four drops of Bovine serum albumin (1 mg/ml) solution. The tubes were centrifuged at 2,000 rpm (MSE Mistral 6L) for 15 min, the supernatant was discarded and the pellet dissolved in one drop of 0.2M NaOH. 2 ml 10% TCA was added to reprecipitate the proteins and the tubes centrifuged at 2,000 rpm for 15 min. The supernatant was discarded and the TCA wash was repeated. The resultant protein pellet was dissolved in 0.2 ml 2M NaOH for determination of ^{32}P content using Cerenkov counting.

Slab S.D.S. Polyacrylamide Gel Electrophoresis (P.A.G.E.)

The method used was based on that described by Laemmli. (1970). The solutions used were the same solutions previously described in Disc S.D.S. P.A.G.E. (Characterisation of islet cyclic AMP-dependent protein kinases).

Procedure

Running gels were prepared by the addition of 20 ml Solution A, 22 ml solution B, 17 ml distilled water and 0.29 ml

solution C to a 500 ml Buchner flask. This gave a total volume of 59 ml, sufficient for two slab gels (0.75 cm x 17.7 cm x 12.0 cm). If only one slab gel was being prepared a smaller total volume was prepared using the same proportion of reagent solutions to give a final concentration of 10% Acrylamide, 0.3% Bis-Acrylamide, 0.1% S.D.S. in 370 mM Tris/HCl, pH 8.8. If 7% Acrylamide, 0.2% Bis-Acrylamide gels were required, an appropriately smaller volume of solution A was added to the Buchner flask and the total volume was made up by the addition of distilled water. The Buchner flask was stoppered, connected to the laboratory vacuum line and the contents of the flask degassed for 10 min. The flask was removed from the vacuum line, 188 µl of solution D and 35 µl of solution E was added to give a final concentration of 0.032% Ammonium persulphate and 0.06% TEMED. The flask was gently swirled to mix the contents. For smaller volumes of running gel mixture appropriately smaller volumes of solution D and E were added to give the same final concentration. Immediately after the addition of solutions D and E the contents of the flask were poured into glass plates, which had been previously washed with Chromic acid, rinsed with distilled water and dried. The glass plates were mounted in a Vertical Slab electrophoresis cell (Bio-Rad Model No. 220) and the gels were poured by means of a disposable 20 ml syringe which pierced the bottom gasket of the cell. Toothless spacers were pushed down between the plates to exclude all air bubbles. The gel solutions were left for approximately 30 min to polymerise.

The stacking gels were prepared by the addition of

2.66 ml of Solution A, 2.5 ml of solution F, 14.72 ml of distilled water and 0.1 ml of solution C to a 100 ml Buchner flask, this gave a final concentration of 4.0% Acrylamide, 0.1% Bis-acrylamide, 0.1% S.D.S. in 125 mM Tris/HCl, pH 6.8. The Buchner flask was stoppered and the contents of the flask degassed for 5 min. The toothless spacer over the running gel was removed and the tops of the running gels were rinsed with distilled water and blotted dry with tissue. The flask was removed from the vacuum line, 100 µl of solution D and 30 µl of solution E to give a final concentration of 0.05% Ammoniun persulphate and 0.15% TEMED was added to the flask which was gently swirled to mix the contents. The flask contents were then immediately poured on top of the running gel and a toothcomb containing ten teeth was slowly inserted excluding all air bubbles. If air bubbles were formed the toothcomb was removed and reinserted, this could not be repeated too many times as the stacking gel at this stage polymerised very quickly. The stacking gels were left for 60 min to polymerise.

The toothcomb in the slab gel was then carefully removed and the teeth slots were rinsed with distilled water, poured off and blotted dry with tissue paper. 10 µl of 0.1% Bromophenol blue was added to each electrophoresis sample and an aliquot (100-150 µl, 30-60 µg protein) was applied by means of a microsyringe to the slots. Standard solutions of proteins, RNA polymer se, Bovine serum albumin, Ovalbumin and Chymotrypsinogen (75 µg/ml) previously prepared for electrophoresis were routinely run on each slab gel. Electrophoresis was performed at 50 volts until the tracker dye (Bromophenol blue) had entered the running gel and then at 100 volts for four

hours, using the water cooling system of the electrophoresis cell. The gels were then removed from the plates, stained for protein as previously described using 100 ml of staining and destaining solutions and then prepared for autoradiography.

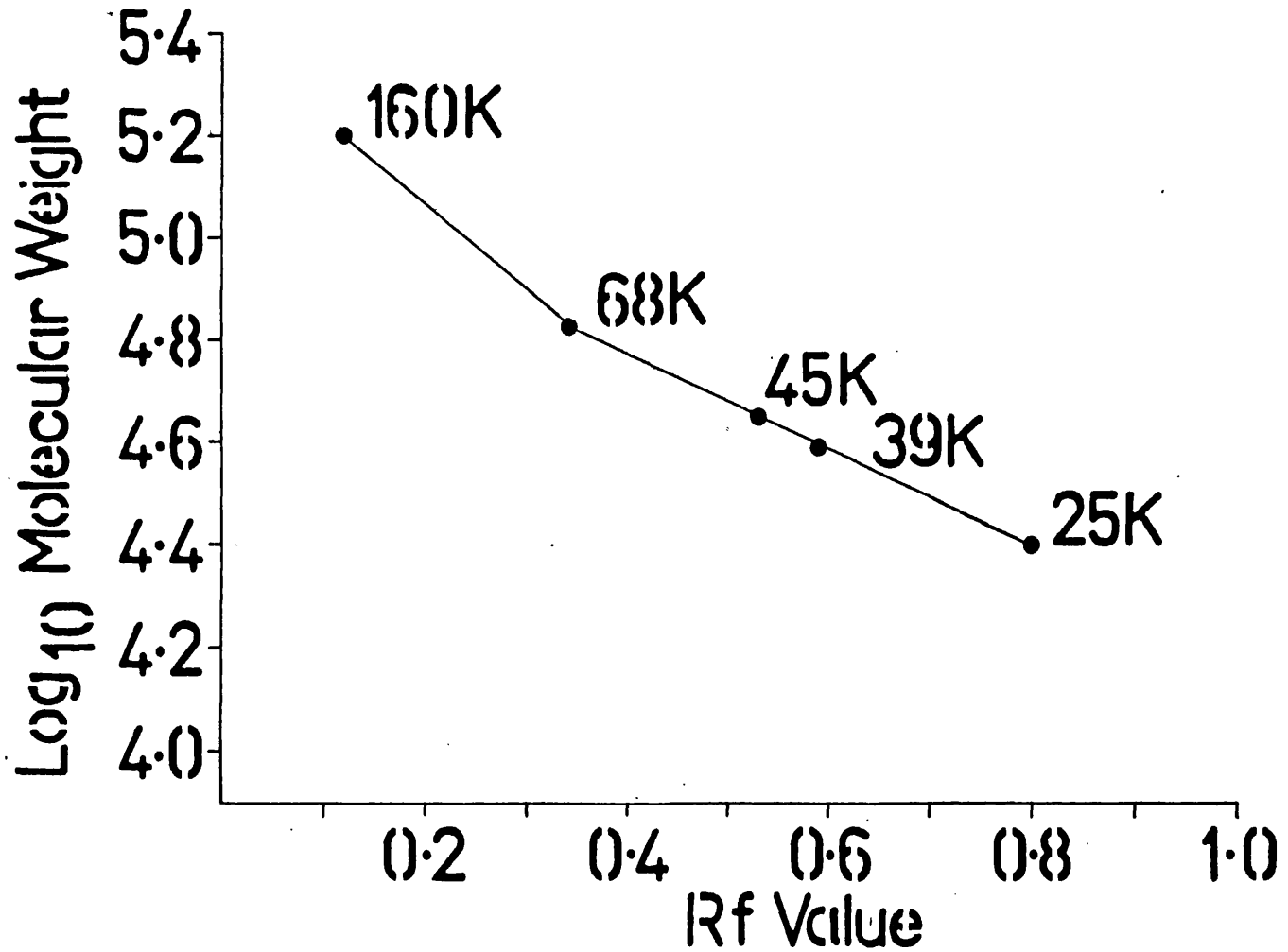
Molecular Weight Calibration

A typical standard calibration curve which was used for the molecular weight determination of polypeptides separated by S.D.S. polyacrylamide gel electrophoresis is shown in Figure 2.10. The molecular weights of the standard proteins were logarithmically related in the Rf value. Log-linear relationships were apparent between molecular weights of 25,000 dalton (Chymotrypsinogen) and 68,000 daltons (Bovine Serum Albumin), at 68,000 dalton the gradient of the line changed. It was assumed that a log-linear relationship existed between 68,000 dalton and 160,000 dalton (R.N.A. Polymerase).

Autoradiography of Slab S.D.S. Polyacrylamide Gels

Slab gels were placed onto hardened filter papers (Whatman 3M) leaving a 2 cm border around the gel, then covered by a layer of 'Cling-film'^{R}. The gels were dried onto the filter paper using a Bio-Rad Gel Slab dryer (Model No. 224), a drying time of 2 hours was found to be sufficient. The layer of Clingfilm^{R} was stretched out to remove any wrinkles, and the gel was exposed face down to a sheet of X-ray film (Kodak RP-54) in the dark. The gel was taped at the corner to secure the filter paper to the film and to provide marks on the emulsion for identifying the orientation of the gel on the film. The gel and film were placed in a black light-tight plastic bag, constructed from a dustbin bag and tape and then placed in a press contained in a light-tight wooden box. The exposure time was dependent upon

Figure 2.10. S.D.S. Polyacrylamide Gel Electrophoresis Molecular Weight Calibration Curve.
Rf values of standard proteins R.N.A. polymerase (160,000 and 39,000 daltons), Bovine serum albumin (68,000 daltons), Ovalbumin (45,000 daltons) and Chymotrypsinogen (25,000 daltons) were plotted against the logarithm (base 10) of their molecular weights.


Log10 Molecular Weight
5·4
5·2
5·0
4·8
4·6
4·4
4·2
4·0
160K
68K
45K
39K
25K
0·2
0·4
0·6
0·8
1·0
Rf Value

the specific activity of the (γ -^{32}P)-ATP used, it was found that with a specific activity of 5-20 Ci/m mole an exposure time of 3 weeks was sufficient, while with a specific activity of 0.4 Ci/m mole an exposure time of 8 weeks was used. Using an exposure time greater than 3 weeks was not satisfactory, as due to the short half-life of ^{32}P (14.7 days) maximum exposure is usually reached at approximately 3 weeks (Rudolph and Kreuger, 1979). Thus in order to obtain good autoradiographs of ^{32}P incorporation into islet cell protein a high specific activity (γ -^{32}P)-ATP was required. After the appropriate exposure time had been completed the film was removed from the gel, in the dark, and developed using Kodak D-19 developer and Kodak FX-40 fixer, prepared as per the instructions on the box. The films were rinsed with tap water for one hour and dried.

CHAPTER III

The Characterisation of the Cyclic AMP-dependent Protein Kinase Activity of Islets of Langerhans

Introduction

Changes in the intracellular concentration of cyclic AMP in the islets of Langerhans produce changes in rates of insulin secretion (Turtle and Kipnis, 1967), although little is known of the exact mechanism by which this nucleotide may influence the secretory process. In mammalian tissues, the only well-defined action of cyclic AMP is to stimulate protein phosphorylation via activation of cyclic AMP-dependent protein kinase.

Cyclic AMP-dependent protein kinase activity has been identified in islets of Langerhans (Dods and Burdowski, 1973), the majority of the total islet activity being located in the S-100 subcellular fraction (Montague and Howell, 1972; Sussman and Leitner, 1977). In these studies however, no attempt was made to solublise membrane-bound proteins. This study was undertaken to investigate the possible occurrence of cyclic AMP-dependent protein kinase activity that might be released by the non-ionic detergent Nonidet-P40. The effect of various exogenous protein substrates on islet cell protein kinase activity was also investigated.

Isozymes of cyclic AMP-dependent protein kinase have been identified in a variety of mammalian tissues (Uno et al., 1976; Corbin et al., 1975) but until recently no attempt had been made to identify the presence of different molecular forms in islet tissue. Sugden et al. (1979) demonstrated the presence of two cyclic AMP-dependent protein kinase isozymes

in a rat islet sonicate, although the subcellular localisation of these isozymes was not determined. In this study the subcellular distribution of the islet cell cyclic AMP-dependent protein kinase isozymes has been investigated.

Method

Subcellular Distribution of Protein Kinase Activity

Isolated guinea-pig islets of Langerhans were homogenised in Buffer A (see Appendix), subcellular fractions were prepared by differential centrifugation and diluted with buffer as described in detail in Chapter II.

Protein kinase activity was measured in the absence and presence of cyclic AMP (1 μM) and Walsh protein (approx. 300 units/ml) using 50 μl of sample. Protamine, histone and hydrolysed casein (2 mg/ml) were used as exogenous substrate and the reaction conditions were 20 minutes at 37°C. The reaction was terminated using the method of Glass et al. (1978).

DEAE-Cellulose Chromatography of Total Islet Cell Sonicate

Islets of Langerhans were isolated and treated ultrasonically as previously described in Chapter II. The sonicate was chromatographed on a column (0.5 cm x 3.0 cm) of DEAE-cellulose. Protein kinase activity of the eluant fractions was measured at 37°C for 20 minutes using 50 μl sample and histone (2 mg/ml) as exogenous substrate. Cyclic AMP dependency was measured in the absence and presence of 1 μM cyclic AMP. The reation was terminated using the method of Glass et al. (1978).

DEAE-Cellulose Chromatography of Islet S-100 and P-100 Subcellular Fractions

Islets of Langerhans were isolated and homogenised in Buffer B (see Appendix). The S-100 and P-100 subcellular fractions were prepared by differential centrifugation as previously described in Chapter II. The fractions were chromatographed on DEAE-cellulose, the eluant fractions were

collected and assayed for protein kinase activity in the absence and presence of 1 μM cyclic AMP, using histone or protamine (2 mg/ml) as exogenous substrate. The reaction conditions were 20 minutes at 37°C using 50 μl of sample. The reaction was terminated using either the TCA precipitation method or the Glass method (1978).

Polyacrylamide Gel Electrophoresis

Native (Davis, 1964) and S.D.S. (Laemmli, 1970) disc polyacrylamide gel electrophoresis was performed as described in Chapter II. The method of (^{3}H)-cyclic AMP binding on disc S.D.S. polyacrylamide gels was based on that described by Knight and Skala (1977) and Montague and Howell (1972). The determination of protein kinase activity on native polyacrylamide gel electrophoresis was based on the method described by Montague and Howell (1972). These methods were described in detail in Chapter II.

Results

The results in this chapter are expressed as mean values ± the standard deviation about the mean, the number of observations are shown in parenthesis. Values significantly different from controls (p less than 0.05) are denoted with asterisks. DEAE-cellulose chromatography activity elution profiles were measured in duplicate and are typical of results obtained in at least three separate experiments.

Subcellular Distribution of Islet Cell Protein Kinase Activity

The subcellular distribution of islet cell protein kinase activity measured in the absence and presence of cyclic AMP (1 μM), using protamine as exogenous substrate is shown in Table 3.1a. Protein kinase activity was equally distributed between the P-0.6 (30%), S-100 (32%) and P-100 (28%) subcellular fractions, significant cyclic AMP stimulation of protein kinase activity was observed only in the S-100 and S-0.6 subcellular fractions.

The subcellular distribution of islet cell protein kinase activity measured in the absence and presence of cyclic AMP (1 μM), using histone as exogenous substrate is shown in Table 3.1b. The majority (60%) of the total islet cell protein kinase activity was located in the S-100 subcellular fraction and this was significantly stimulated by the addition of cyclic AMP. The protein kinase activity observed in the P-0.6 subcellular fraction (24%) was also stimulated by cyclic AMP, while that observed in the P-100 subcellular fraction (16%) was unaffected by the addition of cyclic AMP.

The subcellular distribution of islet cell protein kinase activity measured in the absence and presence of cyclic AMP (1 μM), using hydrolysed casein as exogenous

TABLE 3.1 - Subcellular Distribution of Islet Cell Protein Kinase Activity

Islet cell subcellular fractions were prepared and assayed for protein kinase activity as described in the text, in the absence and presence of 1 μM cyclic AMP. Protamine a), Histone b), and hydrolysed casein c), (2 mg/ml) were used as exogenous substrate.

Subcellular Fraction	Protein Kinase Activity (p moles ^{32}P/min/mg substrate/islet)		
	- cyclic AMP	+ cyclic AMP	
a)			
Homogenate	1.68 (± 0.32)	2.09 (± 0.65)	(6)
S-0.6	1.80 (± 0.39)	2.22 (± 0.47)*	(6)
P-0.6	0.95 (± 0.39)	0.95 (± 0.10)	(6)
S-100	0.76 (± 0.27)	1.02 (± 0.13)*	(6)
P-100	0.93 (± 0.29)	0.89 (± 0.26)	(6)
b)			
Homogenate	0.21 (± 0.07)	0.35 (± 0.04)*	(6)
S-0.6	0.28 (± 0.08)	0.38 (± 0.08)*	(6)
P-0.6	0.09 (± 0.02)	0.12 (± 0.02)*	(6)
S-100	0.17 (± 0.03)	0.30 (± 0.06)*	(6)
P-100	0.09 (± 0.02)	0.08 (± 0.03)	(6)
c)			
Homogenate	0.06 (± 0.01)	0.12 (± 0.05)*	(4)
S-0.6	0.10 (± 0.06)	0.16 (± 0.02)*	(4)
P-0.6	0.02 (± 0.01)	0.03 (± 0.01)	(4)
S-100	0.03 (± 0.02)	0.02 (± 0.02)	(4)
P-100	0.02 (± 0.03)	0.03 (± 0.01)	(4)

* Values assessed by Student t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$)

substrate, is shown in Table 3.1c. Low levels of protein kinase activity were observed in the subcellular fractions. Protein kinase activity was equally distributed among the P-0.6 (35%), S-100 (29%) and P-100 (36%) subcellular fractions. No significant change in protein kinase activity was observed in the P-0.6, S-100 and P-100 subcellular fractions when cyclic AMP was added, although significant cyclic AMP stimulation of protein kinase activity was observed in the islet homogenate and S-0.6 subcellular fraction.

Effect of Substrate on Islet Cell Protein Kinase Activity

Irrespective of the exogenous substrate used, the protein kinase activity observed in the S-0.6 subcellular fraction was greater than that observed in the total islet homogenate (Table 3.2).

TABLE 3.2 - Protein Kinase Activity in the Islet Homogenate and S-0.6 Subcellular Fractions of Guinea-Pig Islets of Langerhans, Using Protamine, Histone and Hydrolysed Casein as Exogenous Substrate

Exogenous Substrate		Protein Kinase Activity (ρ moles ^{32}P/min/mg substrate/islet)		
		Homogenate	S-0.6	
Protamine	- cyclic AMP	1.68 (± 0.32)	1.80 (± 0.39)	(6)
	+ cyclic AMP	2.09 (± 0.65)	2.22 (± 0.47)*	(6)
Histone	- cyclic AMP	0.21 (± 0.07)	0.28 (± 0.08)	(6)
	+ cyclic AMP	0.35 (± 0.04)*	0.38 (± 0.08)*	(6)
Hydrolysed casein	- cyclic AMP	0.06 (± 0.01)	0.10 (± 0.06)	(6)
	+ cyclic AMP	0.12 (± 0.05)*	0.16 (± 0.02)*	(6)

* Values assessed by Student t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

On comparing the protein kinase activity obtained using

different exogenous substrates it was observed that protamine was a better substrate than histone and histone was a better substrate than hydrolysed casein on a weight basis.

The ratio of Protamine:Histone protein kinase activity was found to be higher in the pellet fractions than the S-100 subcellular fraction (Table 3.3).

TABLE 3.3 - Ratio of Protamine:Histone Protein Kinase Activity in Subcellular Fractions of Islets of Langerhans

Subcellular Fraction	- Cyclic AMP	+ Cyclic AMP
P-0.6	10.6	7.9
S-100	4.5	3.4
P-100	10.3	11.1

Subcellular Distribution of Islet Cell Cyclic AMP-independent Protein Kinase Activity

Islet cell protein kinase activity is comprised of cyclic AMP-dependent and independent activities. In order to estimate the contribution of the cyclic AMP-dependent activity to the total protein kinase activity, cyclic AMP-independent protein kinase activity was measured using the Walsh protein, a specific inhibitor of the cyclic AMP-dependent catalytic subunit (Ashby and Walsh, 1972). Cyclic AMP-dependent activity could not be measured directly, as there is no known specific inhibitor of the independent activity.

The subcellular distribution of islet cell cyclic AMP-independent protein kinase activity, using protamine as exogenous substrate, is shown in Table 3.4a.

The majority (45%) of the total islet cell cyclic AMP-independent protein kinase activity was located in the P-100 subcellular fraction. With the exception of the S-0.6 and S-100 subcellular fractions, cyclic AMP had no significant

TABLE 3.4 - Subcellular Distribution of Islet Cell Cyclic AMP-independent Protein Kinase Activity

Islet cell subcellular fractions were prepared and assayed for protein kinase activity in the presence of Walsh protein (> 300 Units/ml), as described in the text, in the absence and presence of 1 μM cyclic AMP. Protamine a) and Histone b) (2 mg/ml) were used as exogenous substrate.

Subcellular Fraction	Protein Kinase Activity (p moles ^{32}P/min/mg substrate/ml)		
a)	- cyclic AMP	+ cyclic AMP	
Homogenate	0.94 (± 0.14)	0.86 (± 0.12)	(4)
S-0.6	1.00 (± 0.18)	1.34 (± 0.18)*	(4)
P-0.6	0.46 (± 0.12)	0.48 (± 0.04)	(4)
S-100	0.31 (± 0.11)	0.46 (± 0.12)*	(4)
P-100	o.74 (± 0.19)	0.78 (± 0.32)	(4)
b)			
Homogenate	0.08 (± 0.03)	0.08 (± 0.01)	(4)
S-0.6	0.09 (± 0.04)	0.09 (± 0.03)	(4)
P-0.6	0.04 (± 0.01)	0.04 (± 0.01)	(4)
S-100	0.04 (± 0.02)	0.04 (± 0.02)	(4)
P-100	0.05 (± 0.01)	0.05 (± 0.01)	(4)

* Values assessed by Student t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

effect on the protein kinase activity observed in the subcellular fractions. Significant cyclic AMP stimulation of protein kinase activity was observed in the S-0.6 and S-100 subcellular fractions and this was thought to be due to the number of free cyclic AMP-dependent protein kinase catalytic subunits exceeding the number of Walsh protein molecules.

The subcellular distribution of islet cyclic AMP-independent protein kinase activity, using histone as exogenous substrate is shown in Table 3.4b. Cyclic AMP-independent protein kinase activity was distributed equally among the P-0.6 (33%), S-100 (36%) and P-100 (31%) subcellular fractions. No significant stimulation of activity was observed in the presence of cyclic AMP, this indicated that no cyclic AMP-dependent protein kinase activity was being measured.

Effect of Substrate on the Subcellular Distribution of Total Islet Cell Cyclic AMP-independent Protein Kinase Activity

Table 3.5, shows how the subcellular distribution of total islet cyclic AMP-independent protein kinase activity varied with the exogenous substrates used. The distribution of the P-0.6 fraction was unaffected by the exogenous substrates use , however, the distribution of the S-100 fraction was decreased and the P-100 was increased when protamine was used instead of histone.

TABLE 3.5 - Subcellular Distribution of Total Islet Cyclic AMP-independent Protein Kinase Activity Using Protamine and Histone as Exogenous Substrate

Subcellular Fraction	Subcellular Percentage Distribution (%)			
	Protamine		Histone	
	--	+cA	--	+cA
P-0.6	30	30	29	33
S-100	20	28	34	36
P-100	49	42	36	31

Results are expressed as a percentage of the sum of the P-0.6, S-100 and P-100 activities.

Greater cyclic AMP-independent protein kinase activity was observed when protamine was used as exogenous substrate, this was more marked in the pellet fractions (Table 3.6), particularly the P-100 subcellular fraction.

TABLE 3.6 - Ratio of Protamine:Histone Cycle AMP-independent Protein Kinase Activity in Subcellular Fractions of Islets of Langerhans

Subcellular Fraction	- Cyclic AMP	+ Cyclic AMP
P-0.6	12.3	12.9
S-100	7.4	7.4
P-100	16.4	20.0

The protein content of the islet cell subcellular fractions was determined and hence the specific activity of the protein kinases in the subcellular fractions was calculated. (Table 3.7a, 3.7b). In the presence of cyclic AMP, using protamine as exogenous substrate, the P-0.6 and P-100 subcellular fractions had the highest protein kinase specific activity, while in the presence of Walsh protein only the P-100 subcellular fraction had the highest protein kinase specific activity.

TABLE 3.7a)-Protein Kinase Specific Activity in Subcellular Fractions of Guinea-Pig Islets of Langerhans Using Protamine as Exogenous Substrate

Subcellular Fraction	Protein (µg/islet)	Specific Activity (^{32}P moles/min/mg substrate/mg islet protein)			
				Walsh	
		- cA	+ cA	- cA	+ cA
Homogenate	4.99	337 (± 64)	419 (± 130)	188 (± 27)	171 (± 24)
P-0.6	1.34	709 (± 291)	709 (± 75)	340 (± 92)	357 (± 28)
S-100	3.35	227 (± 80)	305 (± 39)*	92 (± 33)	137 (± 37)*
P-100	1.15	809 (± 252)	774 (± 226)	644 (± 169)	676 (± 278)

TABLE 3.7b)-Protein Kinase Specific Activity in Subcellular Fractions in Guinea-Pig Islets of Langerhans Using Histone as Exogenous Substrate

Subcellular Fraction	Protein (µg/islet)	Specific Activity (^{32}P moles/min/mg substrate/mg islet protein)			
				Walsh	
		- cA	+ cA	- cA	+ cA
Homogenate	4.99	42 (± 14)	70 (± 8)*	15 (± 5)	17 (± 2)
P-0.6	1.34	67 (± 15)	89 (± 15)*	28 (± 1)	28 (± 2)
S-100	3.35	51 (± 9)	89 (± 18)*	13 (± 6)	12 (± 6)
P-100	1.15	78 (± 17)	70 (± 26)	39 (± 6)	29 (± 9)

* Values assessed by Student t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

In the presence of cyclic AMP, using histone as exogenous substrate, the P-0.6 and S-100 subcellular fractions had the highest protein kinase specific activity, while in the presence of Walsh protein, the P-100 subcellular fraction had the highest protein kinase specific activity.

Subcellular Distribution of Islet Cell Cyclic AMP-dependent Protein Kinase Activity

By subtracting the cyclic AMP-independent protein kinase activity from the total protein kinase activity an estimate of cyclic AMP-dependent protein kinase activity could be obtained. Table 3.8a shows the subcellular distribution of total islet cell cyclic AMP-dependent protein kinase activity, using protamine as exogenous substrate.

The majority (50%) of the total islet cell cyclic AMP-dependent protein kinase activity was located in the S-100 subcellular fraction, the P-0.6 fraction contained 40% and the P-100 fraction contained 10% of the total activity. No significant change in activity was observed in the presence of cyclic AMP in the P-0.6 and P-100 subcellular fractions. In the S-100 subcellular fraction a significant increase in activity was observed in the presence of cyclic AMP.

The subcellular distribution of total cell cyclic AMP-dependent protein kinase activity using histone as exogenous substrate is shown in Table 3.8b. The majority (67%) of the total cyclic AMP-dependent protein kinase activity was located in the S-100 subcellular fraction, the P-0.6 fraction contained 21% and the P-100 subcellular fraction contained 12% of the total activity. Significant cyclic AMP stimulation of protein kinase activity occurred in the P-0.6 and S-100 subcellular fractions, however, the P-100 subcellular fraction showed no significant increase in activity in the presence of cyclic AMP.

TABLE 3.8 - Subcellular Distribution of Islet Cell Cyclic AMP-dependent Protein Kinase Activity

Cyclic AMP-independent activity (Table 3.4) was subtracted from the protein kinase activity (Table 3.1). Protamine a) and Histone b) (2 mg/ml) was used as exogenous substrate.

Subcellular Fraction	Protein Kinase Activity (p moles ^{32}P/min/mg substrate/islet)	
a)	- cyclic AMP	+ cyclic AMP
Homogenate	0.74 (± 0.46)	1.23 (± 0.46)*
S-0.6	0.80 (± 0.57)	0.88 (± 0.65)
P-0.6	0.49 (± 0.51)	0.47 (± 0.14)
S-100	0.45 (± 0.38)	0.56 (± 0.25)
P-100	0.19 (± 0.48)	0.11 (± 0.58)
b)		
Homogenate	0.13 (± 0.10)	0.27 (± 0.05)*
S-0.6	0.19 (± 0.12)	0.29 (± 0.11)
P-0.6	0.05 (± 0.03)	0.08 (± 0.03)
S-100	0.13 (± 0.05)	0.27 (± 0.07)*
P-100	0.04 (± 0.03)	0.03 (± 0.06)

* Values assessed by Student t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

Effect of Substrate on the Subscellular Distribution of Total Islet Cell Cyclic AMP-dependent Protein Kinase Activity

The subcellular distribution of total islet cell cyclic AMP-dependent protein kinase activity varied with the exogenous substrate used. (Table 3.9).

TABLE 3.9 - Subcellular Distribution of Total Islet Cyclic AMP-dependent Protein Kinase Activity Using Protamine and Histone as Exogenous Substrate

Subcellular Fraction	Subcellular Percentage Distribution (%)			
	Protamine		Histone	
	-	+cA	-	+cA
P-0.6	45	35	27	21
S-100	40	50	54	67
P-100	15	14	19	12

Results are expressed as a percentage of the sum of the P-0.6, S-100 and P-100 activities.

The subcellular distribution of the S-100 fraction decreased and that of the P-0.6 fraction increased when protamine was used instead of histone. The subcellular percentage distribution of the P-100 fraction was unaffected by the change in exogenous substrate.

Greater cyclic AMP-dependent protein kinase activity was observed when protamine was used as exogenous substrate, this was more marked in the P-0.6 subcellular fraction (Table 3.10).

TABLE 3.10 - Ratio of Protamine : Histone Cyclic AMP-dependent Protein Kinase Activity in Subcellular Fractions of Islets of Langerhans

Subcellular Fraction	- cAMP	+ cAMP
P-0.6	7.9	6.0
S-100	3.5	2.7
P-100	3.8	4.2

Diethylaminoethyl (DEAE) - Cellulose Chromatography

a) Total Islet Cell Sonicate

Three peaks of cyclic AMP-dependent protein kinase activity were separated by DEAE-cellulose chromagraphy (Figure 3.1). Type I isozyme exhibited stimulation of activity upon the addition of cyclic AMP and was eluted in 0-50mM NaCl. The second peak of protein kinase activity was designated type IIa, as it was eluted from the DEAE-cellulose chromatography column in 100-175 mM NaCl. This isozyme showed a marked stimulation of activity upon the addition of cyclic AMP. Type IIb isozyme was eluted in 175-300 mM NaCl and exhibited stimulation of activity upon the addition of cyclic AMP. Type II (a + b) had a greater total protein kinase activity than Type I cyclic AMP-dependent protein kinase isozyme.

The presence of two Type II cyclic AMP-dependent protein kinase isozymes was thought to have been due to proteolytic degradation.

b) Islet Cell S-100 Subcellular Fraction

Several experiments were performed in an attempt to obtain a cyclic AMP-dependent protein kinase isozyme profile for the islet S-100 subcellular fraction. Examples of typical profiles obtained are presented in this thesis. Problems were encountered in finding a suitable protease inhibitor to eliminate artifactual peaks of cyclic AMP-dependent protein kinase activity.

The use of the (^{3}H)-cyclic AMP binding assay (described in detail in Chapter II) was found to be unreliable and in later experiments the DEAE-cellulose chromatography column eluant fractions were not assayed for (^{3}H)-cyclic AMP binding

Figure 3.1. DEAE-cellulose Chromatography of Islet Sonicates.
Islet sonicates were prepared and applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) as described in the text. Protein kinase activity was assayed in duplicate, in the absence (O) and presence (●) of 1μM cyclic AMP. Histone (2mg/ml) was used as exogenous substrate.

^{32}P Incorporation
($\times 10^3$ cpm)
3
2
1
10
20
Elution Volume (ml)
500
400
300
200
100
0
[NaCl] (mM)

Figure 3.2. DEAE-cellulose Chromatography of Islet S-100 Subcellular Fractions.
Islet S-100 subcellular fractions were prepared and applied to a DEAE-cellulose chromatography column (1.2cm x 5.0cm) as described in the text. Protein kinase activity was assayed in duplicate in the absence (O) and presence (●) of 1μM cyclic AMP. Histone (2mg/ml) was used as exogenous substrate. The reactions were terminated using the TCA precipitation method described in detail in the text.

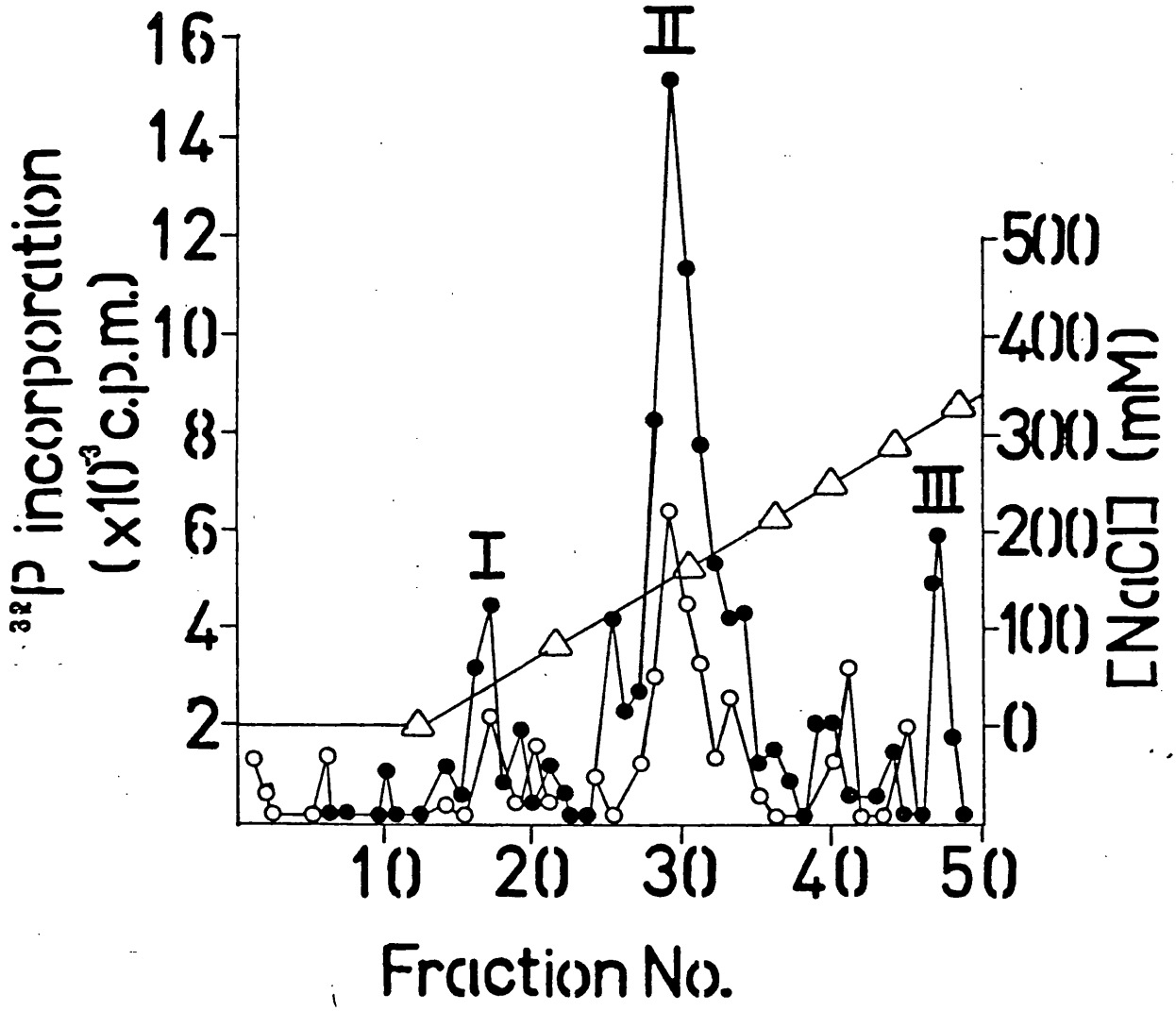
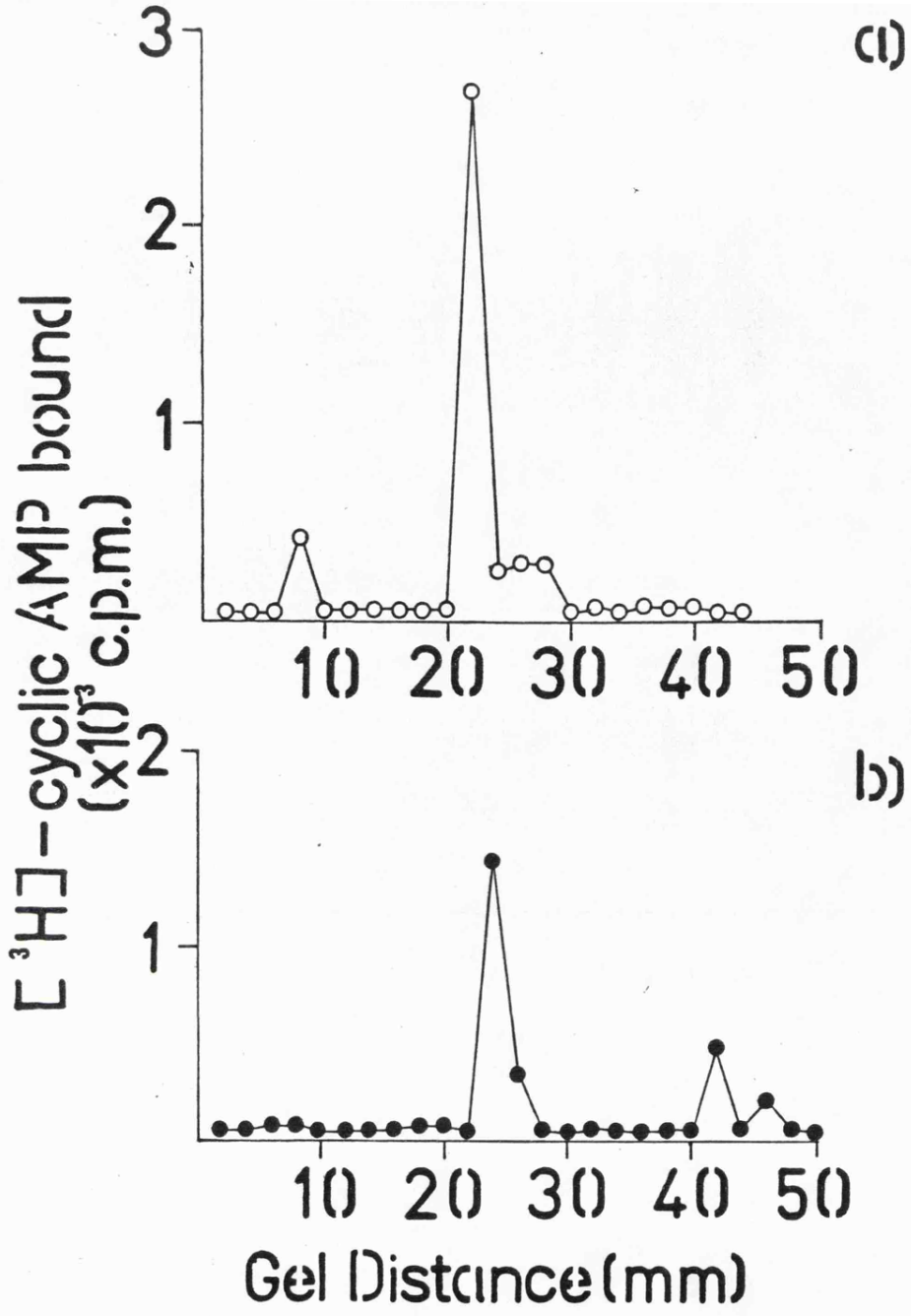

16
14
12
10
8
6
4
2
^{32}P incorporation
(x10^{3}c.p.m.)
I
II
III
500
400
300
200
100
0
[NaCl] (mM)
10
20
30
40
50
Fraction No.

Figure 3.3. S.D.S. Polyacrylamide Gel Electrophoresis of Islet S-100 Type I cyclic AMP binding activity.
The pooled freeze dried fractions of islet S-100 Type I (Fig. 3.2) protein kinase activity were reconstituted and subjected to S.D.S. Polyacrylamide gel electrophoresis. The gels were either assayed for cyclic AMP binding activity in the presence (a) and absence (b) of histone (0.2mg/ml) plus NaCl (800mM) or stained for protein (c) as described in detail in the text.


a)
b)
[³H]-cyclic AMP bound
(x10³ c.p.m.)
3
2
1
10 20 30 40 50
2
1
10 20 30 40 50
Gel Distance (mm)

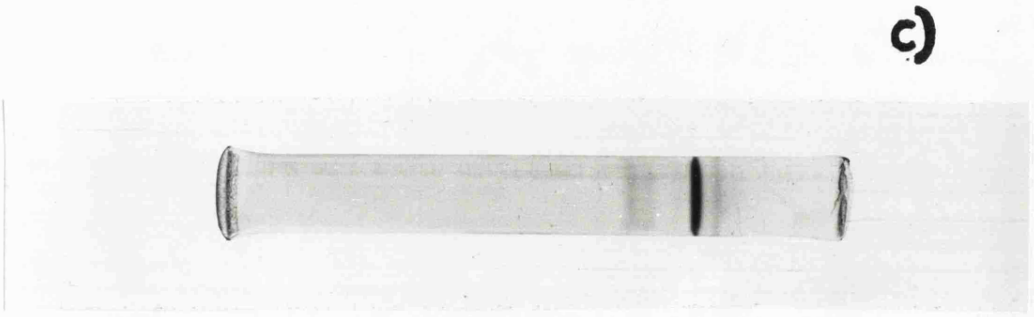
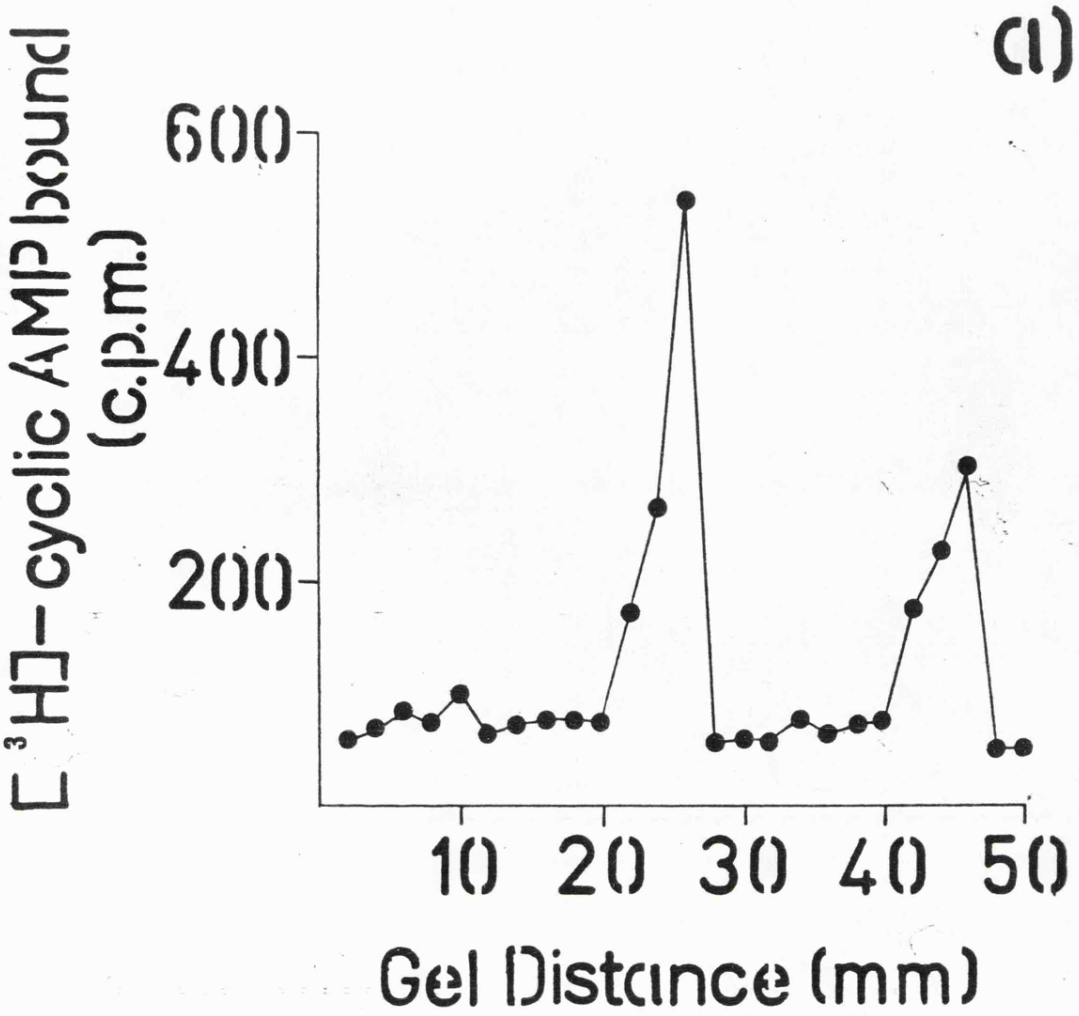

c)

Figure 3.4. S.D.S. Polyacrylamide Gel Electrophoresis of Islet S-100 Type II cyclic AMP binding activity.
The pooled freeze dried fractions of islet S-100 Type II (Fig. 3.2) protein kinase activity were reconstituted and subjected to S.D.S. Polyacrylamide gel electrophoresis. The gels were either assayed for cyclic AMP binding in the presence and absence (a) of histone (0.2mg/ml) plus NaCl (800mM) or stained for protein (b) as described in detail in the text.

a)


[3H]-cyclic AMP bound (c.p.m.)
600
400
200
10
20
30
40
50
Gel Distance (mm)

b)

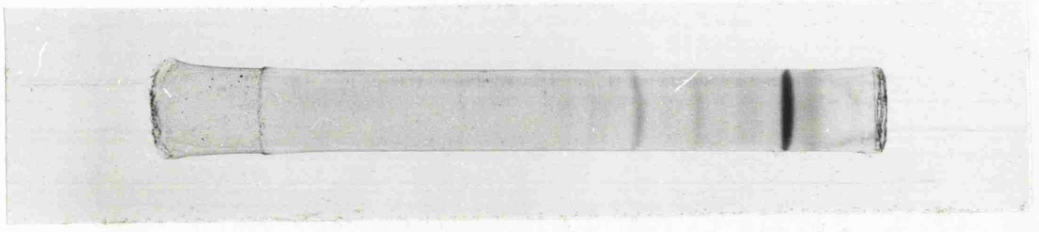
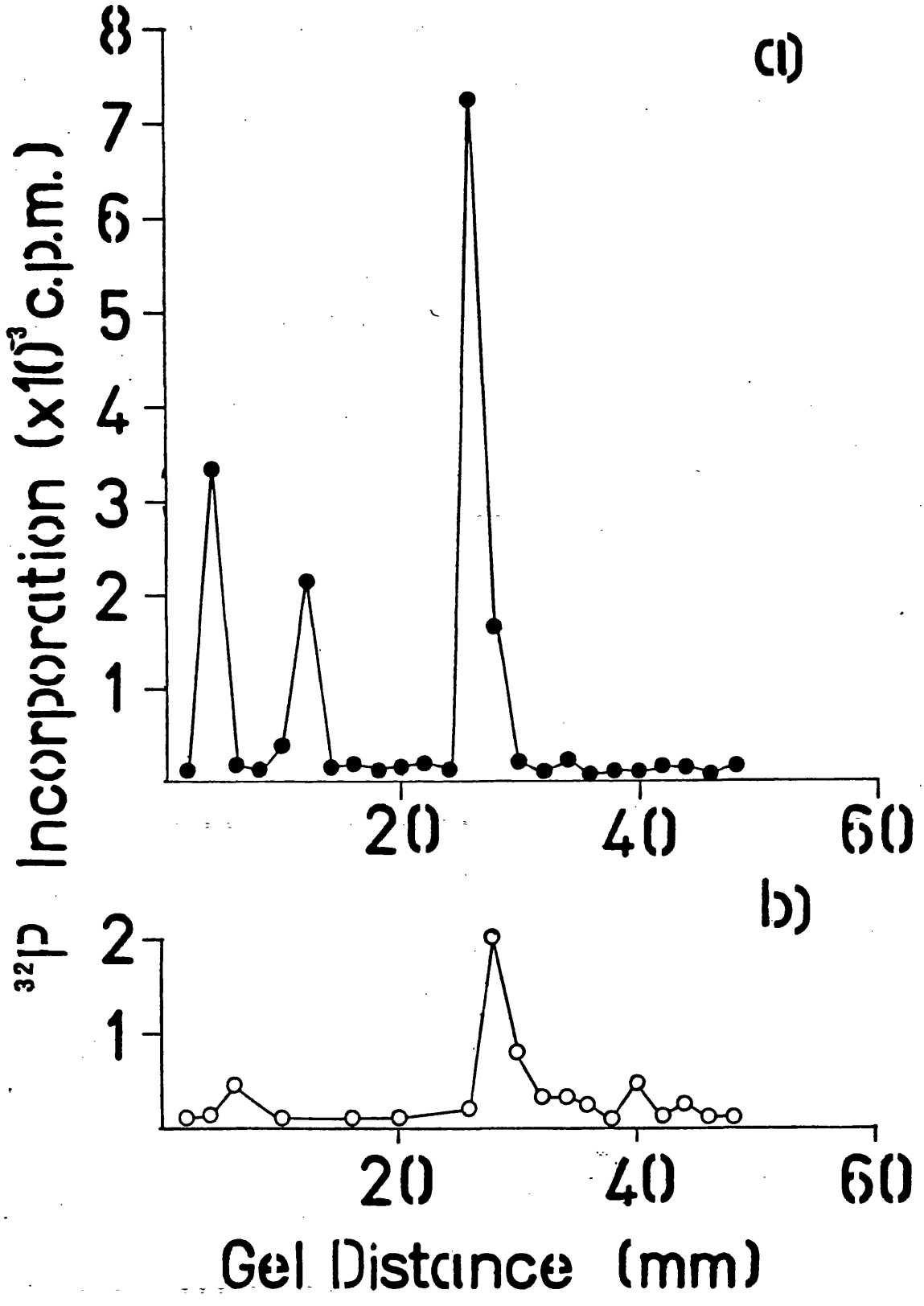


Figure 3.5. Native Polyacrylamide Gel Electrophoresis of Islet S-100 Type III Protein Kinase Activity.
The pooled freeze dried fractions of islet S-100 Type III (Fig. 3.2) protein kinase activity were reconstituted and subjected to Native (Davis) Polyacrylamide gel electrophoresis. The gels were assayed for protein kinase activity in the presence (a) and absence (b) of 1μM cyclic AMP as described in detail in the text.


a)
b)
^{32}P Incorporation (x10^{3} c.p.m.)
Gel Distance (mm)
1
2
3
4
5
6
7
8
20
40
60

activity.

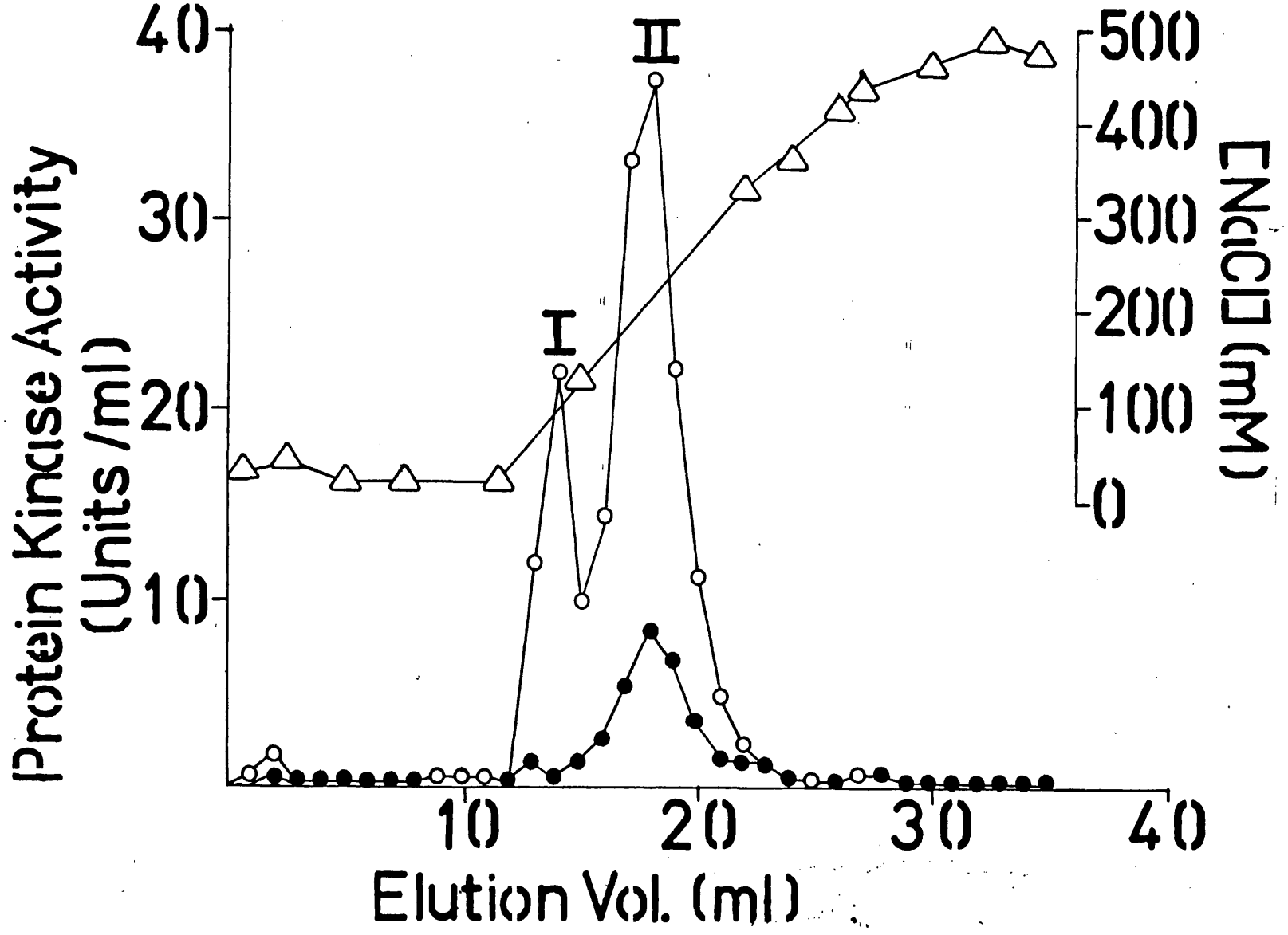
Three protein kinase isozymes were separated by DEAE cellulose chromatography, using a (1.2 cm x 5.0 cm) column (Figure 3.2) Type I isozyme, eluted in 25-50 mM NaCl, exhibited a marked increase in activity upon the addition of cyclic AMP. Type II isozyme consisted of a single major peak eluted in 125-200 mM NaCl and a marked stimulation of protein kinase activity was observed in the presence of cyclic AMP. Type II isozyme contained a greater total amount of activity than the other two isozymes. Type III isozyme eluted in 300 mM NaCl, exhibited no basal protein kinase activity in the absence of cyclic AMP.

The peak fractions of protein kinase activity were pooled, freeze dried, and reconstituted for disc polyacrylamide gel electrophoresis. S.D.S. disc polyacrylamide electrophoresis gels were run for Type I and II isozymes. Single major peaks of (^{3}H)-cyclic AMP binding were observed (Figure 3.3 and 3.4). The (^{3}H)-cyclic AMP binding activity of the Type I isozyme was stimulated in the presence of Histone (0.2 mg/ml) plus NaCl (800 mM) (Figure 3.3a) but no stimulation of Type II (^{3}H)-cyclic AMP binding activity was observed. No major peak of (^{3}H)-cyclic AMP binding activity corresponded to a major protein band and no protein kinase activity was detected.

Native (Davis) disc polyacrylamide gels were run for the Type III isozyme. A major peak of cyclic AMP-dependent protein kinase activity was demostrated (Figure 3.5), but no (^{3}H)-cyclic AMP binding activity could be detected.

When Benzamidine (a protease inhibitor) and dithiothreitol (D.T.T.) were added to the homogenisation buffer, two S-100 cyclic AMP-dependent protein kinase isozymes were separated

Figure 3.6. DEAE-cellulose Chromatography of Islet S-100 Subcellular Fractions.
Islet S-100 subcellular fractions were prepared and applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) as described in the text. Protein kinase activity was assayed in duplicate in the presence of 1μM cyclic AMP, using histone (●) and protamine (○) (2mg/ml) as exogenous substrate. The reactions were terminated using the Glass (1978) method described in detail in the text.


Protein Kinase Activity (Units/ml)
40
30
20
10
I
II
500
400
300
200
100
0
[NaCl] (mM)
10
20
30
40
Elution Vol. (ml)

by DEAE-cellulose chromatography using a (0.5 cm x 3.0 cm) column (Figure 3.6). Protein kinase activity was measured in the presence of cyclic AMP, using either histone or protamine as exogenous protein substrate. Type I isozyme, eluted in 65-125 mM NaCl, showed little activity when histone was used as the exogenous protein substrate. Type II isozyme, eluted in 150-300 mM NaCl, could be detected when either protamine or histone was used as exogenous protein substrate. Table 3.11 shows the S-100 Type I cyclic AMP-dependent protein kinase isozyme had a much higher Protamine : Histone activity ratio than the Type II isozyme.

TABLE 3.11 - Protein Kinase Activity Ratios (Protamine : Histone) for Type I and Type II S-100 Cyclic AMP-dependent Protein Kinases in the Presence of Cyclic AMP (1 μM)

Cyclic AMP-dependent Protein Kinase Isozyme	Activity Ratio
Type I	14.5
Type II	4.5

c) Islet P-100 Subcellular Fraction

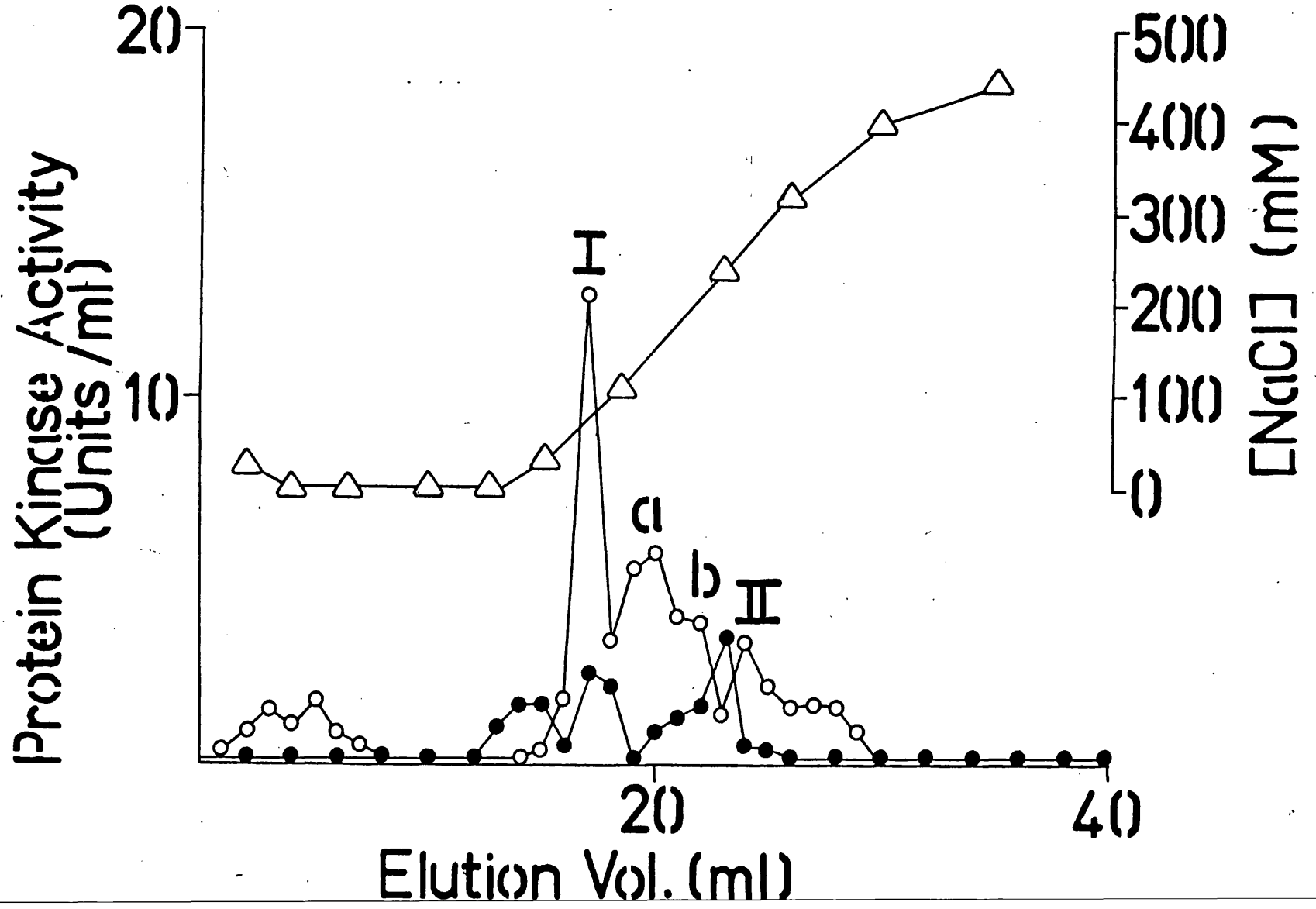
In this study no attempt was made to extract the solublised islet cell P-100 proteins by centrifugation because of the small amounts of tissue involved.

Four peaks of protein kinase activity from an islet cell P-100 subcellular fraction, using protamine as exogenous substrate, were separated by DEAE-cellulose chromatography, using a (0.5 cm x 3.0 cm) column (Figure 3.7). The first and major peak of protein kinase activity, eluted in 0-20 mM NaCl, was stimulated by the addition of cyclic AMP (1 μM) and was designated Type I cyclic AMP-dependent protein kinase isozyme. Peaks 2 and 3, eluted in 50-70 mM and

Figure 3.7. DEAE-cellulose Chromatography of Islet P-100 Subcellular Fractions.
Islet P-100 subcellular fractions were prepared and applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) as described in the text. Protein kinase activity was assayed in duplicate in the absence (O) and presence (●) of 1μM cyclic AMP. Protemine (2mg/ml) was used as exogenous substrate and the reactions were terminated using the Glass (1978) method described in detail in the text.

[NaCl] (mM)
300
200
100
0
Protein Kinase Activity
(Units /ml)
30
20
10
I
a
b
II
10
20
30
Elution Vol. (ml)

Figure 3.8. DEAE-cellulose Chromatography of Islet P-100 Subcellular Fractions.
Islet P-100 subcellular fractions were prepared and applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) as described in the text. Protein kinase activity was assayed in duplicate in the presence of 1μM cyclic AMP, using histone (●) and protamine (O) (2mg/ml) as exogenous substrate. The reactions were terminated using the Glass (1978) method described in detail in the text.


20
10
Protein Kinase Activity (Units/ml)
500
400
300
200
100
0
[NaCl] (mM)
I
a
b
II
20
40
Elution Vol. (ml)

100-120 mM NaCl respectively, exhibited no stimulation of activity upon the addition of cyclic AMP and were designated cyclic AMP-independent protein kinase a and b respectively. Peak 4, eluted in 130-160 mM NaCl, was stimulated by the addition of cyclic AMP and was designated Type II cyclic AMP-dependent protein kinase.

The effect of varying the exogenous substrate on islet cell P-100 protein kinase activities separated by DEAE-cellulose chromatography was examined (Figure 3.8). On changing the exogenous substrate to histone a reduction in the protein kinase activity in the DEAE—cellulose chromatography eluant was observed.

The ratio of protamine : histone P-100 protein kinase activity in the presence of cyclic AMP (1 μM) of the four peaks of activity separated by DEAE-cellulose chromatography was determined (Table 3.12).

TABLE 3.12 - The Ratio of Protamine : Histone Protein Kinase Activity of Peaks of Activity Separated by DEAE-cellulose Chromatography From an Islet P-100 Subcellular Fraction

Peak	Ratio Protamine : Histone Protein Kinase Activity
1) (Type I cyclic AMP-dependent)	5.14
2) (Type a cyclic AMP-independent)	7.11
3) (Type b cyclic AMP-independent)	2.44
4) (Type II cyclic AMP-dependent)	6.33

Both cyclic AMP-dependent protein kinase isozymes had activity ratios that were similar to that observed for the S-100 cyclic AMP-dependent protein kinase Type II isozyme.

The activity ratios for the two cyclic AMP-independent protein kinases were markedly different, type a kinase had a greater preference for protamine as exogenous substrate.

Discussion

Subcellular Distribution of Islet Cell Protein Kinase Activity

In this study, using the method described by Glass et al. (1978) for the determination of protein kinase activity, it was observed that on a weight basis protamine was preferred to histone and histone was preferred to hydrolysed casein as exogenous protein substrate, irrespective of the addition of test substances to the reaction medium. This may in part be due to protamine being a more basic protein than histone and histone being a more basic protein than hydrolysed casein. The more basic the protein is, the better it is adsorbed onto phosphocellulose (P81) paper (a cation exchange).

Irrespective of the additions of test substances to the reaction medium, the protein kinase activity observed in the S-0.6 fraction was greater than that observed in the total islet homogenate. This was probably due to the inefficiency of the first homogenisation to release all of the soluble protein kinase activity present in the islet cell.

Effect of Substrate on Subcellular Distribution of Total Islet Cell Protein Kinase Activity

The apparent subcellular distribution of total islet cell protein kinase activity was dependent upon the exogenous substrate used (Table 3.1). This was a reflection of the ability of the different subcellular fractions to utilise different substrates and not due to the artifactual differences in protein kinase activity observed in the presence of the different exogenous protein substrates caused by the method of assay. The majority of protein kinase activity was located in the islet cell S-100 fraction when histone was used, with protamine and hydrolysed casein the protein kinase activity was equally distributed among the islet cell

P-0.6, S-100 and P-100 subcellular fractions. This suggested that either protamine and hydrolysed casein were being preferentially phosphorylated by the protein kinases in the membrane containing subcellular fractions or that histone was being preferentially phosphorylated by the protein kinases in the S-100 fraction or both situations were occurring concurrently. This correlated with a previous study which demonstrated that membrane bound cyclic AMP-dependent protein kinases preferred protamine to histone and that soluble cyclic AMP-dependent protein kinases preferred histone to protamine as exogenous substrate (Uno et al., 1976). Indeed it was observed that the P-0.6 and P-100 subcellular fractions did have a much higher protamine : histone activity ratio than the S-100 fraction. In this study any preferential phosphorylation of histone by the S-100 fraction would be masked because of the assay method used.

Montague and Howell (1972) and Sussman and Leitner (1977), using rat islets of Langerhans, have both shown that the P-0.6 fraction contained 25%, the P-100 fraction 20% and the S-100 fraction 55% of the total islet cell cyclic AMP stimulated protein kinase activity, using histone as exogenous substrate.

This compared favourably with that found in this study. This suggested that either the solublisation of the membrane containing fractions with Nonidet - P40 did not release significant amounts of protein kinase activity or that Nonidet-P40 inhibited the activity released.

No cyclic AMP stimulation of protein kinase activity in the islet cell P-0.6 fraction was reported in previous studies, while in this study significant cyclic AMP stimulation

of activity was observed when histone was used as exogenous substrate. This perhaps suggested Nonidet-P40 may have released a small amount of nuclear cyclic AMP-dependent protein kinase activity. In the islet cell P-100 fraction no cyclic AMP stimulation of protein kinase activity (irrespective of the exogenous substrate used) was observed in this and previous studies. (Although Sussman and Leitner (1977) did observe a marked cyclic AMP stimulation of protein kinase activity in a secretory graunule (P-24) fraction). This correlated with the observation that the majority (80%) of protein kinase activity present in the P-100 fraction was cyclic AMP-independent (Table 3.7).

In previous studies in rat islets (Lipson et al., 1979; Sugden et al., 1979), an approximate three fold cyclic AMP stimulation of protein kinase activity was observed in the islet homogenate, however this study demonstrated a two fold stimulation. This suggested that either the cyclic AMP-dependent protein kinase present in islet cells were partially dissociated before the addition of cyclic AMP or that the cyclic AMP protein kinases activity ratio was higher in rat islets than in guinea-pig islets. It is more likely that the former occurred in this study. The dissociation of the cyclic AMP-dependent protein kinases could have been due to the relatively long reaction time (20 minutes), facilitating the dissociation of the R_2C_2 holoenzyme. It was thought that the NaF (20 mM) present in the reaction medium was unable to generate sufficient cyclic AMP (by activating adenyl cyclase activity (Martin et al., 1979) to allow dissociation of the R_2C_2 holoenzyme.

Effect of Substrate on the Subcellular Distribution of Total Islet Cell Cyclic AMP-dependent Protein Kinase Activity

The subcellular distribution of islet cell cyclic AMP-independent protein kinase activity was also dependent upon the exogenous protein substrate used. (Table 3.4). The protamine : histone activity ratio (Table 3.6) was found to be much greater in the islet cell P-100 (and to a lesser extent in the P-0.6) subcellular fraction. This suggested protamine was the preferred substrate for the islet cell P-100 cyclic AMP-independent protein kinase. This correlated with a previous observation of a protein kinase which utilised histone but preferred protamine as an exogenous substrate, but was unaffected by the presence of cyclic AMP (Kuehn, 1971).

By comparing the islet cell subcellular distribution of cyclic AMP-independent protein kinase activity with that of the total protein kinase activity, an estimate of the distribution and type of protein kinase activity could be obtained. Using protamine as exogenous protein substrate, 50% of the total islet protein kinase activity was found to be cyclic AMP-independent. The majority (42%) of the total islet cyclic AMP-independent activity was located in the P-100 subcellular fraction, while the majority (50%) of the total islet cyclic AMP-dependent protein kinase activity was located in the S-100 subcellular fraction. Using histone as exogenous substrate, 25% of the total islet protein kinase activity was found to be cyclic AMP-independent. Total islet cyclic AMP-independent protein kinase activity was equally distributed among the P-0.6, S-100 and P-100 subcellular fractions. The majority (75%) of total islet cyclic AMP-dependent protein kinase activity was located in the S-100 subcellular

fraction.

Islet Cell Sonicate DEAE-Cellulose Chromatography

Three cyclic AMP-dependent protein kinase isozymes, designated Type I, Type IIa and Type IIb, were separated by DEAE-cellulose chromatography from guinea-pig islet cell sonicates. Type I and Type II cyclic AMP-dependent protein kinase isozymes have been partially characterised in rat islet sonicates. (Sugden et al., 1979; Oliver and Kemp, 1980). In agreement with these studies, the Type II cyclic AMP-dependent protein kinase was found to be the predominant isozyme.

Although the position of the Type IIa cyclic AMP-dependent protein kinase corresponded to that where a rat islet cyclic AMP-independent protein kinase was found (Sugden et al., 1979). It was thought the Type IIa protein kinase was cyclic AMP-dependent because the rat islet cyclic AMP-independent protein kinase could only phosphorylate hydrolysed casein and not histone. The presence of two Type II isozymes suggested the possibility that one may have originated from the P-100 subcellular fraction and the other from the S-100 subcellular fraction. As very small amounts of the Type II isozyme were found in the P-100 subcellular fraction, this indicated that even in the presence of P.M.S.F. proteolytic degradation had been the probable cause for the appearance of two Type II isozymes.

The interpretation of an isozyme pattern obtained from a tissue sonicate should be treated with caution, as it may not represent the total protein kinase activity of that tissue. Because of the formation of membrane vesicles during sonication some membrane bound protein kinases may go undetected.

Islet S-100 DEAE-Cellulose Chromatography

Two cyclic AMP-dependent protein kinases, designated Type I and Type II were separated by DEAE-cellulose chromatography from islet S-100 subcellular fractions. Type II cyclic AMP-dependent protein kinase was found to be the predominant isozyme. (Figure 3.6).

The data presented in this study highlights the importance of the inclusion of a suitable protease inhibitor, such as Benzamidine, in the preparation of cyclic AMP-dependent protein kinases. In the absence of Benzamidine, three cyclic AMP-dependent protein kinases were separated by DEAE-cellulose chromatography from islet S-100 subcellular fractions. This suggested that the appearance of more than two cyclic AMP-dependent protein kinases was due to proteolytic degradation.

Two endogenous proteases specific for cyclic AMP-dependent protein kinases have been detected. In bovine heart it has been shown that an endogenous protease, specific for the Regulatory subunit of the Type II cyclic AMP-dependent protein kinase isozyme, was stimulated by Ca^{2+} ions and inhibited by Benzamidine (Corbin et al., 1977). In brush border membranes isolated from rat small intestine, an endogenous protease specific for the Catalytic subunit of cyclic AMP-dependent protein kinase has been identified. The specific, restricted and limited action of the protease, together with the prevention of its action by the substrate and the Regulatory subunit has suggested that this membranal protease may have a distinct physiological (possibly regulatory) role (Alhanaty and Shaltiel, 1979). Thus there is a possibility that an endogenous protease specific for cyclic AMP-dependent

protein kinases may be present in islets of Langerhans.

The possibility of proteolytic degradation by exogenous proteases can not be ignored. Islets of Langerhans are surrounded by pancreatic acinar tissue rich in proteolytic enzymes. Contamination of the islet tissue with small amounts of acinar tissue was very likely to occur during the isolation procedure and this could have caused the proteolysis observed.

The observed proteolytic degradation products of the islet cell cyclic AMP-dependent kinases retained their catalytic and regulatory activity and were eluted in higher NaCl concentrations. This was thought to be due to the proteolytic action removing small amounts of the polypeptides thereby exposing more charged sites but enabling the protein kinase to retain its regulatory and catalytic activity. In rat brown adipose tissue, the presence of eight cyclic AMP-dependent protein kinases has been claimed. Each kinase has been shown to exhibit different catalytic properties and their activities have been shown to change during post-natal development (Knight and Skala, 1977). In view of the findings of this study, it was thought that such a study must be subjected to some sceptism.

Polyacrylamide gel electrophoresis of the pooled fractions from the DEAE-cellulose chromatography columns (Figures 3.3 and 3.4) showed the fractions were heterogenous with respect to proteins and the cyclic AMP-dependent protein kinases were not the major proteins present.

(^{3}H)-cyclic AMP binding of the pooled fractions could be detected on S.D.S. polyacrylamide gel electrophoresis but not on native polyacrylamide gel electrophoresis. This was thought be have been due to the comigration of the R_2C_2 holenzyme

within the matrix of the native polyacrylamide gel which prevented the detection of receptor subunits. Type I cyclic AMP-dependent protein kinase, but not Type II, exhibited enhanced (^{3}H)-cyclic AMP binding in the presence of histone (0.2 mg/ml) plus NaCl (800 mM) (Figure 3.3a), this correlated with previous observations (Sugden and Corbin, 1976; Tao and Hackett, 1973). It has been shown Type I isozyme is dissociated by histone or high salt concentrations more rapidly than the Type II isozyme (Corbin et al., 1975).

Protein kinase activity could be detected on native electrophoresis gels but not on S.D.S. electrophoresis gels. This was thought to be due to the inefficiency of the washing procedure to remove sufficient S.D.S. to allow renaturation to occur.

The Protamine : Histone activity ratio of the two cyclic AMP-dependent protein kinases identified in the islet S-100 fraction were very different. Type I cyclic AMP-dependent protein kinase isozyme had a much greater preference for protamine as an exogenous substrate, than did the Type II isozyme. This perhaps suggested that the Type I isozyme may have been originally a membrane bound enzyme. Translocations of catalytic subunits of protein kinase have been reported, the most common being translocation from the cytosol to the nucleus (Palmer et al., 1975; Costa et al., 1976) but recently translocations from the particulate to the cytosol have also been reported (Corbin et al., 1977; Hayes et al., 1980).

Islet P-100 DEAE-Cellulose Chromatography

Two cyclic AMP-dependent protein kinase isozymes were separated by DEAE-cellulose chromatography from islet cell

P-100 subcellular fractions solublised with Nonidet-P40 (0.5%). Type I cyclic AMP-dependent protein kinase was found to be the predominant isozyme with a high level of basal activity. This was the reverse of the isozyme profile found in the islet cell S-100 fraction. Two cyclic AMP-independent protein kinases were also separated by DEAE-cellulose chromatography. These were designated Type a and b in order of their elution from the DEAE-cellulose chromatography column. From the previous studies on the subcellular distribution of total islet protein kinase activity it was shown that 80% of the total P-100 fraction protein kinase activity was cyclic AMP-independent. (Figure 3.7). In contrast, the majority of protein kinase activity observed in the DEAE-cellulose chromatography column eluant, was cyclic AMP-dependent. This perhaps suggested that higher NaCl concentrations were required to elute further cyclic AMP-independent protein kinases (Baggio et al , 1970).

The Protamine : Histone activity ratios for the islet cell P-100 cyclic AMP-dependent protein kinases were similar to that obtained for the islet cell S-100 Type II cyclic AMP-dependent protein kinase. The protamine : histone activity ratio for the two cyclic AMP-independent protein kinases were quite different, Type a showed a preference for protamine as exogenous protein substrate. This suggested that the Type a protein kinase was similar to that described by Kuehn (1971). Type b protein kinase showed a twofold preference for protamine as exogenous protein substrate. This was probably due to the increased adsorption of protamine onto P81 paper and not due to an increase in phosphorylation. This suggested the possibility that this protein kinase may

preferentially phosphorylate hydrolysed casein or phosvitin.

Summary

The data presented in this chapter indicate that the majority of the islet cell cyclic AMP-dependent protein kinase activity is located in the S-100 subcellular fraction. Type II cyclic AMP-dependent protein kinase isozyme was found to be the predominant molecular form in the islet S-100 fraction. The islet P-100 subcellular fraction did contain a small amount of cyclic AMP-dependent protein kinase activity and the predominant molecular form was found to be the Type I isozyme. The significance of these findings in respect of the role of cyclic AMP in insulin secretion will be discussed in Chapter VI.

The majority of the total islet cell cyclic AMP-independent protein kinase activity was located in the P-100 subcellular fraction. A partial characterisation of two molecular forms was made.

No significant increase in protein kinase activity was detected in the P-0.6 and P-100 subcellular fractions solubilised with the non-ionic detergent, Nonidet-P40.

CHAPTER IV

Studies on the Effect of Nonidet-P40 on Islet Cell Protein Kinase Activity

INTRODUCTION

The results presented in Chapter III indicated that there was no demonstrable increase in the protein kinase activity of the membrane fractions from islet cells, after solublising the membranes with Nonidet-P40 (0.5%). The studies reported in this chapter were undertaken to investigate the possibility that Nonidet-P40 might have inhibited islet cell protein kinase activity and thereby prevented such an increase. In addition, studies were undertaken to investigate the possibility that Nonidet-P40 might inhibit islet cell ATPase activity, since indirect evidence has suggested that this may occur in rat synaptic membranes (Thérien & Mushynski, 1979).

The effect of Nonidet-P40 upon DEAE-cellulose chromatography of islet cell S-100 cyclic AMP-dependent protein kinases was also examined together with the effect of Nonidet-P40 on the time course of protein kinase activity, using islet cell S-100 protein kinases and partially purified rabbit skeletal muscle cyclic AMP-dependent protein kinases. Rabbit skeletal muscle cyclic AMP-dependent protein kinases were used to investigate the possibility that the Nonidet-P40 effect upon protein kinase activity might be unique to the islet cell.

Methods

Preparation of Islet Subcellular Fractions

Islets of Langerhans were isolated using the methods described in Chapter II. Islets were homogenised in Buffer A (Appendix), subcellular fractions were prepared by differential centrifugation (described in detail in Chapter II), and the fractions obtained were diluted with homogenisation buffer to give the following concentrations:- Homogenate (106-125 islets/ml), P-0.6 (318-375 islets/ml), S-0.6 (48-75 islets/ml), P-100 (53-225 islets/ml) and S-100 (48-125 islets/ml). The P-0.6 and P-100 subcellular fractions were homogenised in homogenisation buffer containing Nonidet-P40 (0.5%). Nonidet-P40 was added to aliquots of the homogenate, S-0.6 and S-100 subcellular fractions to give a final concentration of 0.5ml/100ml.

Determination of Protein Kinase Activity

Protein kinase activity was measured using the method described in detail in Chapter II. 50µl of sample was used and the reaction conditions were 20 min at 37°C. Protamine (2mg/ml) and histone (2mg/ml) were used as exogenous substrates and cyclic AMP dependency was measured in the absence and presence of 1µM cyclic AMP.

Determination of (γ-^{32}P)- ATP Hydrolysis and ^{32}P Production

(γ -^{32}P) - ATP hydrolysis and ^{32}P production were measured using the method described in detail in Chapter II. The reaction conditions were the same as that used in the assay of protein kinase activity.

Islet S-100 DEAE-Cellulose Chromatography

Islet S-100 subcellular fractions were prepared as previously described (Chapter II). The S-100 fractions were divided into two equal volumes, to one Nonidet-P40 was added to give a final concentration of 0.5%, to the other an equal volume of

homogenisation buffer minus sucrose was added. The two samples were incubated at 4°C for 18 hours, then applied separately to a DEAE-cellulose chromatography column (0.5cm x 3.0cm). The DEAE-cellulose used for the Nonidet-P40 treated S-100 fraction was equilibrated with Equilibration buffer containing 0.5% Nonidet-P40. The DEAE-cellulose chromatography columns were eluted with a linear NaCl (0-400mM) gradient (20 ml total volume) and 0.5ml fractions were collected. Protein kinase activity was determined using 50µl of sample and an incubation of 20 min at 37°C. Cyclic AMP dependency was measured in the absence and presence of 1µM cyclic AMP. Protamine (2mg/ml) was used as the exogenous substrate.

Time Course of Nonidet-P40 'activation' of Islet S-100 Protein Kinase Activity

Islet S-100 subcellular fractions (375-400 islets/ml) were prepared as described in detail in Chapter II. Protein kinase activity was measured in duplicate, in the absence and presence of 0.5% Nonidet-P40, using 100µl sample and 100µl of assay buffer containing 200µM(γ-^{32}P)-ATP and Protamine (4mg/ml). 15µl aliquots were removed at stated time intervals and ^{32}P incorporation into protein was determined using the Glass method (1978). Cyclic AMP dependency was measured in the absence and presence of 1µM cyclic AMP.

Time Course of Nonidet-P40 'activation' of Rabbit Skeletal Muscle Cyclic AMP-dependent Protein Kinase Isozymes

1mg of the Type I or Type II Rabbit skeletal muscle protein kinase isozyme (Sigma) was reconstituted to 10ml with distilled water. Protein kinase activity was measured in triplicate in the absence and presence of 0.5% Nonidet-P40. 200µl of protein kinase assay buffer containing 200µM (γ-^{32}P)-ATP and Protamine (4mg/ml) was added to 200µl of

the enzyme solution. 50µl aliquots were removed at stated time intervals and ^{32}P incorporation into protein was determined as previously described using the Glass method (1978). Cyclic AMP depencency was measured in the absence and presence of 1µM cyclic AMP.

Results

The results in this Chapter are expressed as mean values ± the standard deviation about the mean, the number of observations are shown in parenthesis. Values significantly different from controls (P less than 0.05) are denoted with asterisks. DEAE-cellulose chromatography activity elution profiles were measured in duplicate and are typical of results obtained in at least three separate experiments.

Effect of Nonidet-P40 on Protein Kinase Activity in the Islet Homogenate, S-0.6 and S-100 Subcellular Fractions

The effect of Nonidet-P40 (0.5%) on protein kinase activity in the islet homogenate and S-0.6 and S-100 subcellular fractions, using histone as exogenous substrate, is shown in Table 4.1a). Nonidet -P40 stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions in the absence and presence of cyclic AMP (1μM). 81%, 36% and 88% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively in the absence of cyclic AMP. 60%, 24% and 47% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively in the presence of cyclic AMP (1μM).

The effect of Nonidet-P40 (0.5%) on protein kinase activity in the islet homogenate and S-0.6 and S-100 subcellular fractions, using protamine as exogenous substrate, is shown in Table 4.1b). Nonidet-P40 stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions in the absence and presence of cyclic AMP (1μM). 70%, 42% and 66% stimulation of protein

TABLE 4.1. Effect of Nonidet-P40 on Islet Cell Protein Kinase Activity

Protein kinase activity of the untreated and Nonidet-P40 treated islet homogenate and S-0.6 and S-100 subcellular fractions was measured as described in the text, in the absence and presence of 1μM cyclic AMP. Histone a) and Protamine b) (2mg/ml) was used as exogenous substrate

Subcellular Fraction	Protein Kinase Activity (ρ moles ^{32}P/min/mg substrate/islet)		
	- cyclic AMP	+ cyclic AMP	
a)			
Homogenate	0.21(± 0.07)	0.35(± 0.04)	(6)
Homogenate + Nonidet-P40	0.38(± 0.13)*	0.56(± 0.01)*	(6)
S-0.6	0.28(± 0.08)	0.38(± 0.08)	(6)
S-0.6 + Nonidet-P40	0.38(± 0.08)*	0.47(± 0.06)*	(6)
S-100	0.17(± 0.03)	0.30(± 0.06)	(6)
S1100 + Nonidet-P40	0.32(± 0.08)*	0.44(± 0.03)*	(6)
b) Homogenate	1.68(± 0.32)	2.09(± 0.65)	(6)
Homogenate + Nonidet-P40	2.86(± 0.15)*	3.75(± 0.42)*	(6)
S-0.6	1.80(± 0.39)	2.22(± 0.47)	(6)
S-0.6 + Nonidet-P40	2.56(± 0.41)*	2.98(± 0.35)*	(6)
S-100	0.76(± 0.27)	1.02(± 0.13)	(6)
S-100 + Nonidet-P40	1.26(± 0.21)*	1.58(± 0.29)*	(6)

*Values assessed by Students t test to be significantly different from that observed in the absence of Nonidet-P40 ($p<0.05$)

kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively in the absence of cyclic AMP. 79%, 34% and 54% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively, in the presence of cyclic AMP (1μM).

Effect of Nonidet-P40 on Cyclic AMP-independent protein kinase activity in the islet homogenate, S-0.6 and S-100 subcellular fractions.

In order to investigate the possibility that the Nonidet-P40 stimulation of islet protein kinase activity was specific for the cyclic AMP-dependent protein kinases, the effect of Nonidet-P40 on protein kinase activity measured in the presence of Walsh protein (a specific inhibitor of cyclic AMP-dependent protein kinases) was examined. Table 4.2a) shows the effect of Nonidet-P40 (0.5%) on protein kinase activity measured in the presence of Walsh protein in the islet homogenate, S-0.6 and S-100 subcellular fractions, using histone as exogenous substrate. In the absence and presence of cyclic AMP, Nonidet-P40 was found to have no significant effect on the protein kinase activity observed in the islet homogenate, S-0.6 and S-100 subcellular fractions.

The effect of Nonidet-P40 on protein kinase activity measured in the presence of Walsh protein in the islet homogenate, S-0.6 and S-100 subcellular fractions, using protamine as exogenous substrate is shown in Table 4.2b). Cyclic AMP stimulation of protein kinase activity (although not significant) was observed in every case, with the exception of the untreated islet homogenate. Significant Nonidet-P40 stimulation of protein kinase activity occurred

TABLE 4.2 Effect of Nonidet-P40 on Islet Cell Cyclic AMP- Independent Protein Kinase Activity

Cyclic AMP-independent protein kinase activity was measured in the untreated and Nonidet-P40 treated islet homogenate and S-0.6 and S-100 subcellular fractions in the presence of Walsh protein (approx. 300 Units/ml) as described in the text. Cyclic AMP dependency was measured in the absence and presence of 1μM cyclic AMP. Histone a) and Protamine b) (2mg/ml) was used as exogenous substrate.

Subcellular Fraction	Protein Kinase Activity (ρ moles ^{32}P/min/mg substrate/ml)		
	-cyclic AMP	+ cyclic AMP	
a)			
Homogenate	0.08(± 0.03)	0.08(± 0.01)	(4)
Homogenate + Nonidet-P40	0.09(± 0.02)	0.10(± 0.02)	(4)
S-0.6	0.09(± 0.04)	0.09(± 0.03)	(4)
S-0.6 + Nonidet-P40	0.09(± 0.04)	0.11(± 0.02)	(4)
S-100	0.04(± 0.02)	0.07(± 0.01)	(4)
S-100 + Nonidet-P40	0.05(± 0.02)	0.06(± 0.01)	(4)
b)			
Homogenate	0.94(± 0.14)	0.86(± 0.12)	(4)
Homogenate + Nonidet-P40	1.57(± 0.14)*	1.72(± 0.21)*	(4)
S-0.6	1.00(± 0.18)	1.34(± 0.18)	(4)
S-0.6 + Nonidet-P40	1.43(± 0.13)*	1.65(± 0.17)*	(4)
S-100	0.31(± 0.11)	0.46(± 0.12)	(4)
S-100 + Nonidet-P40	0.69(± 0.13)*	0.91(± 0.31)*	(4)

* Values assessed by Student t test to be significantly different from that observed in the absence of Nonidet-P40 ($p<0.05$)

in all the fractions examined. 67%, 43% and 123% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively in the absence of cyclic AMP. In the presence of cyclic AMP (1μM), 100%, 23% and 98% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively.

By deducting the protein kinase activity measured in the presence of Walsh protein from that observed in its absence, a measure of cyclic AMP-dependent protein kinase activity was obtained. Table 4.3a) shows the effect of Nonidet-P40 (0.5%) on cyclic AMP-dependent protein kinase activity in the islet homogenate, S-0.6 and S-100 subcellular fractions, using histone as exogenous substrate. Significant Nonidet-P40 stimulation of cyclic AMP-dependent protein kinase activity was observed in the islet homogenate and S-100 subcellular fractions. 110%, 55% and 116% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively, in the absence of cyclic AMP. In the presence of cyclic AMP (1μM), 72%, 22% and 50% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively.

Table 4.3b) shows the effect of Nonidet-P40 (0.5%) on cyclic AMP-dependent protein kinase activity in the islet homogenate, S-0.6 and S-100 subcellular fractions using protamine as exogenous substrate. Significant Nonidet-P40 stimulation of cyclic AMP-dependent protein kinase activity was observed in the islet homogenate. 76%,44% and 22% stimulation of protein kinase activity was observed in the

TABLE 4.3 Effect of Nonidet-P40 on Islet Cell Cyclic AMP-dependent protein kinase activity

Cyclic AMP-independent activity (Table 4.2) was subtracted from protein kinase activity (Table 4.1). Histone a) and Protamine b) (2mg/ml) was used as exogenous substrate.

Subcellular Fraction	Protein Kinase Activity (p moles ^{32}P/min/mg substrate/islet)	
	-cyclic AMP	+ cyclic AMP
a)		
Homogenate	0.13(± 0.10)	0.27(± 0.05)
Homogenate + Nonidet-P40	0.29(± 0.16)*	0.46(± 0.03)*
S-0.6	0.19(± 0.12)	0.29(± 0.11)
S-0.6 + Nonidet-P40	0.29(± 0.12)	0.36(± 0.08)
S-100	0.13(± 0.05)	0.27(± 0.07)
S-100 + Nonidet-P40	0.27(± 0.10)*	0.38(± 0.04)*
b)		
Homogenate	0.74(± 0.46)	1.23(± 0.77)
Homogenate + Nonidet-P40	1.29(± 0.29)*	2.03(± 0.63)*
S-0.6	0.80(± 0.57)	0.88(± 0.65)
S-0.6 + Nonidet-P40	1.13(± 0.54)	1.33(± 0.52)
S-100	0.45(± 0.38)	0.56(± 0.25)
S-100 + Nonidet-P40	0.57(± 0.34)	0.67(± 0.60)

* Values assessed by Student t test to be significantly different from that observed in the absence of Nonidet-P40 ($P < 0.05$).

islet homogenate, S-0.6 and S-100 subcellular fractions respectively, in the absence of cyclic AMP. While in the presence of cyclic AMP (1μM), 71%, 54% and 17% stimulation of protein kinase activity was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions respectively.

Effect of Nonidet-P40 on Phosphoprotein Adsorption onto P-81 (phosphocellulose) paper.

The effect of Nonidet-P40 on phosphoprotein adsorption onto P-81 (phosphocellulose) paper was investigated in order to ensure that the Nonidet-P40 stimulation of protein kinase activity was not due to artifactual increases in phosphoprotein adsorption. As the percentage of Nonidet-P40 in the terminated reaction mixture was increased, a decrease in adsorption of phosphoprotein was observed (Fig. 4.1). The percentage loss of phosphoprotein from P-81 paper was linear with respect to the percentage of Nonidet-P40. In all experiments where the effect of Nonidet-P40 was being examined an appropriate reagent blank was always included.

Effect of Nonidet-P40 on (γ-^{32}P)-ATP hydrolysis and ^{32}P production in islet cell subcellular fractions

The possibility that the observed Nonidet-P40 stimulation of islet cell protein kinase activity may have been due to inhibition of islet cell ATPase activity, thereby preventing the depletion of (γ-^{32}P)-ATP substrate in the reaction mix, was investigated.

Fig. 4.2 shows the effect of Nonidet-P40 treatment upon ^{32}P production in the islet homogenate, S-0.6 and S-100 subcellular fractions.

Inhibition of ^{32}P production occurred in the Nonidet-P40 treated subcellular fractions. Fig. 4.3 shows the effect of Nonidet-P40 treatment upon (γ-^{32}P) - ATP hydrolysis in the islet

Figure 4.1. Effect of Nonidet-P40 on Phosphoprotein Adsorption onto P-81 (phosphocellulose) paper.
Islet S-0.6 and S-100 subcellular fractions were prepared and assayed for protein kinase activity as described in the text. The reactions were terminated using the Glass (1978) method and appropiate volumes of Nonidet-P40 were added. Aliquots (50µl) of the terminated reaction mixtures were pipetted onto P-81 (phosphocellulose) paper (2cm x 2cm) and P protein incorporation was determined as described in the text. Each point represents the mean value ± S.D. (n=4) of the percentage loss of ^{32}P protein incorporation with respect to the control with no addition of Nonidet-P40.

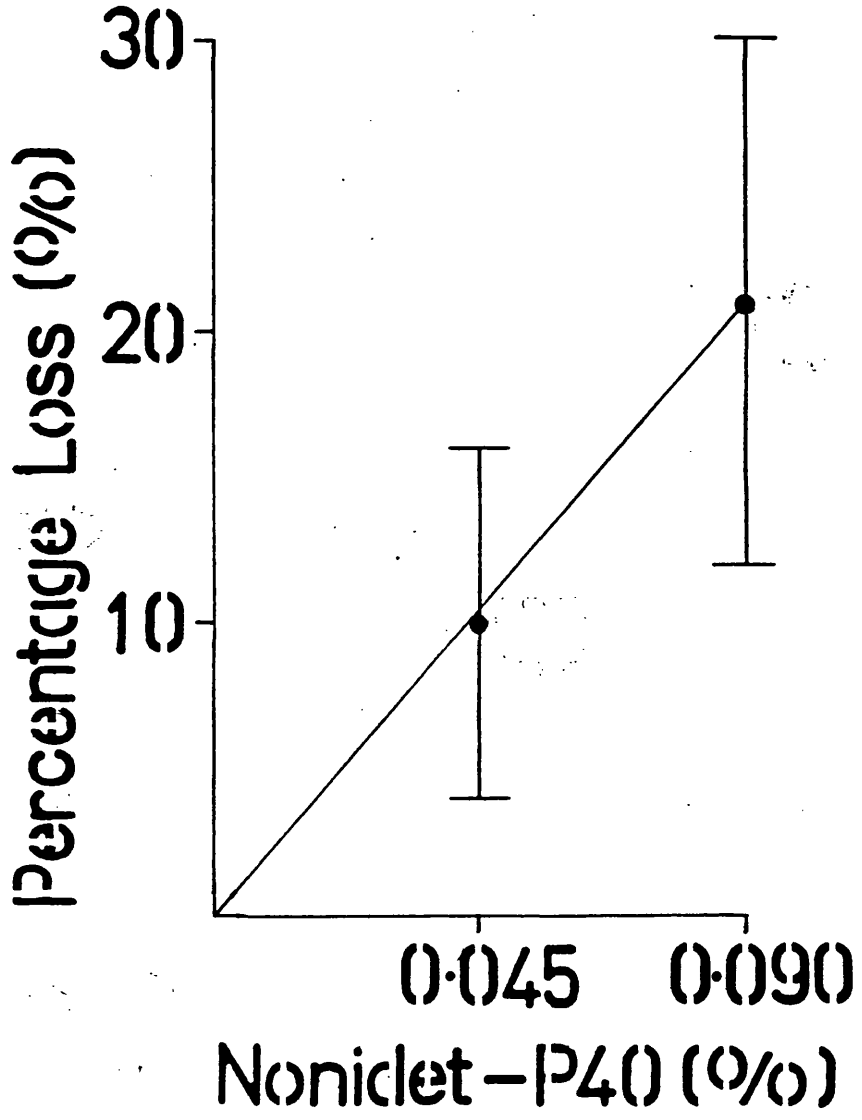

30
20
10
Percentage Loss (%)
0·045
0·090
Noniclet–P40 (%)

Figure 4.2. Effect of Nonidet-P40 on ^{32}P Production in the Islet Homogenate, S-0.6 and S-100 Subcellular Fractions. Islet subcellular fractions were prepared as described in the text. ^{32}P production in the islet (a) homogenate, (b) S-0.6 and (c) S-100 subcellular fractions was assayed in duplicate in the absence (O) and presence (●) of 0.5% Nonidet-P40, using (γ-^{32}P)-ATP substrate, as described in the text. The reaction conditions were the same as that described for the protein kinase assay. The results are typical of those obtained in three seperate experiments.

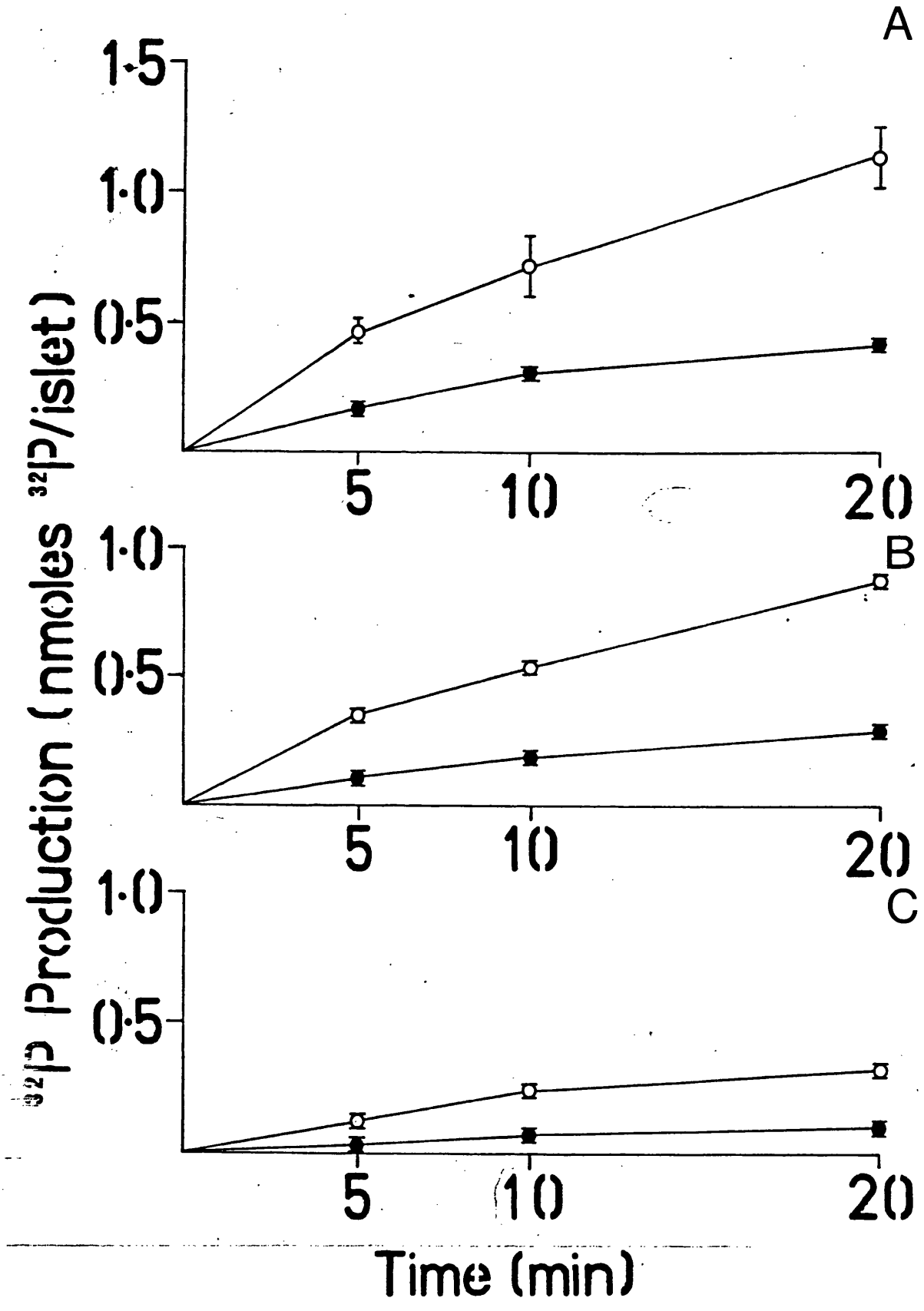

A
B
C
1·5
1·0
0·5
5
10
20
^{32}P Production (nmoles ^{32}P/islet)
Time (min)

Figure 4.3. Effect of Nonidet-P40 on (γ^{-32}P)-ATP Hydrolysis in the Islet Homogenate, S-0.6 and S-100 Subcellular Fractions.

Islet subcellular fractions were prepared as described in the text. (γ^{-32}P)-ATP hydrolysis in the islet (a) homogenate, (b) S-0.6 and (c) S-100 subcellular fractions was assayed in duplicate in the absence (O) and presence (●) of 0.5% Nonidet-P40, as described in the text, using the same reaction conditions as that described for the protein kinase assay. The results are typical of those obtained in three seperate experiments.

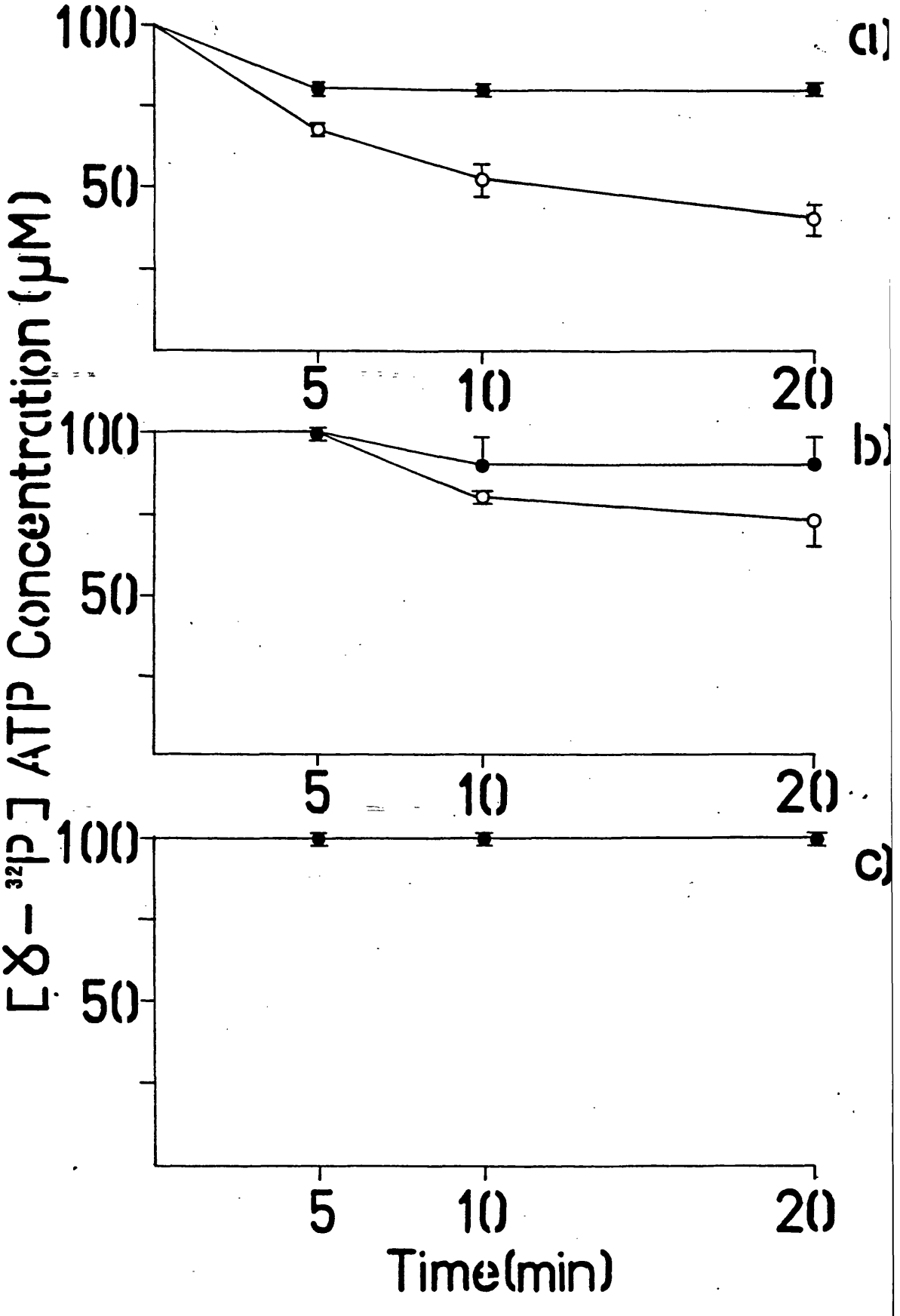

100
50
a)
5
10
20
100
50
b)
5
10
20
100
50
c)
5
10
20
Time(min)
[γ-32P] ATP Concentration (μM)

TABLE 4.4. Subcellular Distribution of Islet Cell ^{32}P Production Activity

^{32}P production activity of islet homogenates and subcellular fractions was measured in the absence and presence of Nonidet-P40 (0.5%) as described in the text.

Subcellular Fraction	Activity (p Moles ^{32}P/min/islet)	Protein (μg/islet)	Specific Activity (p moles ^{32}P/min/mg islet protein)	
Homogenate	57.2(± 4.4)	4.78	11.97(± 0.92)	(4)
Homogenate + Nonidet-P40	21.7(± 1.7)*	4.78	4.54(± 0.36)*	(4)
P-0.6 + Nonidet-P40	8.1(± 4.4)	1.56	5.21(± 2.87)	(6)
S-0.6	42.9(± 7.9)	4.88	8.78(± 0.57)	(6)
S-0.6 + Nonidet-P40	20.7(± 5.0)*	4.88	4.24(± 1.03)*	(6)
P-100 + Nonidet-P40	11.3(± 1.0)	1.03	10.97(± 0.98)	(6)
S-100	16.0(± 1.0)	3.81	4.20(± 0.26)	(6)
S-100 + Nonidet-P40	6.9(± 0.9)*	3.81	1.82(± 0.24)*	(6)

* Values assessed by Student t test to be significantly different from that observed in the absence of Nonidet-P40 ($p<0.05$)

homogenate, S-0.6 and S-100 subcellular fractions. In the islet homogenate and S-0.6 subcellular fraction, treatment with Nonidet-P40 (0.5%) produced a decrease in (γ-^{32}P) -ATP hydrolysis. However, in the S-100 subcellular fraction no (γ-^{32}P)-ATP hydrolysis was observed in the absence and presence of Nonidet-P40.

The subcellular distribution and specific activity of ^{32}P production in the islet cell was determined (Table 4.4) In the presence of Nonidet-P40, the majority (52%) of ^{32}P production activity was located in the P-100 subcellular fraction.

The previous results presented in this chapter demonstrate Nonidet-P40 stimulation of islet cell protein kinase activity could not be directly due to its inhibition of islet cell ATPase activity, as no (γ-^{32}P)-ATP hydrolysis was observed in the islet cell S-100 subcellular fraction. The following studies were undertaken in order to characterise the Nonidet-P40 'activation' of cyclic AMP-dependent protein kinases.

Islet Cell S-100 DEAE-Cellulose Chromatography

Two peaks of protein kinase activity from an islet S-100 subcellular fraction, incubated at 4^{o}C for 18 hours in the presence of Nonidet-P40 (0.5%), were separated by DEAE-cellulose chromatography (Fig. 4.4a). The first major peak of protein kinase activity, eluted in 10-70mM NaCl, exhibited cyclic AMP inhibition. The position of the first peak of protein kinase activity corresponded to that of the S-100 cyclic AMP-dependent protein kinase Type I isozyme (Chapter 3, Fig. 3.6). The second peak of protein kinase, eluted in 120-200mM NaCl, was much smaller and broader than the first peak and also exhibited cyclic AMP inhibition. The position of the second

Figure 4.4a). DEAE-cellulose Chromatography of Nonidet-P40 Treated Islet S-100 Subcellular Fractions.
Islet S-100 subcellular fractions were prepared and treated with Nonidet-P40 (0.5%) as described in the text. The S-100 subcellular fractions were applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) previously equilibrated with 0.5% Nonidet-P40. Protein kinase activity was assayed in duplicate in the absence (o) and presence (●) of 1μM cyclic AMP, using protamine (2mg/ml) as exogenous substrate. The reaction was terminated using the Glass (1978) method.

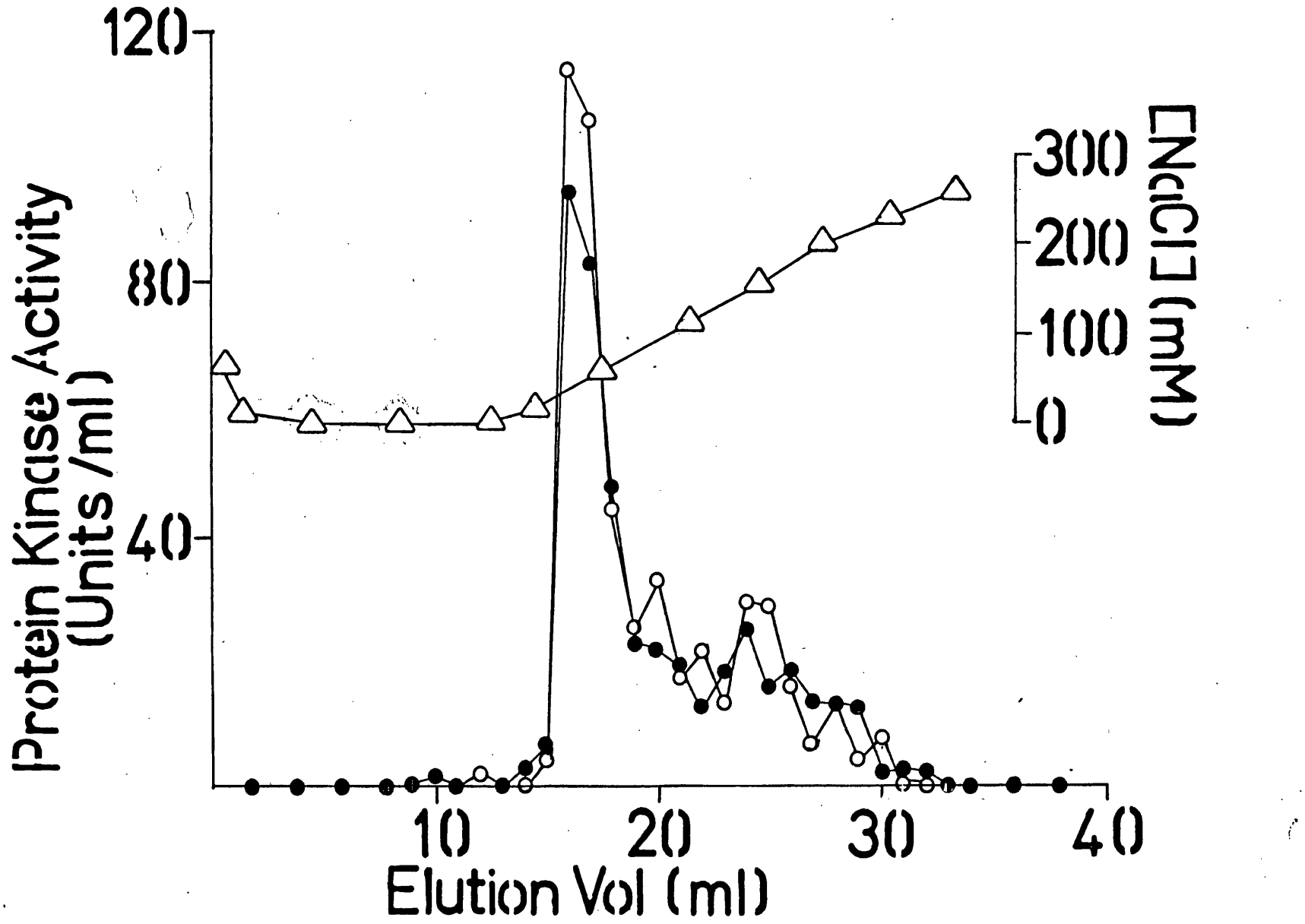
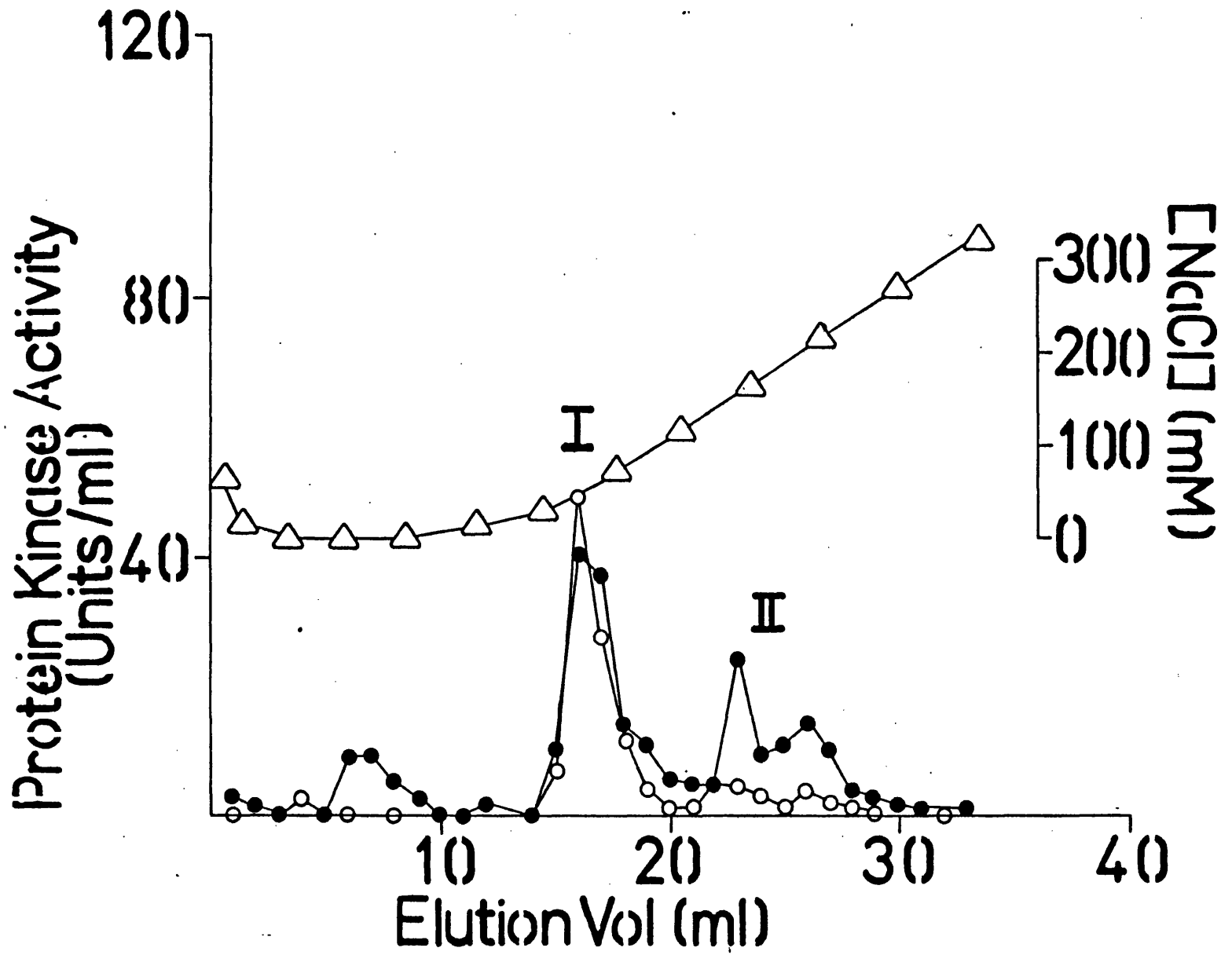

120
80
40
Protein Kinase Activity
(Units /ml)
300
200
100
0
[NaCl] (mM)
10
20
30
40
Elution Vol (ml)

Figure 4.4b). DEAE-cellulose Chromatography of Islet S-100 Subcellular Fractions.
Islet S-100 subcellular fractions were prepared and applied to a DEAE-cellulose chromatography column (0.5cm x 3.0cm) as described in the text. Protein kinase activity was assayed in duplicate in the absence (O) and presence (●) of 1μM cyclic AMP, using protamine (2mg/ml) as exogenous substrate. The reaction was terminated using the Glass (1978) method.


120
80
40
Protein Kinase Activity (Units/ml)
I
II
300
200
100
0
[NaCl] (mM)
10
20
30
40
Elution Vol (ml)

peak of protein kinase activity corresponded to that of the S-100 cyclic AMP-dependent protein kinase Type II isozyme (Chapter 3, Fig. 3.6).

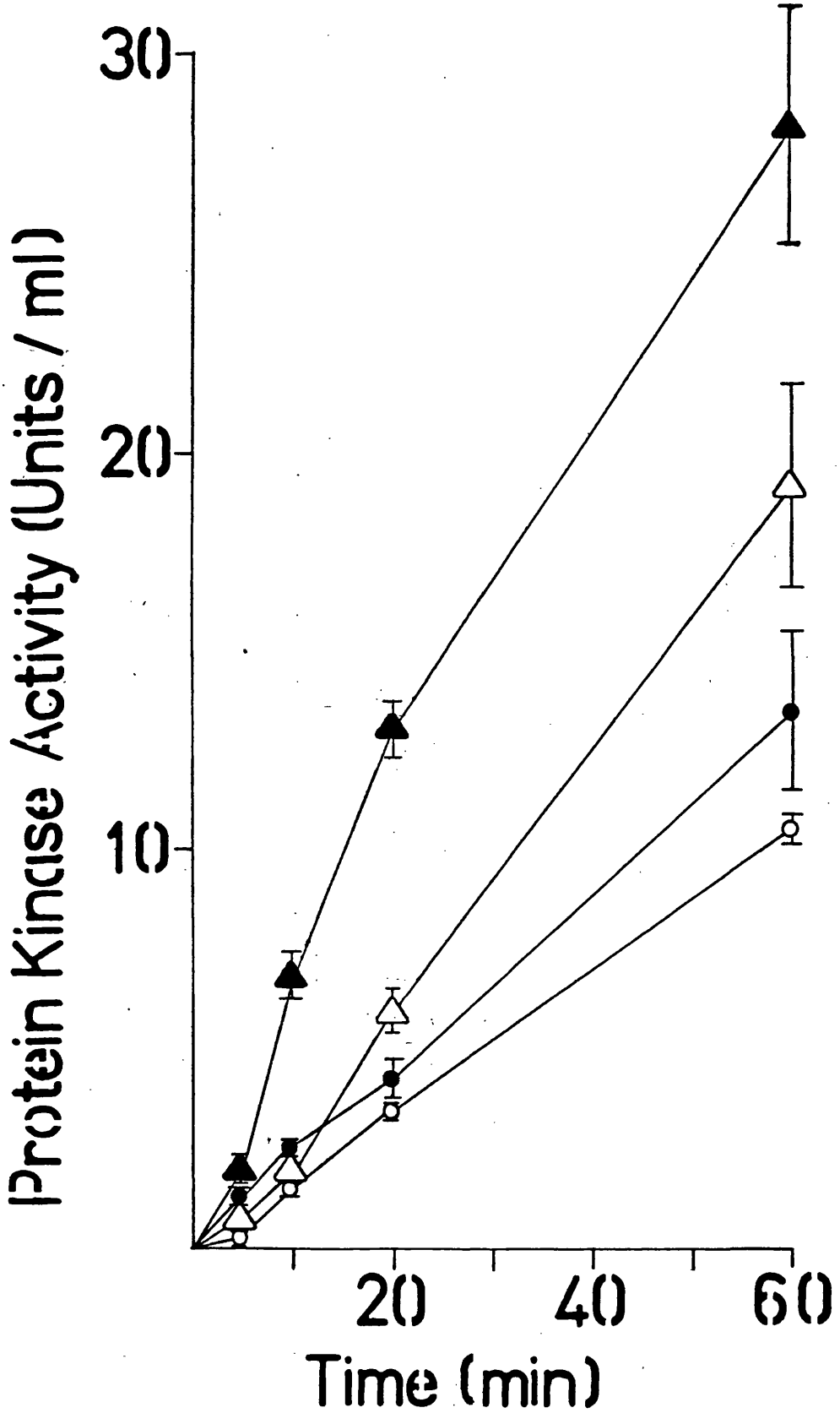
Three peaks of protein kinase activity from an islet S-100 subcellular fraction, incubated at $4^{o}C$ for 18 hours in the absence of Nonidet-P40, were separated by DEAE-cellulose chromatography (Fig. 4.4b). The first peak of protein kinase activity, observed in the column flow-through was stimulated by cyclic AMP (1μM). The second major peak of protein kinase activity, eluted in 30-70mM NaCl exhibited cyclic AMP inhibition. The position of the second peak of protein kinase activity corresponded to that of the S-100 cyclic AMP-dependent protein kinase Type I isozyme. The corresponding peak of protein kinase activity in the Nonidet-P40 treated S-100 fraction exhibited a three-fold greater activity. The third peak of protein kinase activity, eluted in 120-220mM NaCl, was much smaller and broader than the second peak. The activity of this peak of protein kinase was stimulated by the addition of cyclic AMP and its position corresponded to that of the S-100 cyclic AMP-dependent protein kinase Type II isozyme. The corresponding peak of protein kinase activity found in the Nonidet-P40 treated S-100 fraction exhibited the same level of activity, but was unaffected by the addition of cyclic AMP.

Time Course of Nonidet-P40 'activation' of Protein Kinase Activity

a) Islet S-100 Protein Kinase Activity

The time course of Nonidet-P40 'activation' of islet cell S-100 protein kinase activity in the absence and presence of cyclic AMP (1μM) is shown in Fig. 4.5. In the absence of cyclic AMP, Nonidet-P40 'activation' of protein kinase activity

Figure 4.5. Time Course of Nonidet-P40 'activation' of Islet S-100 Protein Kinase Activity.
Islet S-100 protein kinase activity was assayed in duplicate in the absence (○,●) and presence (△,▲) of 0.5% Nonidet-P40 as described in detail in the text. Cyclic AMP dependency was measured in the absence (○,△) and presence (●,▲) of 1μM cyclic AMP. The results are typical of those obtained in three seperate experiments.


30
20
10
Protein Kinase Activity (Units / ml)
20
40
60
Time (min)

was observed after 20 min incubation at 37°C. The protein kinase activity observed in the presence of Nonidet-P40 was greater than that observed in the presence of cyclic AMP. In the presence of cyclic AMP, Nonidet-P40 'activation' was observed after 5 min incubation at 37°C. Protein kinase activity was linear with respect to time in the absence of Nonidet-P40. In the presence of Nonidet-P40, protein kinase activity was linear with respect to time after 'activation', although in the presence of cyclic AMP non-linearity was observed after 20 min incubation.

b) Partially purified Rabbit Skeletal Muscle Type I and Type II Cyclic AMP-dependent Protein Kinases

The effect of Nonidet-P40 on partially purified Rabbit skeletal muscle Type I and Type II cyclic AMP-dependent protein kinases (Beavo et al., 1974) was investigated in order to examine the possibility that Nonidet-P40 'activation' of protein kinase activity may be a phenomenon unique to the islet cell. Using the Type I isozyme (Fig. 4.6a), Nonidet-P40 'activation' of protein kinase activity was observed after 20 min incubation at 37°C, in the absence of cyclic AMP. In the presence of cyclic AMP (1μM), Nonidet-P40 'activation' of Type I protein kinase activity was observed after 10 min incubation. Protein kinase activity was approximately linear with respect to time in the absence of cyclic AMP. In the presence of cyclic AMP protein kinase activity was initially linear with respect to time, but after 20 min non-linearity was observed.In the presence of Nonidet-P40 and cyclic AMP, protein kinase activity was initially linear with respect to time after activation had occurred (5 min), but after 20 min non-linearity was observed.

Figure 4.6a). Time Course of Nonidet-P40 'activation' of Rabbit Skeletal Muscle Type I Cyclic AMP-dependent Protein Kinase.

Protein kinase activity was assayed in triplicate in the absence (○,●) and presence (△,▲) of 0.5% Nonidet-P40 as described in the text. Cyclic AMP dependency was measured in the absence (○,△) and presence (●,▲) of 1μM cyclic AMP. The results are the mean value of protein kinase activity ± S.D. (n = 3).

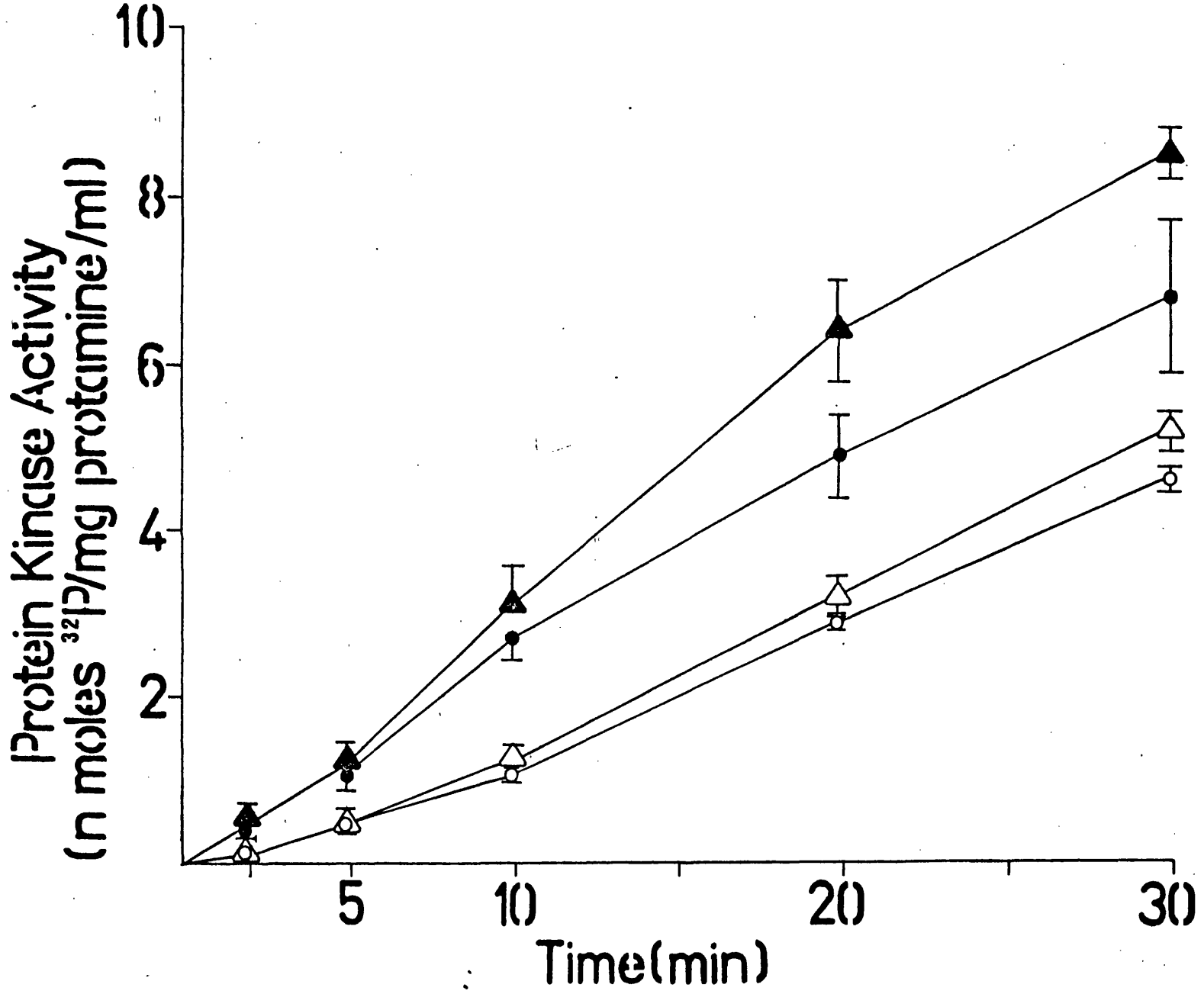
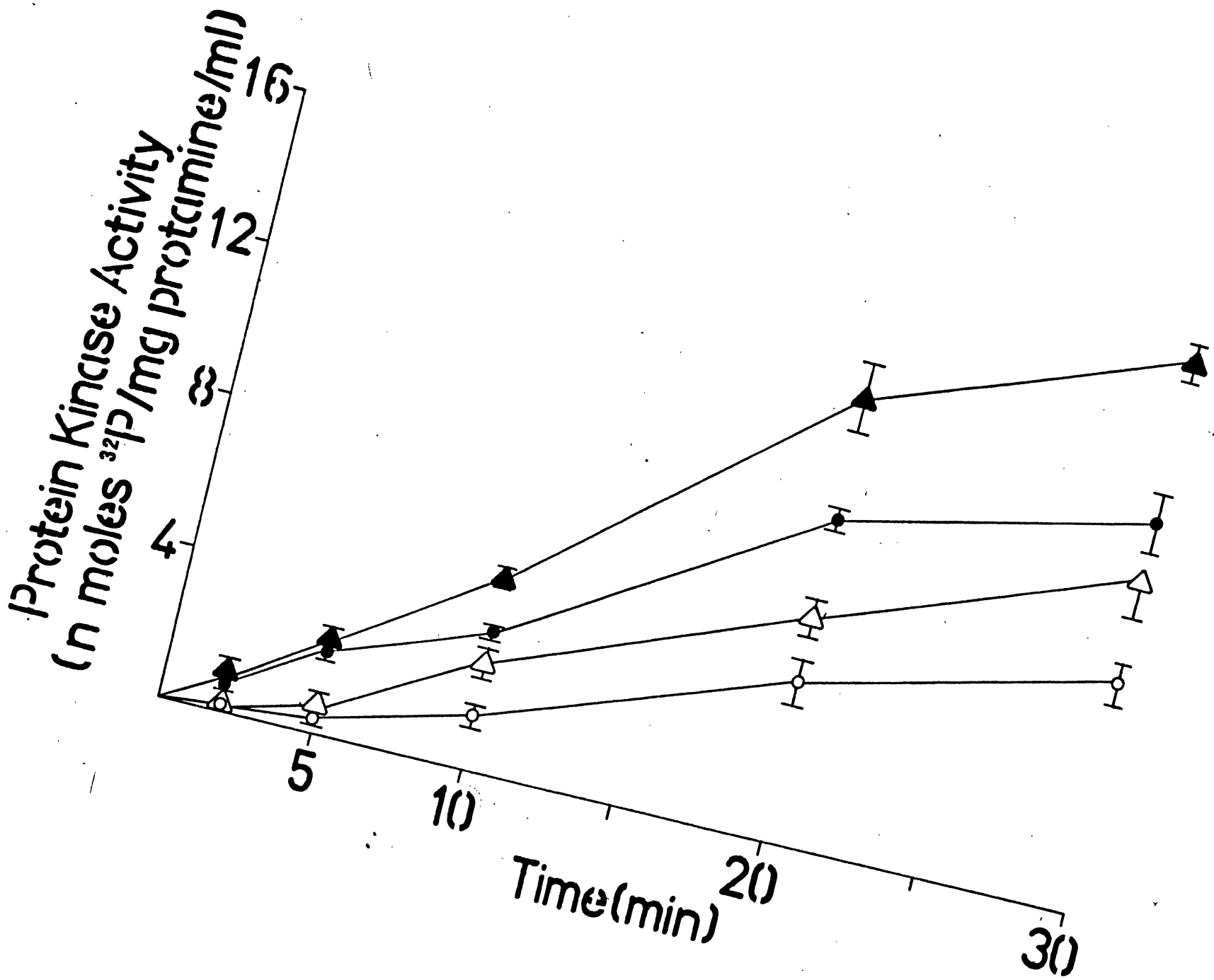

Protein Kinase Activity
(n moles ^{32}P/mg protamine/ml)
10
8
6
4
2
5
10
20
30
Time(min)

Figure 4.6b). Time Course of Nonidet-P40 'activation' of Rabbit Skeletal Muscle Type II Cyclic AMP-dependent Protein Kinase.

Protein kinase activity was assayed in triplicate in the absence (○,●) and presence (△,▲) of 0.5% Nonidet-P40 as described in the text. Cyclic AMP dependency was measured in the absence (○,△) and presence (●,▲) of 1μM cyclic AMP. The results are the mean value of protein kinase activity ± S.D. (n = 3).


Protein Kinase Activity
(n moles ^{32}P/mg protamine/ml)
16
12
8
4
5
10
20
30
Time(min)

Using the type II isozyme (Fig.4.6b) Nonidet-P40 'activation' of protein kinase activity was observed after 10 min incubation at 37°C in the absence and presence of cyclic AMP. In the absence of cyclic AMP and Nonidet-P40, protein kinase activity was approximately linear with respect to time. In the presence of Nonidet-P40, protein kinase activity was linear with respect to time after activation (10 min)In the presence of cyclic AMP, protein kinase was initially linear to time, but after 20 min non-linearity was observed. In the presence of Nonidet-P40 and cyclic AMP, protein kinase activity was initially linear with respect to time after activation (5 min) but after 20 min incubation non-linearity was observed.

Discussion

Nonidet-P40 stimulation of protein kinase activity was observed in the islet homogenate, and S-0.6 and S-100 subcellular fractions, irrespective of the exogenous substrate used (Table 4.1). No artifactual increase of protein bound ^{32}P adsorption onto P81 (phosphocellulose) paper was observed in the presence of Nonidet-P40 (Fig.4.1). This suggested Nonidet-P40 'activated' islet cell S-100 protein kinases. This correlated with a previous study in which increases in rat liver cytosolic protein kinase activity were observed in the presence of the non-ionic detergent, Triton X-100 (Sommarin and Jergil, 1978). The effect of Nonidet-P40 on protein kinase activity measured in the presence of Walsh protein (a specific inhibitor of cyclic AMP-dependent protein kinases), using histone as exogenous substrate (Table 4.2a), appeared to suggest that the Nonidet-P40 activation of protein kinase activity was specifically cyclic AMP-dependent (Ducommun et al, 1979). However, the increases in protein kinase activity observed in the presence of Nonidet-P40 and Walsh protein, when protamine was used as exogenous substrate (Table 4.2b), suggested that this was not the case. Since stimulation of protein kinase activity was observed in the presence of cyclic AMP and Walsh protein, it was thought that the Walsh protein concentration used was insufficient to inhibit all of the free catalytic subunits of the cyclic AMP-dependent protein kinases present in the reaction mix.

Islet Cell ^{32}P Production and (γ-^{32}P)-ATP Hydrolysis

Using synaptic membranes solublised with Triton X-100 (a non-ionic detergent), it has been observed that there is a negative correlation between protein kinase activity and protein concentration. This has led to the suggestion that

Triton X-100 may inhibit or inactivate ATPase activity (Thérien & Mushynski, 1979). In order to investigate the possibility that the Nonidet-P40 'activation' of islet protein kinase activity may have been indirectly due to its inhibition of ATPase activity, a study on islet cell ATPase activity was undertaken. Nonidet-P40 inhibition of ^{32}P production was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions (Fig.4.2). This suggested Nonidet-P40 inhibited an ATPase or phosphatase activity. The observed Nonidet-P40 inhibition of (γ-^{32}P) -ATP Hydrolysis in the islet homogenate and S-0.6 subcellular fraction (Fig.4.3) suggested Nonidet-P40 inhibited ATPase activity. This correlated with the observation that a wide variety of detergents (ionic and non-ionic) inactivated the Na/K ATPase activity of the electric eel (Brotherus et al., 1979). No (γ-^{32}P) -ATP hydrolysis was observed in the absence and presence of Nonidet-P40 in the islet S-100 subcellular fraction. Hence the increase in the islet cell S-100 protein kinase activity observed in the presence of Nonidet-P40 was due to 'activation' of the protein kinase and not indirectly due to inhibition of ATPase activity, preventing depletion of (γ-^{32}P) -ATP substrate.

Islet Cell S-100 DEAE-cellulose Chromatography

Evidence to suggest Nonidet-P40 may directly affect the islet cell S-100 cyclic AMP-dependent protein kinases came from studies on the partially purified enzymes. In the absence of Nonidet-P40, Type I cyclic AMP-dependent protein kinase was the predominant isozyme and was found to be unaffected by the presence of cyclic AMP. This was in complete contrast to previous observations, (Chapter 3, Fig. 3.6). It was thought storage at 4°C for 18 hours may have resulted in the

loss of Type II isozyme activity and the dissociation of the Type I isozyme after DEAE-cellulose chromatography. If dissociation of the Type I subunits had occurred prior to the DEAE-cellulose chromatography, the catalytic subunit of the Type I isozyme would not have been retained by the column (Nimmo and Cohen, 1977). Dissociation of the Type I isozyme after DEAE-cellulose chromatography has been previously reported in rat islets of Langerhans (Oliver and Kemp, 1980).

In the presence of Nonidet-P40, Type I isozyme was found to be the only molecular form to exhibit greater (three-fold) activity than that observed in the absence of Nonidet-P40. Both isozymes were dissociated after DEAE-cellulose chromatography. It was thought Nonidet-P40 might exert two different effects on the isozymes. As the catalytic subunits of the different isozymes are reported to the identical in most, if not all, properties (Corbin et al., 1976), the differential effect of Nonidet-P40 on the isozymes would probably be due to the different properties of the receptor subunits. The possibility that the loss of activity of the Type II isozyme through storage at $4^{o}C$ for 18 hours may have prevented its 'activation' by Nonidet-P40 in the presence of cyclic AMP cannot be ignored.

Time Course of Nonidet-P40 'activation' of Protein Kinase Activity

Nonidet-P40 'activation' of islet cell S-100 protein kinase activity was found to occur fairly rapidly at 37^{o}. In the presence of cyclic AMP, Nonidet-P40 'activation' of protein kinases was faster and this was thought to be due to the cyclic AMP dissociation of the cyclic AMP-dependent protein kinases facilitating the Nonidet-P40 'activation'.

The ability of Nonidet-P40 to activate the partially purified Type I and Type II rabbit skeletal muscle cyclic

AMP-dependent protein kinases (Fig.4.6) demonstrated that Nonidet-P40 activation of cyclic AMP-dependent protein kinases was a general phenomenon and not unique to the islet tissue. Nonidet-P40 'activation' of the rabbit skeletal muscle Type I isozyme was faster and greater in the presence of cyclic AMP, although the Type II isozyme showed no significant difference in Nonidet-P40 'activation' in the absence and presence of cyclic AMP. Overall, faster 'activation' was obtained with the Type II isozyme. These results appear to suggest that Nonidet-P40 may have a differential effect on the two cyclic AMP-dependent protein kinase isozymes.

The data presented in this chapter demonstrates increases in protein kinase activity are observed in the presence of Nonidet-P40. These observations raise the questions of how much of the membrane protein kinase activity, solublised by non-ionic detergents, is due to 'activation', and are membrane bound protein kinases activated to the same extent as the soluble protein kinases? Uno et al., (1976) have reported Triton X-100 has almost no effect on the activity of the membrane derived protein kinases although it does increase the activity of the cytosol protein kinases. Thus it appears the effect of Nonidet-P40 on protein kinase activity may be spedific for the soluble protein kinases.

Summary

The data presented in this chapter demonstrated Nonidet-P40 did not inhibit islet cell protein kinase activity. (Hence the low levels of protein kinase activity observed in the islet membrane containing fractions were not due to the presence of Nonidet-P40). In contrast it was shown Nonidet-P40 'activated' islet S-100 protein kinases. This 'activation' was not directly due to Nonidet-P40 inhibition of islet ATPase activity. The possibility that the Nonidet-P40 'activation' may be specific for the cyclic AMP-dependent protein kinases was suggested.

The phenomenon of Nonidet-P40 'activation' was not found to be unique to islet tissue, since it was observed using partially purified cyclic AMP-dependent protein kinases from rabbit skeletal muscle. Nonidet-P40 had a differential effect on the protein kinase isozymes, although the specificity and mechanism of the 'activation' has yet to be defined.

CHAPTER V

The Partial Characterisation of Islet Cell Endogenous Protein Phosphorylation

Introduction

In this study the phosphorylation of islet cell proteins by islet cell protein kinases, using a cell free system was investigated. Individual phosphorylated islet proteins were separated according to their molecular size using S.D.S.-Polyacrylamide electrophoresis and localised by autoradiography. Two methods of study were used in this investigation, the first utilised the same reaction conditions used in the characterisation of islet cyclic AMP-dependent protein kinases. The second method was based on that described by Rudolph and Kreuger (1979). The difference in the results obtained with these two methods will be discussed.

This study was aimed at investigating the relationship between the phosphorylation of islet proteins and insulin secretion. Hence in this study the phosphorylation of non-nuclear proteins and the effects on the phosphorylation, of substances known to affect the secretory mechanism, e.g., cyclic AMP (Turtle and Kipnis, 1967), calcium (Grodsky and Bennett, 1966) and colchicine (Montague et al., 1975) were investigated. The effects of agents, known to affect cyclic AMP-dependent protein kinases such as Walsh protein (Ashby and Walsh, 1972) and Nonidet-P40 (Chapter IV), on protein phosphorylation was also examined. The dephosphorylation of phosphorylated islet proteins and the effect of cyclic AMP on dephosphorylation were also examined.

Cyclic AMP stimulation of ^{32}P incorporation into an islet cell tumour ribosomal protein (molecular weight

28,000 dalton) has been demonstrated (Schubart et al., 1977). In order to examine the possibility of cyclic AMP mediated phosphorylation of islet cell ribosomal proteins, ^{32}P incorporation into guinea-pig islet and liver ribosomal proteins was examined. Liver ribosomal proteins were used in this study as it is reported, that within a species, ribosomal proteins of different tissues are very nearly identical (Sherton and Wool, 1974).

Methods, Results and Discussion

In this chapter the methods are incorporated with the corresponding results and discussion for easier reference.

The results presented in this chapter are expressed as mean values ± the standard deviation about the mean, the number of observations is shown in parenthesis. The autoradiograph photographs are typical of the results obtained in at least three separate experiments.

Method A (pH 6.0)

Subcellular Distribution of Islet Cell Endogenous Protein Phosphorylation

Method

Subcellular fractions of isolated islets of Langerhans were prepared by differential centrifugation as described in detail in Chapter II. Nonidet-P40 was added to aliquots of the islet homogenate, S-0.6 and S-100 subcellular fractions to give a final concentration of 0.5 ml/100 ml. Endogenous protein phosphorylation was measured in the absence and presence of cyclic AMP (1 μM) using the method described in the procedure for the determination of Protein Kinase Activity, with the omission of the exogenous protein substrate. Reaction conditions were 20 min at 37^{o}C. The reaction was terminated using the Glass method (1978).

Results

The subcellular distribution of islet cell endogenous protein phosphorylation measured in the absence and presence of cyclic AMP (1 μM) is shown in Table 5.1. With the exception of the islet P-100 subcellular fraction, cyclic AMP had no significant effect on the endogenous protein phosphorylation observed in the islet homogenate and sub-

TABLE 5.1 - Subcellular Distribution of Islet Cell Endogenous Protein Phosphorylation

Islet subcellular fractions were prepared and assayed for endogenous protein phosphorylation, in the absence and presence of cyclic AMP (1 μM), as described in the text.

Subcellular Fraction	Endogenous Protein Phosphorylation (f moles ^{32}P/min/islet)		
	- cyclic AMP	+ cyclic AMP	
Homogenate	58 (± 17)	41 (± 19)	(4)
S-0.6	41 (± 13)	26 (± 10)	(4)
P-0.6	3 (± 2)	4 (± 2)	(4)
S-100	20 (± 9)	18 (± 8)	(4)
P-100	27 (± 10)	12 (± 7)*	(4)

* Value assessed by Student's t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

TABLE 5.2 - Effect of Nonidet-P40 on Endogenous Protein Phosphorylation in the Islet Homogenate and S-0.6 and S-100 Subcellular Fractions

Nonidet-P40 treated and untreated islet homogenates and S-0.6 and S-100 subcellular fractions were prepared and assayed for endogenous protein phosphorylation, in the absence and presence of cyclic AMP (1 μM), as described in the text.

Subcellular Fraction	Endogenous Protein Phosphorylation (f moles ^{32}P/min/islet)		
	- cyclic AMP	+ cyclic AMP	
Homogenate	58 (± 17)	41 (± 19)	(4)
Homogenate + Nonidet-P40	54 (± 9)	75 (± 12)*†	(4)
S-0.6	41 (± 13)	26 (± 10)	(4)
S-0.6 + Nonidet-P40	43 (± 14)	78 (± 9)*†	(4)
S-100	20 (± 9)	18 (± 8)	(4)
S-100 + Nonidet-P40	31 (± 7)†	50 (± 4)*†	(4)

* Values assessed by Student's t test to be significantly different from that observed in the absence of cyclic AMP ($p < 0.05$).

† Values assessed by Student's t test to be significantly different from that observed in the absence of Nonidet-P40 ($p < 0.05$).

cellular fractions. In the islet P-100 subcellular fraction, a significant decrease in endogenous protein phosphorylation was observed in the presence of cyclic AMP. The majority (55%) of total islet cell endogenous protein phosphorylation was located in the P-100 subcellular fraction in the absence of cyclic AMP. However, in the presence of cyclic AMP, the majority (53%) of total islet cell endogenous protein phosphorylation was located in the S-100 subcellular fraction.

Effect of Nonidet-P40 on Endogenous Protein Phosphorylation in the Islet Homogenate and S-0.6 and S-100 Subcellular Fractions

The results presented in Chapter IV demonstrated Nonidet-P40 'activated' islet cell protein kinase activity. The effect of Nonidet-P40 on endogenous protein phosphorylation in the islet homogenate and S-0.6 and S-100 subcellular fractions was examined (Table 5.2) in order to investigate the possibility of Nonidet-P40 'activation' of islet cell S-100 endogenous protein phosphorylation. In the absence of cyclic AMP, significant Nonidet-P40 activation of endogenous protein phosphorylation was observed only in the islet S-100 subcellular fraction. However, in the presence of cyclic AMP (1 μM), significant Nonidet-P40 activation of endogenous protein phosphorylation was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions. Overall, significant cyclic AMP stimulation of endogenous protein phosphorylation was observed in the Nonidet-P40 treated islet homogenate, S-0.6 and S-100 subcellular fractions.

Identification of Phosphorylated Islet Non-Nuclear Proteins (pH 6.0) S.D.S. PolyAcrylamide Gel Electrophoresis

Method

Islet S-0.6 subcellular fractions prepared in Buffer A

(100 µl) were preincubated in the absence and presence of Nonidet-P40 (1%) for 60 min at 37°C. An equal volume of protein kinase assay buffer containing 100 µM (γ-^{32}P)-ATP, plus and minus cyclic AMP (1 µM) and Walsh protein (approx. 300 Units/ml) was added to the reaction vial and the incubation was continued for a further 60 min at 37°C. The reaction was stopped by the addition of 30 µl of 20% S.D.S. The samples were prepared and subjected to S.D.S.-P.A.G.E. as described in detail in Chapter II. Autoradiographs of the dried slab gels were prepared as described in detail in Chapter II.

Results

Effect of Cyclic AMP, Walsh Protein and Nonidet-P40 on ^{32}P Incorporation into Islet (Non-Nuclear) Proteins

The effect of cyclic AMP, Walsh protein and Nonidet-P40 on ^{32}P incorporation into islet (non-nuclear) polypeptides separated by S.D.S. Polyacrylamide Gel Electrophoresis was investigated in order to investigate the possibility that these test substances may have a specific effect on the phosphorylation state of certain individual islet polypeptides.

Nonidet-P40 (1%) and cyclic AMP (1 µM) had no significant effect on the islet (non-nuclear) polypeptide ^{32}P incorporation pattern (Figure 5.1). ^{32}P incorporation into several islet polypeptides was observed and seven polypeptides (approx. molecular weight, 134,000, 110,000, 96,000, 76,000, 50,000, 37,000 and 26,000 daltons) showed significant ^{32}P incorporation.

Figure 5.2 shows the effect of cylic AMP (1 µM) and Walsh protein (approx. 300 Units/ml) on ^{32}P incorporation

Figure 5.1. Method A: Effect of Nonidet-P40 and Cyclic AMP on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 1μM cyclic AMP and 1% Nonidet-P40 as described in the the text.

Lane A:- Control
Lane B:- + 1μM cyclic AMP
Lane C:- + 1% Nonidet-P40
Lane D:- + 1% Nonidet-P40 + 1uM cyclic AMP

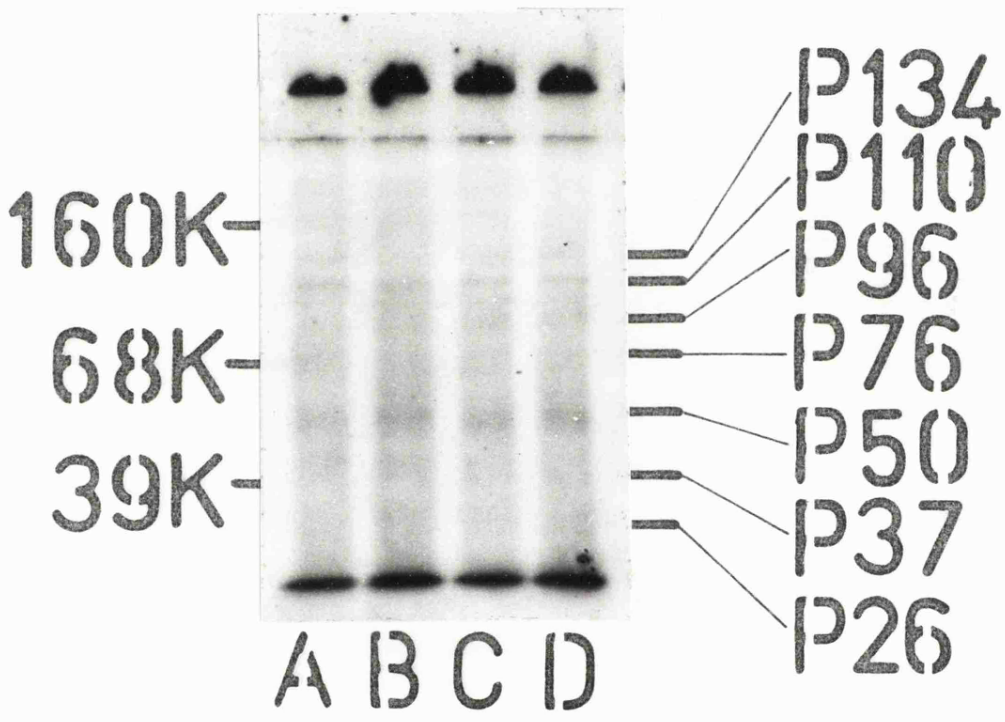
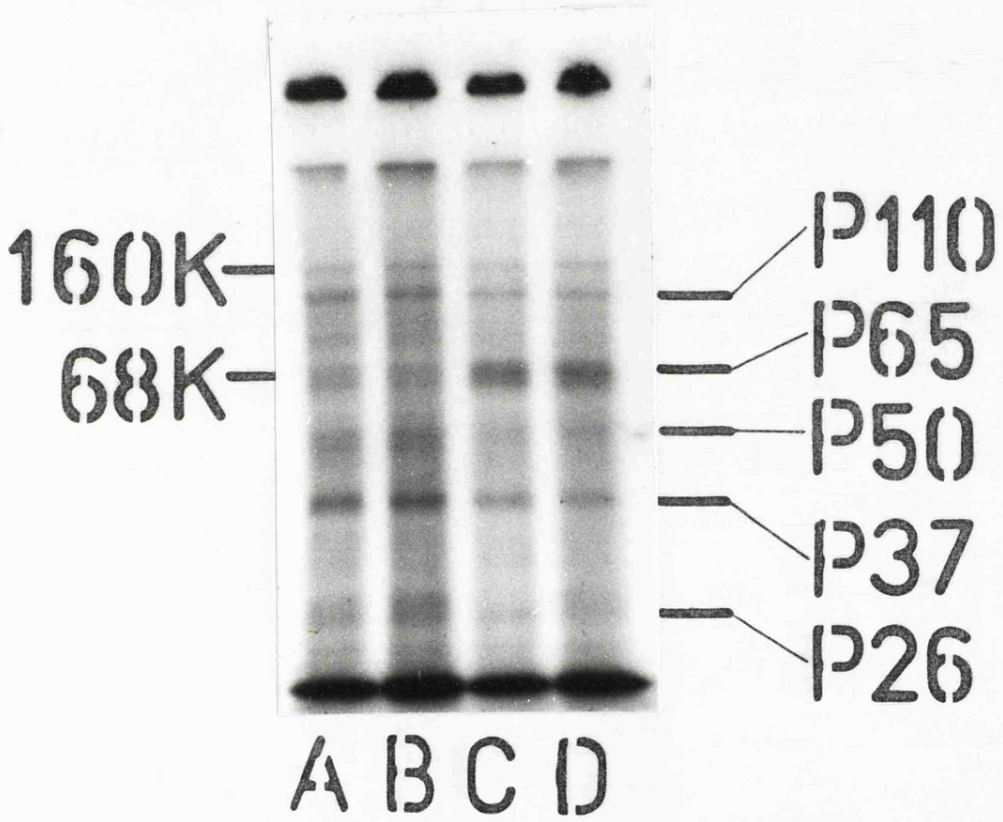

160K
68K
39K
A B C D
P134
P110
P96
P76
P50
P37
P26

Figure 5.2. Method A: Effect of cyclic AMP and Walsh Protein on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.
^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 1μM cyclic AMP and Walsh Protein (approx. 300 Units/ml) after preincubating with 1% Nonidet-P40 as described in detail in the text.

Lane A:- Control
Lane B:- + 1μM cyclic AMP
Lane C:- + Walsh protein (approx. 300 Units/ml)
Lane D:- + Walsh protein (approx. 300 Units/ml)
+ 1μM cyclic AMP.


160K
68K
P110
P65
P50
P37
P26
A B C D

into islet (non-nuclear) polypeptides, after a preincubation with Nonidet-P40 (1%). No significant difference in ^{32}P incorporation was observed in the presence of cyclic AMP. However, in the presence of Walsh protein, a decrease in ^{32}P incorporation was observed in four islet polypeptides (approx. molecular weight, 110,000, 50,000, 37,000 and 26,000 daltons) and an increase in ^{32}P incorporation in the molecular weight range of 68,000 to 62,000 daltons was also observed.

Discussion

Subcellular Distribution of Islet Cell Endogenous Protein Phosphorylation Using Method A.

No cyclic AMP stimulation of endogenous protein phosphorylation was observed in the islet homogenate and islet cell subcellular fractions. These findings extend the observations by Sugden et al. (1979) and Muller et al. (1976) that cyclic AMP had no effect on the phosphorylation of endogenous proteins in rat islet sonicates and homogenates. The majority (53%) of islet cell endogenous phosphorylation was located in the S-100 subcellular fraction in the presence of cyclic AMP (1 μM), however, in the absence of cyclic AMP, the majority (55%) of islet cell endogenous phosphorylation was located in the P-100 subcellular fraction. This correlated with the previous observations of this study (Chapter III) in which the majority of the islet S-100 protein kinase activity was found to be cyclic AMP-dependent and the majority of the islet P-100 protein kinase activity was found to be cyclic AMP-independent. The observation that islet S-100 endogenous phosphorylation was not stimulated by cyclic AMP was thought to be due to an excess of cyclic AMP-independent protein kinase substrates over that

of the cyclic AMP-dependent substrates. The relatively high level of islet cell P-100 endogenous phosphorylation (compared to the level of protein kinase activity present in this fraction) was probably due to the phosphorylation of ribosomal proteins. Ribosomal proteins are basic proteins and therefore would be adsorbed more easily onto the phosphocellulose (P81) papers than the acidic cellular proteins. However, in this study no comparison was made between the subcellular distribution of islet cell endogenous phosphorylation using the Glass method (1978) and the T.C.A. precipitation method.

The Effect of Nonidet-P40 on Endogenous Phosphorylation in the Islet Homogenate, S-0.6 and S-100 Subcellular Fractions

Nonidet-P40 'activation' of islet cell endogenous phosphorylation was observed in the islet homogenate, S-0.6 and S-100 subcellular fractions, in the presence of cyclic AMP. However, in the absence of cyclic AMP, Nonidet-P40 'activation' of islet cell endogenous phosphorylation was only observed in the S-100 subcellular fraction. Overall, significant cyclic AMP stimulation of endogenous phosphorylation was observed in the Nonidet-P40 treated islet homogenate, S-0.6 and S-100 subcellular fractions. This suggested cyclic AMP-dependent protein kinase substrates were present in the islet S-100 subcellular fraction. The Nonidet-P40 'activation' of islet S-100 cyclic AMP-dependent protein kinase activity prevented the phosphorylation of the cyclic AMP-dependent protein kinase substrates from being masked by the phosphorylation of the cyclic AMP-*in*dependent protein kinase substrates. It was thought Nonidet-P40 may prove to be a valuable tool in the identification of

the cyclic AMP-dependent protein kinase substrates, separated by S.D.S. Polyacrylamide Gel Electrophoresis.

S.D.S. Polyacrylamide Gel Electrophoresis (PAGE) of Phosphorylated Islet Non-Nuclear Proteins (Method A)

Cyclic AMP and Nonidet-P40 had no significant effect on ^{32}P incorporation into islet non-nuclear polypeptides separated by S.D.S. P.A.G.E. This was thought to have been due to the long incubation times (1 hour) used. Incubation times of one hour were necessary in order to obtain satisfactory autoradiographs. However, in the presence of Walsh protein, decreases in the ^{32}P incorporation into four islet (non-nuclear) polypeptides (approx. molecular weights 110,000, 50,000, 37,000 and 26,000 daltons) were observed. This indicated that the phosphorylation of these four polypeptides was catalysed by cyclic AMP-dependent protein kinases. In the presence of Walsh protein, an increase in ^{32}P incorporation into a group of islet polypeptides (approx. molecular weights 62,000-68,000 daltons) was also observed. This was thought to be due to the increase of available (γ-^{32}P)-ATP substrate (due to the inhibition of the cyclic AMP-dependent protein kinase catalysed phosphorylation) increasing the ^{32}P incorporation into the cyclic AMP-<u>inde</u>pendent protein kinase substrates. The presence of ^{32}P unable to enter the stacking gel of the S.D.S. P.A.G.E. indicated ^{32}P incorporation into high molecular weight proteins, such as glycoproteins, was occurring.

As cyclic AMP and Nonidet-P40 were found to have no direct effect on the ^{32}P incorporation into islet (non-nuclear) polypeptides using Method A, it was decided to adopt the method of Rudolph and Kreuger (1979). The advantage of this method (B) over method A was thought to

be the use of lower concentrations of cold ATP, thereby increasing the specific activity of the (γ-^{32}P)-ATP present in the reaction mix. As high levels of ATPase activity were present in the islet S-0.6 subcellular fraction (Chapter IV), it was thought that by reducing the reaction time to 5 min, the depletion of (γ-^{32}P)-ATP substrate would be minimal.

Method B (Rudolph & Kreuger, 1979) pH 7.0

Islet S-0.6 Endogenous Protein Phosphorylation

The effects of cyclic AMP and Nonidet-P40 on the total endogenous protein phosphorylation of the islet S-0.6 subcellular fraction, using Method B, were examined to ensure cyclic AMP and Nonidet-P40 had an effect on islet cell endogenous protein phosphorylation under these conditions.

Method

Islet S-0.6 subcellular fractions were prepared and endogenous protein phosphorylation was measured (as described in detail in Chapter II) in the absence and presence of cyclic AMP (10 μM) and Nonidet-P40 (1%). The reaction was terminated by the addition of 2 ml of 10% TCA after an incubation of 5 min at 37°C.

Results

Effect of Cyclic AMP and Nonidet-P40 on Endogenous Protein Phosphorylation in the Islet S-0.6 Subcellular Fraction

Cyclic AMP (10 μM) and Nonidet-P40 (1%) significantly inhibited total endogenous protein phosphorylation in the islet S-0.6 subcellular fraction (Table 5.3). The inhibition of islet S-0.6 endogenous protein phosphorylation by cyclic AMP and Nonidet-P40 was additive.

TABLE 5.3 - Effect of Cyclic AMP and Nonidet-P40 on Endogenous Protein Phosphorylation in the Islet S-0.6 Subcellular Fraction

^{32}P incorporation into TCA precipitated proteins was measured as described in detail in the text, in the absence and presence of cyclic AMP (10 μM) and Nonidet-P40 (10%).

Addition to the Incubation Medium	Endogenous Protein Phosphorylation (f moles ^{32}P/min/islet)
--	5.20 (± 1.84) (4)
10 μM cyclic AMP	3.28 (± 0.89)* (4)
1% Nonidet-P40	1.73 (± 0.51)* (4)
1% Nonidet-P40 + 10 μM cyclic AMP	0.92 (± 0.38)** (4)

* Values assessed by Student's t test to be significantly different from the control with no additions to the medium ($p < 0.05$).

** Values assessed by Student's t test to be significantly different from the addition of either 10 μM cyclic AMP or 1% Nonidet-P40 ($p < 0.05$).

Effect of Cyclic AMP and Nonidet-P40 on the Time Course of Islet S-0.6 Endogenous Protein Phosphorylation

The possibility Nonidet-P40 and cyclic AMP inhibition of islet S-0.6 endogenous protein phosphorylation may only occur after 5 min incubation was investigated by determining the effects of cyclic AMP and Nonidet-P40 on the time course of islet S-0.6 endogenous protein phosphorylation.

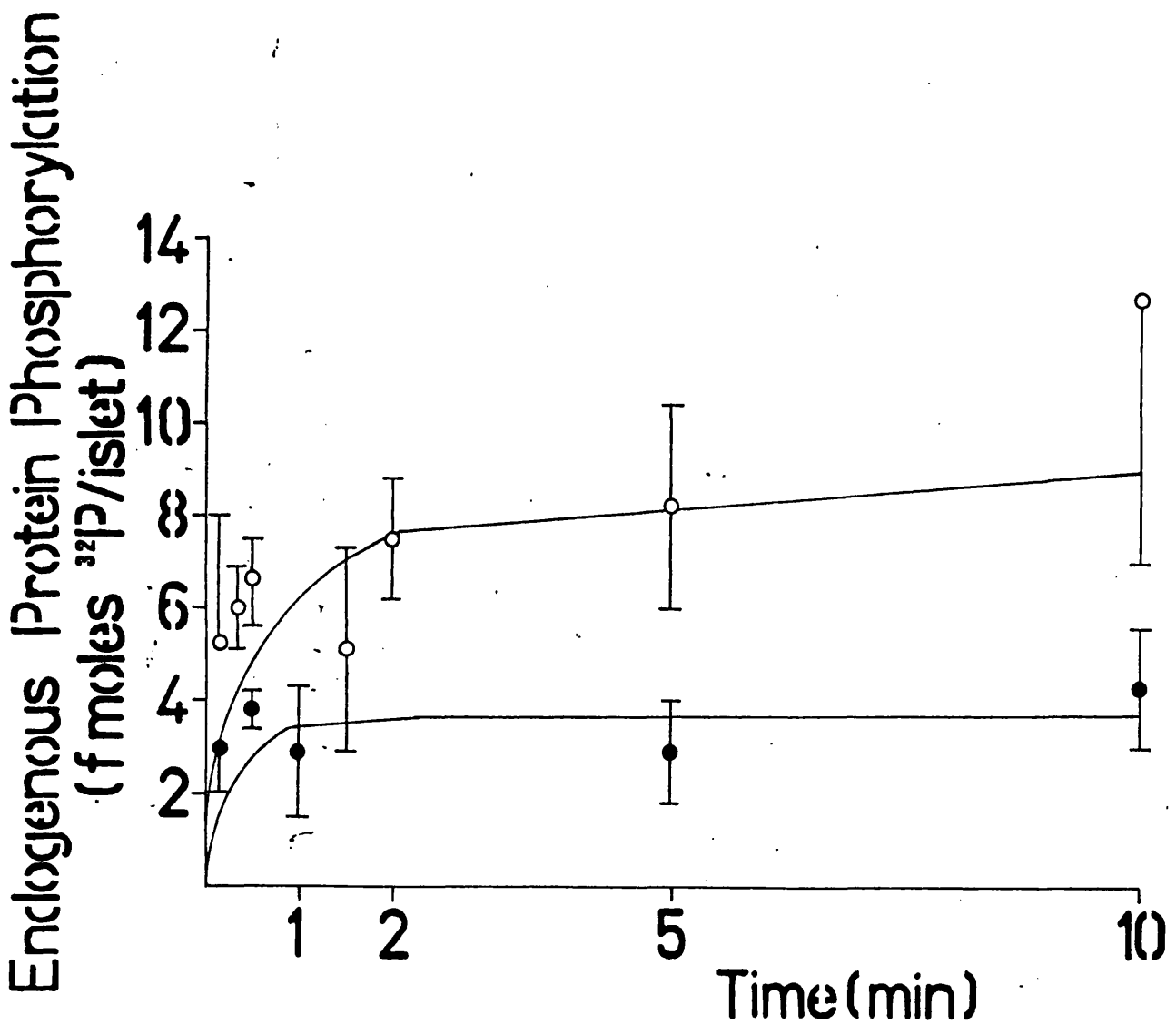
Method

Islet S-0.6 subcellular fractions were prepared and the time course of endogenous protein phosphorylation was measured in triplicate, in the absence and presence of cyclic

Figure 5.3a). Method B: Effect of Cyclic AMP on the Time Course of Islet S-0.6 Endogenous Protein Phosphorylation. Islet S-0.6 endogenous protein phosphorylation was assayed in triplicate, at specific time intervals, in the absence (o) and presence (●) of 10μM cyclic AMP as described in detail in the text. The results are typical of those obtained in three seperate experiments.

Endogenous Protein Phosphorylation
(f moles 32P/ islet)
60
40
20
1
2
5
10
Time (min)

Figure 5.3b). Method B: Effect of Nonidet-P40 on the Time Course of Islet S-0.6 Endogenous Protein Phosphorylation. Islet S-0.6 endogenous protein phosphorylation was assayed in triplicate, at specific time intervals, in the presence of 1% Nonidet-P40 as described in detail in the text. Cyclic AMP dependency was measured in the absence (o) and presence (•) of 10μM cyclic AMP. The results are typical of those obtained in three seperate experiments.


Endogenous Protein Phosphorylation
(f moles ^{32}P/islet)
14
12
10
8
6
4
2
1
2
5
10
Time (min)

AMP (10 μM) and Nonidet-P40 (1%), using a total reaction volume of 400 μl. At various time intervals 40 μl aliquots were removed and added to 2 ml of 10% TCA. Proteins were separated from the reaction mixture using the method described in Chapter II.

Results

In the absence of Nonidet P-40, initial islet S-0.6 endogenous protein phosphorylation was very rapid and was maximal after 30 second incubation (Figure 5.3a). After 30 second, protein dephosphorylation was observed, in the presence of cyclic AMP the rate of dephosphorylation was greater. After 5 min incubation no further change in endogenous protein phosphorylation was observed. Cyclic AMP inhibition of endogenous protein phosphorylation was observed after 5 min incubation.

In the presence of Nonidet-P40 (Figure 5.3b), initial islet S-0.6 endogenous protein phosphorylation was rapid but no dephosphorylation was observed. Greater endogenous protein phosphorylation was obtained in the absence of cyclic AMP. After 2 min and 1 min (in the presence of cyclic AMP) no further changes in islet S-0.6 endogenous phosphorylation were observed.

S.D.S. Polyacrylamide Gel Electrophoresis (PAGE) pH 7.0

Method

Islet S-0.6, S-100 and P-100 subcellular fractions were prepared as described in detail in Chapter II. The samples (100 μl) were incubated with 1 μM (γ-^{32}P)-ATP in the absence and presence of cyclic AMP (10 μM), Walsh protein (approx. 300 Units/ml) and Nonidet-P40 (1%) for 5 min at

37°C. The reaction was terminated by the addition of 30 µl of 20% S.D.S. The samples were prepared and subjected to S.D.S. PAGE as described in detail in Chapter II. Autoradiographs of the dried slab gels were prepared as described in detail in Chapter II.

Results

(i) Effect of Cyclic AMP and Nonidet-P40

Although it appeared that cyclic AMP and Nonidet-P40 inhibited total islet S-0.6 endogenous protein phosphorylation, the possibility cyclic AMP and Nonidet-P40 might stimulate ^{32}P incorporation into specific islet (non-nuclear) polypeptides separated by S.D.S. PAGE was examined.

In the absence of cyclic AMP and Nonidet-P40, ^{32}P was largely incorporated from (γ-^{32}P)-ATP into five islet polypeptides of approximate molecular weights 144,000, 125,000, 88,000, 63,000 and 54,000 daltons (P144, P125, P88, P63 and P54 respectively) after 5 min incubation (Figure 5.4c). After 15 second incubation (Figure 5.4a) ^{32}P incorporation into three islet polypeptides (P125, P88 and P54) was observed. Irrespective of the incubation time, cyclic AMP inhibition of ^{32}P incorporation into the P54 polypeptide and Nonidet-P40 inhibition of ^{32}P incorporation into the P88 polypeptide was observed. High levels of ^{32}P incorporation were observed in the P54 polypeptide and to a lesser extent in the P88 polypeptide. No stimulation of polypeptide ^{32}P incorporation was observed in the presence of either cyclic AMP or Nonidet-P40.

(ii) Time Course of ^{32}P Incorporation

Figure 5.5 shows the time course of ^{32}P incorporation

Figure 5.4. Method B: Effect of Cyclic AMP and Nonidet-P40 on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography, in the absence and presence of 10μM cyclic AMP and 1% Nonidet-P40, after an incubation of 15 seconds (a), 1 min (b) and 5 min (c) as described in detail in the text.

Lane :- Control
Lane B:- + 10μM cyclic AMP
Lane C:- + 1% Nonidet-P40
Lane :- + 1% Nonidet-P40 + 10μM cyclic AMP

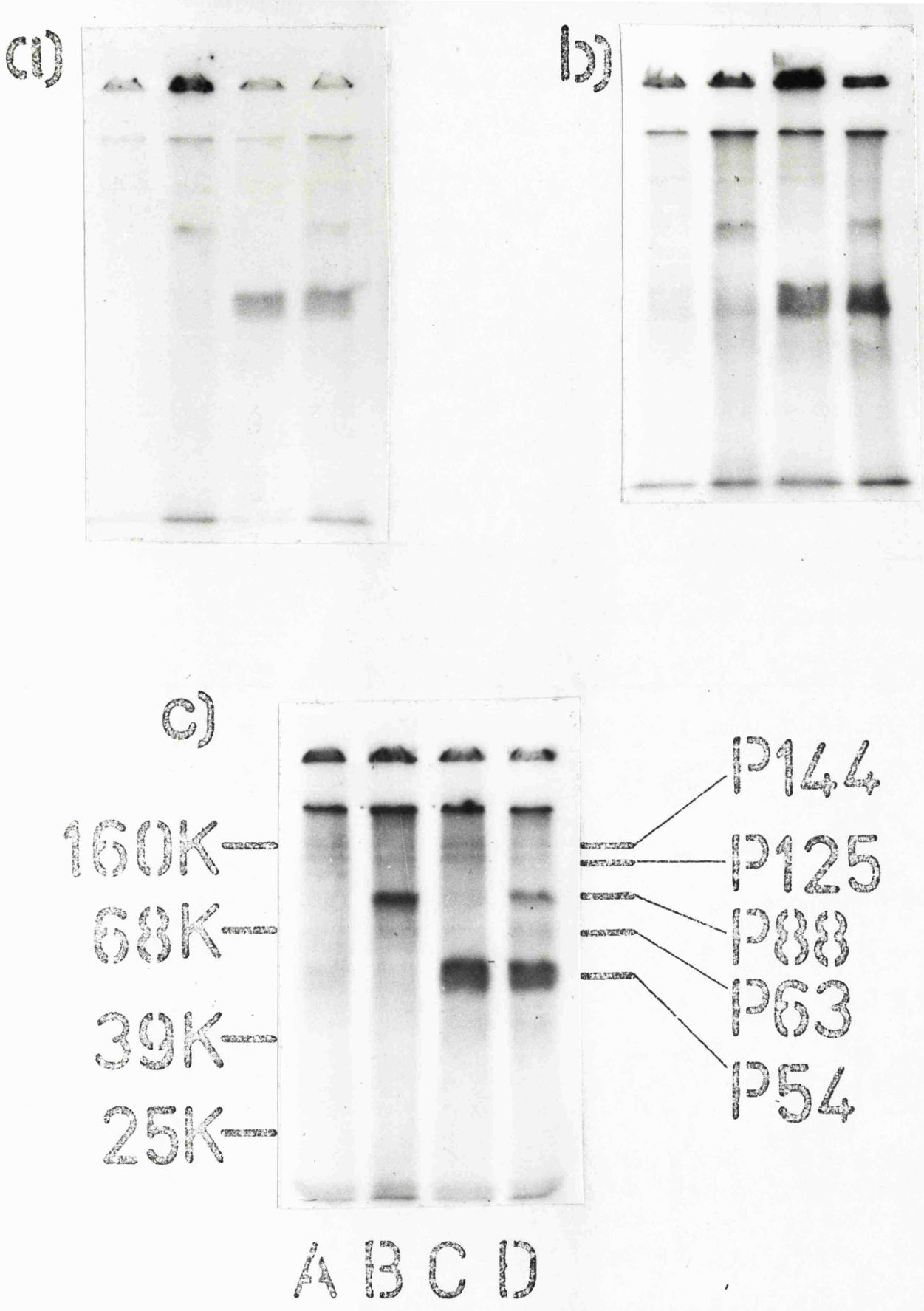
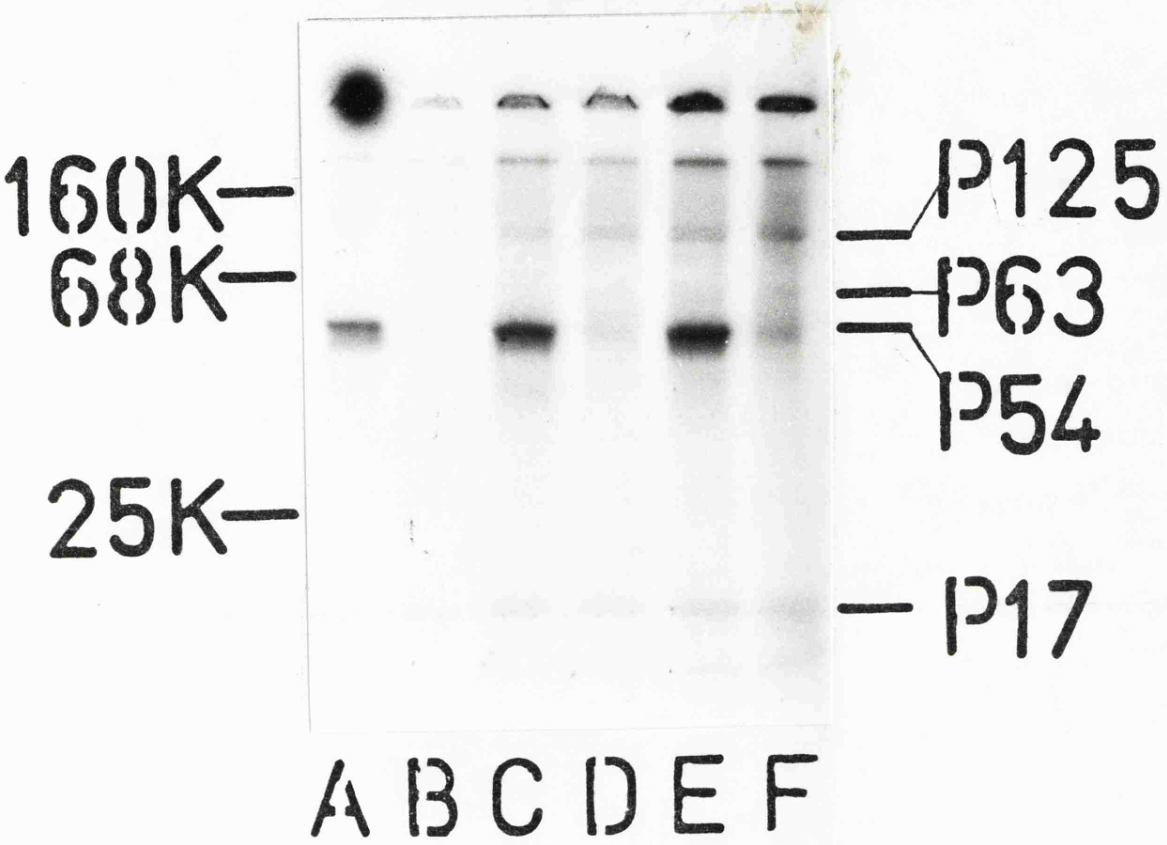

a)
b)
c)
160K
68K
39K
25K
P144
P125
P88
P63
P54
A B C D

Figure 5.5. Time Course of Islet (non-nuclear) Polypeptide ^{32}P Incorporation. Effect of Cyclic AMP.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the presence of 1% Nonidet-P40 and in the absence and presence of 10μM cyclic AMP, after an incubation of 15 seconds, 1 min and 5 min, as described in detail in the text.

Lane A:- Control	) 15 seconds incubation
Lane B:- + 10μM cyclic AMP	)
Lane C:- Control	) 1 min incubation
Lane D:- + 10μM cyclic AMP	)
Lane E:- Control	) 5 min incubation
Lane F:- + 10μM cyclic AMP	)


160K
68K
25K
P125
P63
P54
P17
A B C D E F

into islet non-nuclear polypeptides in the presence of Nonidet-P40 (1%). After 15 second incubation, one major phosphorylated polypeptide (P54) was observed. After 1 minute incubation, three phosphorylated polypeptides (P125, P54 and P17) were observed. After 5 minutes incubation, four major phosphorylated polypeptides (P125, P63, P54 and P17) were observed. Cyclic AMP inhibited ^{32}P incorporation into the P54 polypeptide after 15 second, 1 minute and 5 minutes incubation.

iii) Effect of Walsh Protein and Cyclic AMP

The effect of Walsh protein (approx. 300 Units/ml) on ^{32}P incorpation into islet polypeptides, in the absence and presence of cyclic AMP (10 μM), was investigated in order to examine the possibility that a cyclic AMP-dependent protein kinase may either be involved in the phosphorylation of the islet P54 polypeptide or involved in the cyclic AMP inhibition of phosphorylation of the islet P54 polypeptide.

Walsh protein had no significant effect on the ^{32}P incorporation into the islet P54 polypeptide or the cyclic AMP inhibition of ^{32}P incorporation into the islet P54 polypeptide (Figure 5.6).

iv) Effect of Cyclic AMP Concentration

Although cyclic AMP (10 μM) was found to inhibit ^{32}P incorporation into an islet P54 polypeptide, the possibility cyclic AMP, at concentrations less than 10 μM, may have a stimulatory effect on ^{32}P incorporation into islet polypeptides was examined (Figure 5.7). Cyclic AMP inhibited ^{32}P incorporation into the islet P54 polypeptide at concentrations of 1 μM, 5 μM and 10 μM. Cyclic AMP at the concentrations used had no stimulatory effect on islet polypeptide ^{32}P

Figure 5.6. Method B: Effect of Walsh Protein and Cyclic AMP on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the presence of 1% Nonidet-P40 and in the absence and presence of 10μM cyclic AMP and Walsh protein (approx. 300 units/ml), after an incubation of 1 min, as described in detail in the text.

Lane A:- + 10μM cyclic AMP + Walsh protein (approx. 300 Units/ml)
Lane B:- + 10μM cyclic AMP
Lane C:- + Walsh protein (approx. 300 Units/ml)
Lane D:- Control

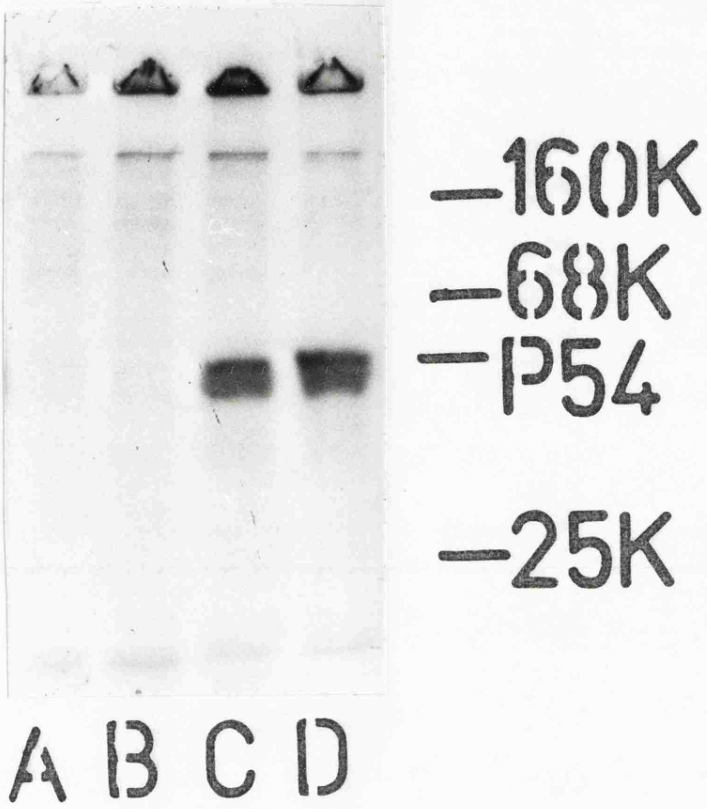
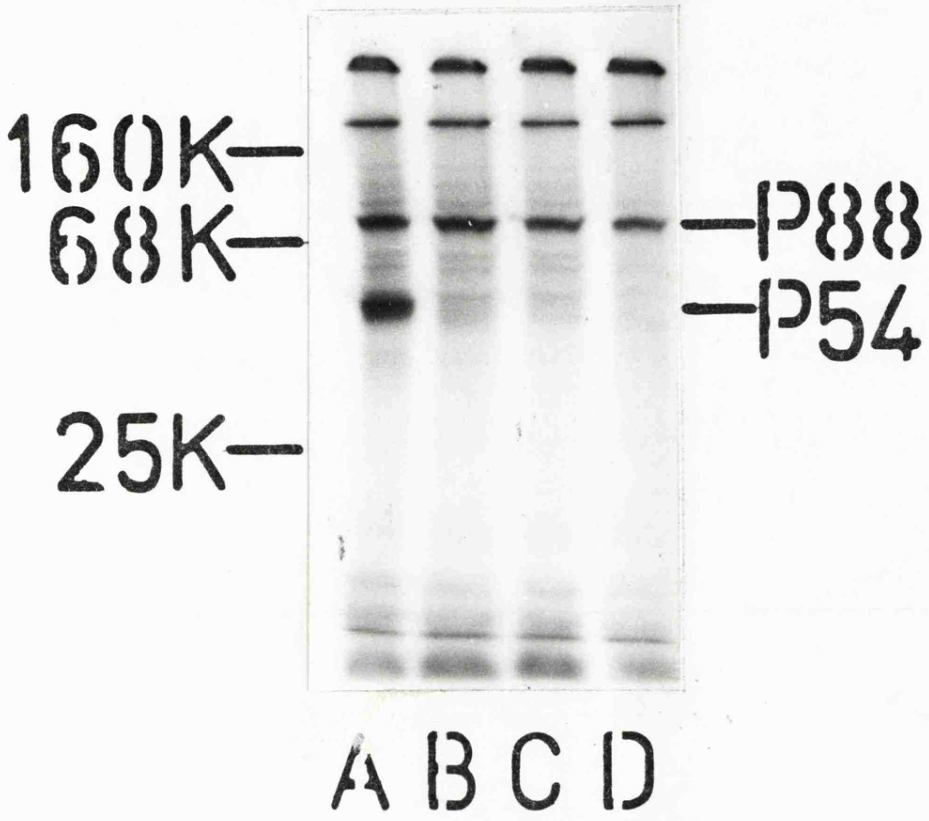

160K
68K
P54
25K
A B C D

Figure 5.7. Method B: Effect of Cyclic AMP Concentration on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.
^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 1μM, 5μM and 10μM cyclic AMP, after an incubation of 5 min, as described in detail in the text.

Lane A:- Control
Lane B:- + 1μM cyclic AMP
Lane C:- + 5μM cyclic AMP
Lane D:- + 10μM cyclic AMP


160K
68K
25K
P88
P54
A B C D

incorporation.

v) Subcellular Distribution of Islet S-0.6 ^{32}P Incorporation

The subcellular distribution of ^{32}P incorporation into islet non-nuclear proteins was determined in order to locate the subcellular fraction containing the P54 polypeptide. In the absence of cyclic AMP, the majority of ^{32}P incorporation occurred in the S-100 islet subcellular fraction (Figure 5.8a). ^{32}P incorporation into six polypeptides (P144, P125, P88, P63, P54 and P31) was observed in the islet S-100 subcellular fraction. In the islet P-100 subcellular fraction, ^{32}P incorporation into one polypeptide (P54) was observed. The majority of ^{32}P incorporation into the P54 polypeptide was located in the islet S-100 subcellular fraction.

The subcellular distribution of ^{32}P incorporation into islet non-nuclear proteins, in the presence of cyclic AMP (10 μM), was also determined (Figure 5.8b). The majority of ^{32}P incorporation occurred in the islet S-100 subcellular fraction. ^{32}P incorporation into six polypeptides (P144, P125, P88, P63, P54 and P31) was observed in the islet S-100 subcellular fraction. In the islet P-100 subcellular fraction no major ^{32}P incorporation was observed.

vi) Effect of Cyclic AMP on the Dephosphorylation of Islet S-0.6 Phosphorylated Polypeptides

The possibility cyclic AMP may inhibit ^{32}P incorporation into the islet S-100 P54 polypeptide by activating a protein phosphatase was examined.

Method

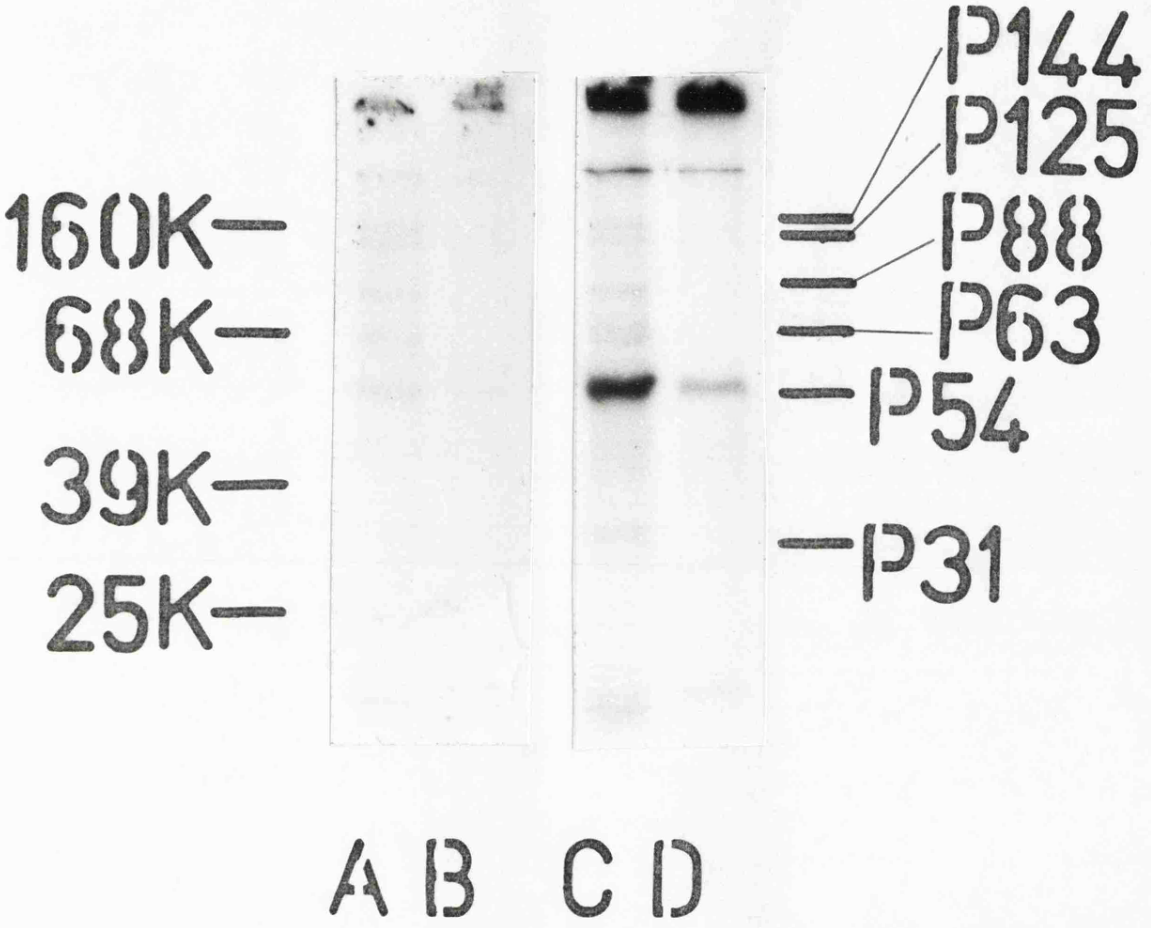
Islet S-0.6 subcellular fractions were prepared as

Figure 5.8. Method B: Subcellular Distribution of Islet S-0.6 ^{32}P Incorporation.

Islet subcellular (S-100 and P-100) fractions were prepared. ^{32}P incorporation into the S-100 and P-100 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 10μM cyclic AMP, after an incubation of 5 min, as described in detail in the text.

Lane A:- S-100 Fraction) + 10μM cyclic AMP
Lane B:- P-100 Fraction)

Lane C:- S-100 Fraction) Control
Lane D:- P-100 Fraction)


160K
68K
39K
25K
P144
P125
P88
P63
P54
P31
A B C D

described in Chapter II. 100 µl samples were incubated with 1 µM (γ-^{32}P)-ATP for 5 min at 37°C. Cold ATP (1 mM) plus and minus 10 µM cyclic AMP was added to the reaction tubes which were further incubated at 37°. The reaction was terminated after time intervals of 15 sec, 1 min and 5 min with the addition of 30 µl of 20% S.D.S. and processed for S.D.S. P.A.G.E. as previously described. S.D.S. P.A.G.E. was performed as described in detail in Chapter II. Autoradiographs of the dried slab gels were then prepared

Results

Cyclic AMP (10 µM) had no demonstrable effect on the rate of dephosphorylation of the islet S-100 P54 polypeptide (Figure 5.9). No significant protein dephosphorylation was observed until after 5 min incubation (Figure 5.9 Lanes F and G). Thus cyclic AMP appeared not to increase the rate of dephosphorylation of the islet S-100 P-54 polypeptide.

vii) Effect of Colchicine on Islet S-0.6 Protein Phosphorylation

Tubulin and microtubule associated proteins (MAPs) are predominantly located in the S-100 subcellular fraction of islets of Langerhans (Montague et al., 1975). Evidence to suggest cyclic AMP and calcium may directly or indirectly regulate the equilibrium between intact microtubules and their subunits in islets of Langerhans has also been reported (Montague and Howell, 1976). In order to examine the possibility that the islet S-100 P54 polypeptide may be tubulin or a MAP, the effect of colchicine, an inhibitor of microtubule subunit polymerisation (Wilson et al., 1974), on the phosphorylation state of this polypeptide was examined in the absence and presence of cyclic AMP (10 µM).

Figure 5.9. Method B: Effect of Cyclic AMP on The Dephosphorylation of Phosphorylated S-0.6 Polypeptides.
The dephosphorylation of ^{32}P labelled islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 10μM cyclic AMP, after an incubation of 15 seconds, 1 min and 5 min, as described in detail in the text.

Lane A:- No dephosphorylation control

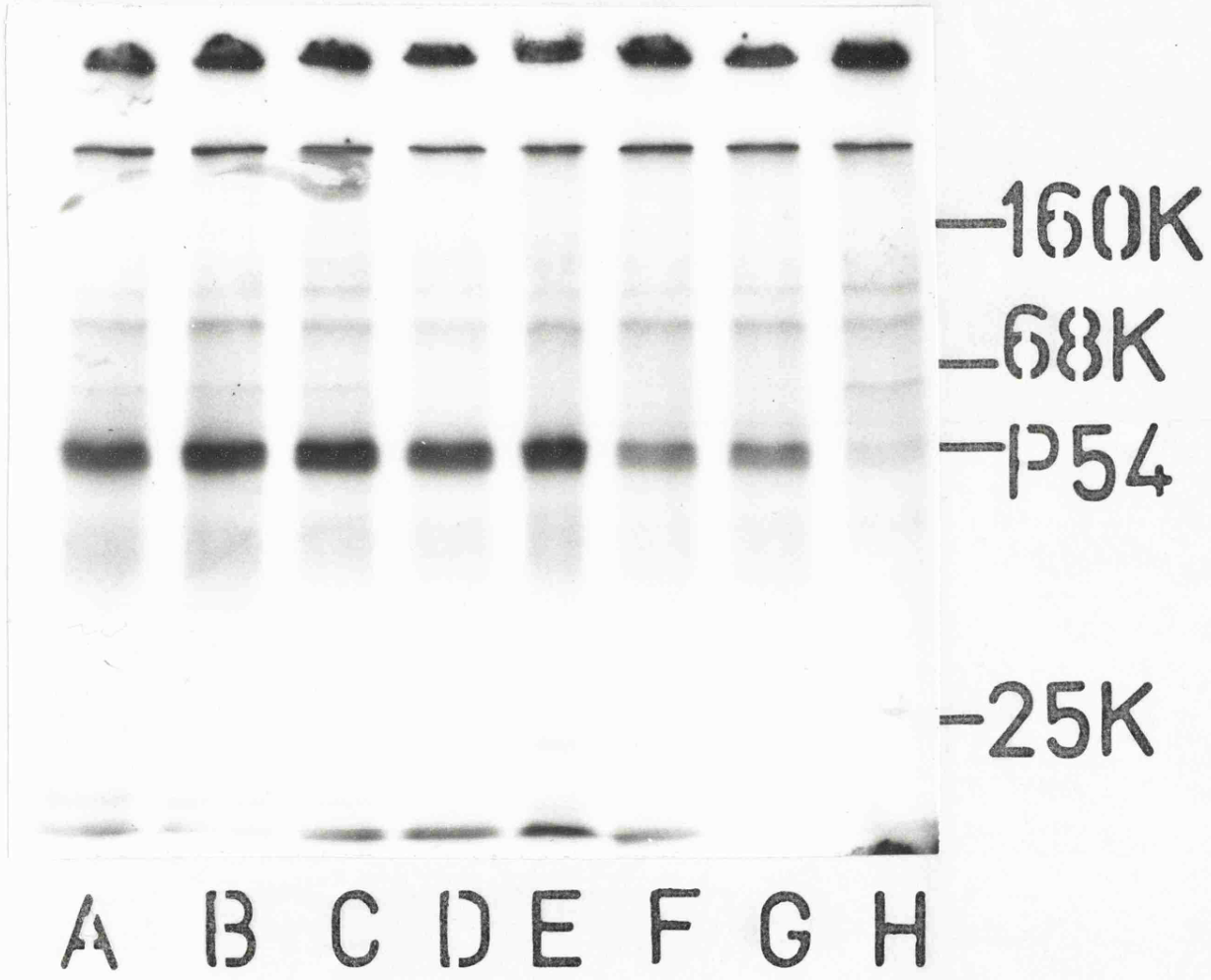
Lane B:- Dephosphorylation) 15
Lane C:- Dephosphorylation + 10μM cyclic AMP) seconds

Lane D:- Dephosphorylation) 1 min
Lane E:- Dephosphorylation + 10μM cyclic AMP)

Lane F:- Dephosphorylation) 5 min
Lane G:- Dephosphorylation + 10μM cyclic AMP)

Lane H:- No dephosphorylation + 10μM cyclic AMP

LANE	ADDITION TO THE PREINCUBATION MIX	DEPHOSPHORYLATION TIME	ADDITION TO THE DEPHOSPHORYLATION MIX
A.	—	—	—
B	—	15 SECS	—
C	—	15 SECS	10μM cyclic AMP
D	—	1 MIN	—
E	—	1 MIN	10μM cyclic AMP
F	—	5 MIN	—
G	—	5 MIN	10μM cyclic AMP
H	10μM cyclic AMP	—	—


160K
68K
P54
25K
A
B
C
D
E
F
G
H

Method

Islet S-0.6 subcellular fractions were prepared as described in Chapter II. 100 µl samples were preincubated at 37°C for 30 min, in the absence and presence of cyclic AMP (10 µM) and colchicine (0.1 mM), in a total volume of 180 µl. 20 µl of 10µM (γ-^{32}P)-ATP, plus and minus cyclic AMP (100µM), was added to the reaction medium and incubated for a further 5 min at 37°C. The reaction was terminated by the addition of 30 µl of 20% S.D.S. and the samples were prepared and subjected to S.D.S. P.A.G.E. as previously described in Chapter II. Autoradiographs of the dried slab gels were prepared as described in detail in Chapter II.

Results

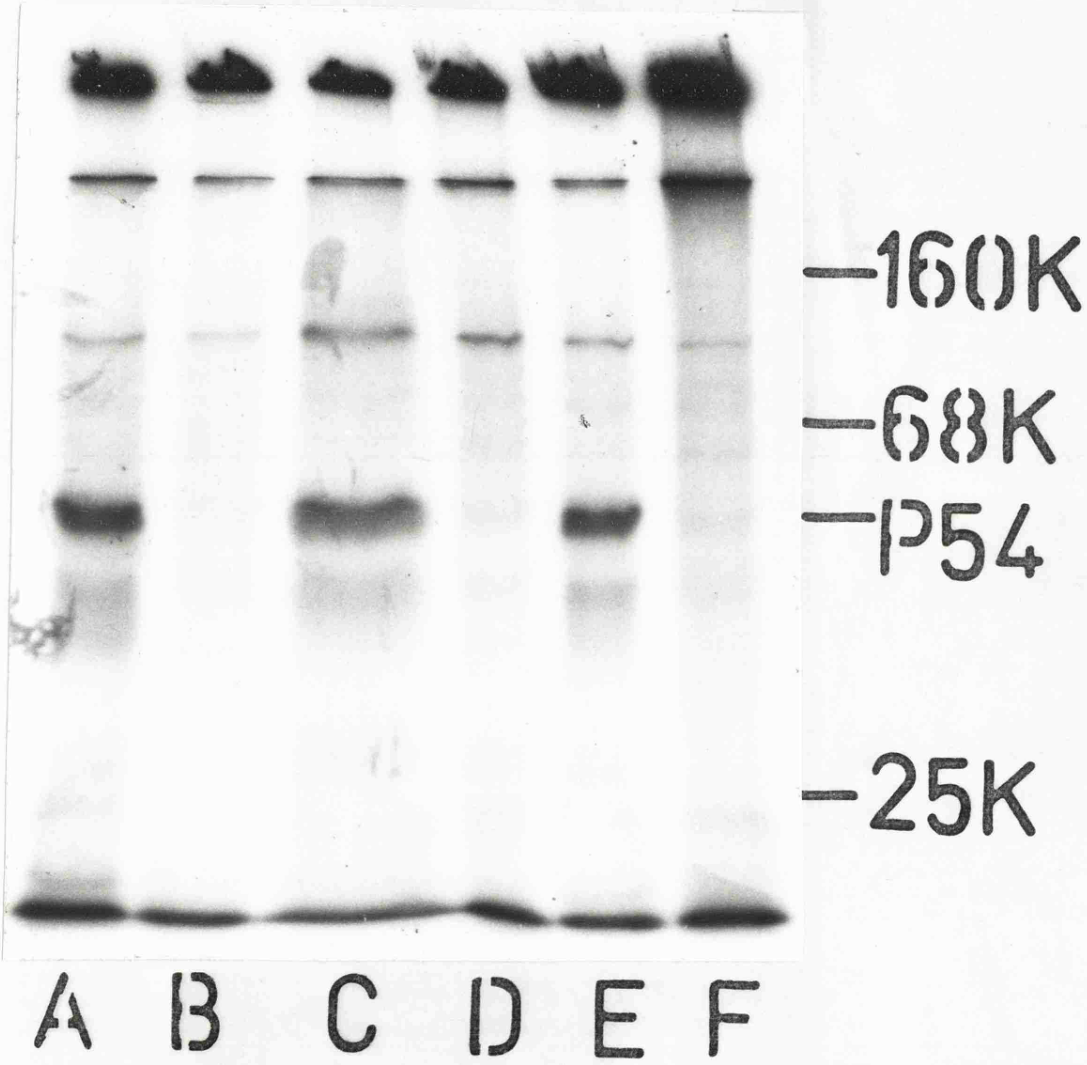
Preincubation of the islet S-0.6 subcellular fraction with 0.1 mM colchicine, in the absence of cyclic AMP, had no effect on the cyclic AMP inhibition of ^{32}P incorporation into the islet S-100 P54 polypeptide (Figure 5.10 Lanes A, B, C and D). When cyclic AMP (10 µM) was added to the preincubation mixture, no inhibition of ^{32}P incorporation into the islet S-100 P54 polypeptide occurred. However, when cyclic AMP (10 µM) and colchicine (0.1 mM) were both added to the preincubation mixture, inhibition of ^{32}P incorporation into the islet S-100 P54 polypeptide was observed.

Figure 5.10. Method B: Effect of Colchicine on Islet (non-nuclear) Polypeptide ^{32}P Incorporation.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 10μM cyclic AMP (5 min incubation), after a 30 min preincubation in the absence and presence of 0.1mM Colchicine and 10μM cyclic AMP as described in detail in the text.

Lane A:- Control
Lane B:- + 10μM cyclic AMP
Lane C:- Preincubation with 0.1mM Colchicine
Lane D:- Preincubation with 0.1mM Colchicine + 10μM cyclic AMP
Lane E:- Preincubation with 10μM cyclic AMP
Lane F:- Preincubation with 10μM cyclic AMP and 0.1mM Colchicine.

LANE	ADDITION TO THE PREINCUBATION MIX	ADDITION TO THE REACTION MIX
A	—	—
B	—	10μM cyclic AMP
C	0.1mM COLCHICINE	—
D	0.1mM COLCHICINE	10μM cyclic AMP
E	10μM cyclic AMP	—
F	0.1mM COLCHICINE + 10μM cyclic AMP	—


160K
68K
P54
25K
A
B
C
D
E
F

Discussion

Islet S-0.6 Endogenous Protein Phosphorylation

Nonidet-P40 (1%) and cyclic AMP (10 μM) inhibited total islet S-0.6 endogenous protein phosphorylation. Nonidet-P40 inhibition and cyclic AMP inhibition of islet S-0.6 endogenous protein phosphorylation was found to be additive. Nonidet-P40 'activation' of islet cell endogenous protein phosphorylation therefore did not occur at pH 7.0.

In the absence of Nonidet-P40, the time course of ^{32}P incorporation into islet S-0.6 proteins was maximal after 30 second incubation, after which dephosphorylation of total islet S-0.6 proteins occurred. However in the presence of Nonidet-P40 no dephosphorylation was observed. Hence, it was thought the dephosphorylation observed in the absence of Nonidet-P40 may have been due to depletion of (γ-^{32}P)-ATP substrate by ATPase activity. In the presence of Nonidet-P40 inhibition of ATPase activity was likely to occur (Chapter IV; Brotherus et al., 1979), thereby preventing depletion of (γ-^{32}P)-ATP substrate. The possibility that Nonidet-P40 may also inhibit a protein phosphatase should not be ignored.

As cyclic AMP inhibited total islet S-0.6 endogenous phosphorylation this suggested the possibility that cyclic AMP may exert its effect in islet cells via a dephosphorylation of a specific protein(s), similar to that observed in Adrenal preperations (Koroscil and Gallant, 1980).

S.D.S. Polyacrylamide Gel Electrophoresis (PAGE) pH 7.0

In the absence of any test substances the majority of ^{32}P incorporation was found to occur in two islet polypep-

tides (approx. molecular weight 88,000 and 54,000 daltons). Phosphorylation of the 88,000 dalton (P88) polypeptide was inhibited by Nonidet-P40 (1%). This suggested the possibility that the islet P88 polypeptide may be an ATPase phosphoenzyme intermediate (Chaloub and de Meis, 1980). Reports that non-ionic detergents inactivate the sodium and potassium (Na^+/K^+) ATPase activity of the electric eel (Brotherus et al., 1979) but activate human erythrocyte calcium and magnesium (Ca^{++}/Mg^{++}) ATPase activity (Gietsen et al., 1980) provides evidence to suggest the possibility that the islet P88 polypeptide may be the phosphoenzyme intermediate of an islet (Na^+/K^+) ATPase.

Cyclic AMP inhibited ^{32}P incorporation into an islet S-100 P54 polypeptide. Cyclic AMP inhibition of protein ^{32}P incorporation has been observed in rat synaptic membranes (Thérien and Mushynski, 1979) and rat adrenal preparations (Koroscil and Gallant, 1980). As in this study, the cyclic AMP inhibition of ^{32}P incorporation into the rat synaptic membrane protein (approx. molecular weight 56,000 daltons) was found to be due to inhibition of phosphorylation rather than an increase in the rate of dephosphorylation. The possibility that the islet S-100 P54 polypeptide may be the regulatory subunit of the Type II cyclic AMP-dependent protein kinase (Ueda and Greengard, 1977) cannot be entirely dismissed.

Autophosphorylation of the cyclic AMP-dependent protein kinase regulatory subunit (molecular weight 54,000 dalton) results in retardation of the rate of reassociation of its isolated subunits in the absence of cyclic AMP (Rangel-Aldao and Rosen, 1976). It is thought likely, in vivo, the

Type II cyclic AMP-dependent protein kinase is phosphorylated (Erlichman et al., 1974) and thus predominately in the dissociated (activated) form. The discovery of a cardiac muscle phosphoprotein phosphatase that acts on the isolated phosphorylated regulatory subunit rather than the phosphorylated holoenzyme (Chou et al., 1977) suggests a mechanism whereby the reassociation of the enzyme can occur at physiological concentrations. Thus physiological elevations of cyclic AMP would result in the activation of the protein kinase. However, the observation that the rat synaptic membrane phosphoprotein phosphatase activity had no apparent specificity in the absence of cyclic AMP, but was almost exclusively directed against the regulatory subunit of the cyclic AMP-dependent protein kinase in the presence of the cyclic nucleotide (Thérien and Mushynski, 1979) suggests the possibility cyclic AMP may exert its effect in some tissues by a specific dephosphorylation process.

In this study Walsh protein had no effect on the phosphorylation of the islet S-100 P54 polypeptide, this therefore excluded the possibility that the ^{32}P incorporation may have been catalysed by a cyclic AMP-dependent protein kinase. The cyclic AMP inhibition of ^{32}P incorporation into the islet S-100 P54 polypeptide was also unaffected by the presence of Walsh protein, thus the inhibition was not catalysed by a cyclic AMP-dependent protein kinase. However, these results did not exclude the possibility that the islet P54 polypeptide may be the regulatory subunit of the cyclic AMP-dependent protein kinase, as Walsh protein will not inhibit catalytic subunits which are bound to the regulatory subunits.

Cyclic AMP inhibition of phosphorylation of the islet P54 polypeptide was prevented by preincubating the islet S-0.6 subcellular fraction with cyclic AMP at 37°C for 30 min. Preincubation with both cyclic AMP and Colchicine, resulted in an inhibition of phosphorylation of the islet P54 polypeptide. Colchicine has been shown to disrupt microtubular function in a wide variety of cell types (Borisy and Taylor, 1967; Bensch and Malawista, 1969) and to inhibit insulin secretion (Lacy et al., 1968; Montague et al., 1975). The findings of this study indicated that the phosphorylation of the islet cell P54 polypeptide may be connected with the islet cell microtubular system. The observation that purified microtubular protein is phosphorylated in a reaction which is stimulated by cyclic AMP (Goodman et al., 1970; Sloboda et al., 1975; Sheterline, 1977) has generated considerable interest in phosphorylation as a possible mechanism for the regulation of microtubule-mediated functions. In microtubule preparations purified by cycles of assembly and disassembly, the major substrate for the cyclic AMP-stimulated phosphorylation reaction was found to be a group of high molecular weight microtubule-associated proteins (MAPs) (Rappaport et al., 1976; Sheterline, 1977). A recent study has indicated that the phosphorylation of MAPs inhibits both the rate and extent of microtubule assembly (Jameson et al., 1980). Furthermore, the MAPs were found to be the major phosphorylated components of microtubular protein, with very little ^{32}P incorporation into tubulin (approx. molecular weight 54,000 daltons). Thus it is unlikely that the islet S-100 P54 polypeptide is indeed tubulin, but the possibility that this polypeptide may be a low molecular

weight tubulin associated protein such as that identified in neuroblastoma cells (Nagle et al., 1977) and HeLa cells (Weatherbee et al., 1978) still remains.

The possibility that the effect of Colchicine on the phosphorylation of the islet S-100 P54 polypeptide may be related to some other effect of this reagent, such as the binding to cell membrane proteins (Wunderlich et al., 1973) cannot be dismissed. Colchicine has been shown to inhibit islet cell cyclic AMP efflux much more than insulin release (Grill and Cerasi, 1977) and the effect was shown to be shared by probenecid (an inhibitor of membrane transport processes (Brazeau, 1975)). This has led to the hypothesis that colchicine exerts its effect on insulin release by inhibiting membrane transport processes and not by inhibiting tubulin polymerisation(Grill and Cerasi, 1977).

Comparison Between the Two Methods (A and B) Used to Characterise ^{32}P Incorporation into Islet (Non-Nuclear) Proteins

Different islet (non-nuclear) protein ^{32}P incorporation patterns were obtained with Method A (pH 6.0) and Method B (pH 7.0). The following studies were undertaken in order to make a direct comparison between the two methods.

Figure 5.11 shows the different ^{32}P incorporation patterns obtained with Method A and Method B after 5 min incubation. With Method A (Lanes C and D), using 1 μM (γ-^{32}P)-ATP, cyclic AMP was found to have no effect on the islet (non-nuclear) protein ^{32}P incorporation pattern. ^{32}P incorporation into three islet polypeptides (approx. molecular weight 70,000, 41,000, and 17,000 daltons) was observed, although overall less ^{32}P incorporation, than that observed with Method B, was observed. Using Method B (Lanes A and B), ^{32}P incorporation into five islet polypeptides (approx. molecular weights 88,000, 54,000, 48,000, 36,000 and 14,000 daltons) was observed. As in previous observations, cyclic AMP was found to inhibit ^{32}P incorporation into the islet P54 polypeptide.

Method A

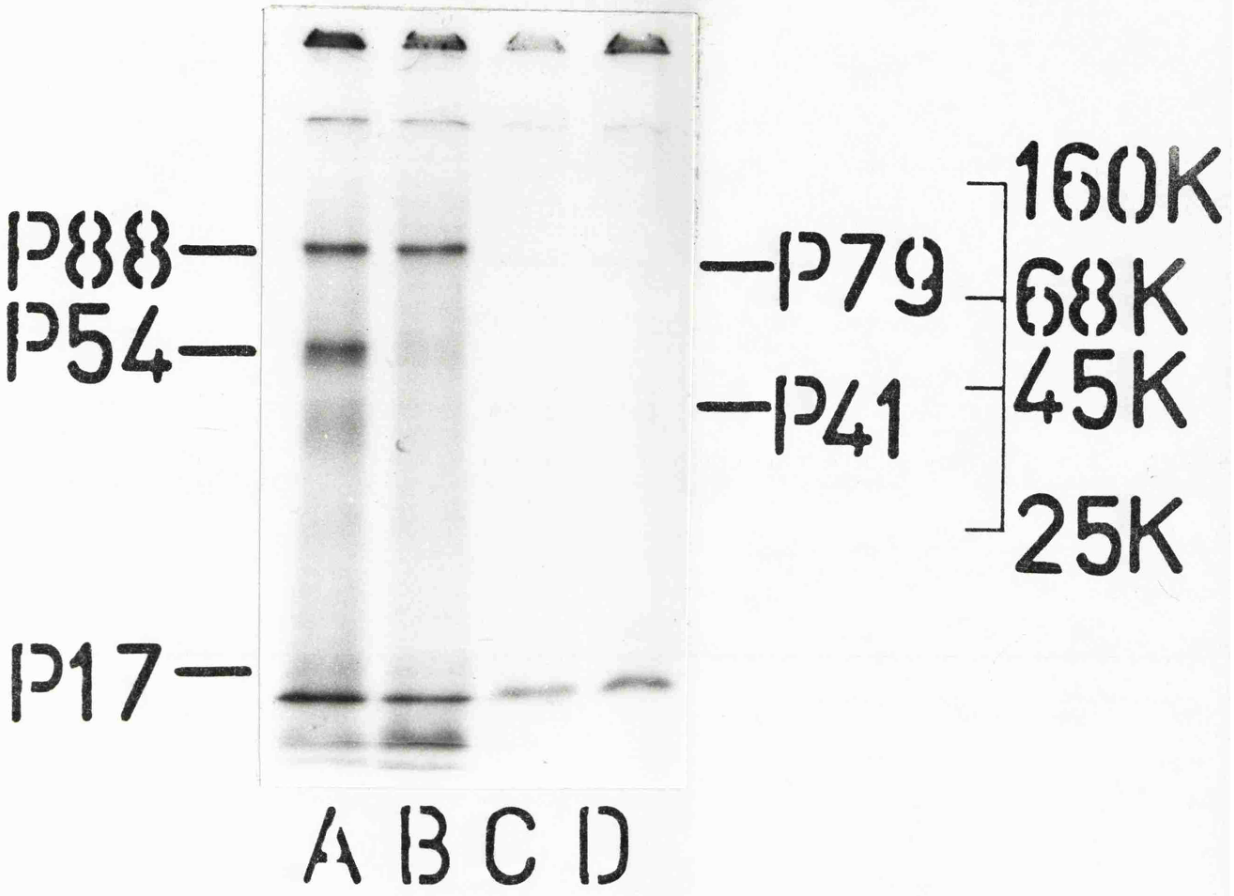
Subcellular Localisation of Phosphorylated Islet Non-Nuclear Proteins

The following studies were undertaken in order to determine the subcellular localisation of the phosphorylated islet (non-nuclear) proteins. In this study the subcellular fractions of the S-0.6 fraction were prepared by differential centrifugation, after the phosphorylation reaction had been terminated by the addition of 20 mM EDTA (this chelated the Mg^{2+} and Ca^{2+} ions and prevented further phosphorylation

Figure 5.11. ^{32}P Incorporation into Islet (non-nuclear) Polypeptides: A Comparison between Method A and Method B.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE was determined by autoradiography in the absence and presence of 1μM cyclic AMP, after a 5 min incubation, using Method A and Method B. Both methods are described in detail in the text.

Lane A:- Method B
Lane B:- Method B + 1μM cyclic AMP
Lane C:- Method A
Lane D:- Method A + 1μM cyclic AMP


P88
P54
P17
P79
P41
160K
68K
45K
25K
A B C D

occurring). Thus soluble proteins which were phosphorylated by membrane protein kinases and membrane proteins which were phosphorylated by soluble protein kinases could be detected.

The effects of using a longer incubation time (1 hour) and a lower concentration of (γ-^{32}P)-ATP (1 μM) on Method A were also examined.

Method

Islet S-0.6 subcellular fractions were prepared in Buffer A as described in Chapter II. 100 μl samples were incubated with 100 μl of Buffer A containing 1 μM (γ-^{32}P)-ATP plus and minus cyclic AMP (1 μM) and calcium (5 mM) for 60 min at 37°C. The reaction was terminated by the addition of either 30 μl of 20% S.D.S. or 20 μlof 200 mM E.D.T.A. and placed in ice. The reaction vials containing S.D.S. were processed for S.D.S. PAGE as previously described. The reaction tubes containing 20 mM E.D.T.A. were centrifuged for 10 min at 20,000 g, (MSE 66, 22,000 r.p.m., 3 x 3 ml swing out rotor) to prepare the P-20 subcellular fractions, containing mitochondria and secretory granules. The supenatant was then centrifuged for 60 min at 100,000 g (MSE 65, 45,000 r.p.m., 3 x 3 ml swing out rotor) to prepare the P-100 and S-100 subcellular fractions. The P-20 and P-100 subcellular fractions were resuspended in 200 μl of Electrophoresis sample buffer plus 30 μl of 20% S.D.S. and prepared for S.D.S. PAGE. 100 μl of Electrophoresis sample buffer plus 30 μl of 20% S.D.S. was added to the S-100 subcellular fractions which were then prepared for S.D.S. PAGE. S.D.S. PAGE was performed and autoradiographs of the dried slab gels were prepared as described in detail in Chapter II.

Results

i) Effect of Cyclic AMP

Inhibition of ^{32}P incorporation into the islet P54 polypeptide was observed in the presence of cyclic AMP (1 μM) in the islet S-0.6 subcellular fraction (Figure 5.12, Lanes A and B). A phosphorylated P54 polypeptide was observed in the P-20 islet subcellular fraction (containing mitochondria and secretory granules) but no cyclic AMP inhibition of phosphorylation occurred (Lanes C and D). The majority of phosphorylated islet non-nuclear polypeptides was located in the P-20 subcellular fraction. No significant ^{32}P incorporation was observed in the islet P-100 subcellular fraction (Lanes E and F). Cyclic AMP inhibition of ^{32}P incorporation into an islet P54 polypeptide was observed in the S-100 subcellular fraction (Lanes G and H).

ii) Effect of Calcium

Ca^{2+}-dependent protein phosphorylation has been reported in several tissues (Sieghart et al., 1978; Schulman and Greengard, 1978). The importance of Ca^{2+} in the regulation of insulin release in the islet cell (see introduction) suggested the possibility that Ca^{2+}-dependent protein phosphorylation may occur in the islet cell.

Calcium (5 mM) had no effect on ^{32}P incorporation in the islet S-0.6 subcellular fraction (Figure 5.13 Lanes A and B). The majority of phosphorylated islet polypeptides were located in both the P-20 and S-100 subcellular fractions. No significant ^{32}P incorporation was observed in the islet P-100 subcellular fraction.

Figure 5.12. Method A: Subcellular Localisation of Phosphorylated Islet (non-nuclear) Polypeptides. Effect of Cyclic AMP.

^{32}P labelled islet S-0.6 polypeptides were fractionated by differential centrifugation and then seperated by S.D.S. PAGE. ^{32}P incorporation into the S-0.6, S-100, P-100 and P-20 polypeptides was determined by autoradiography in the absence and presence of 1μM cyclic AMP, after an incubation of 60 min, as described in detail in the text.

Lane A: S-0.6
Lane B: S-0.6 + 1μM cyclic AMP
Lane C: P-20
Lane D: P-20 + 1μM cyclic AMP
Lane E: P-100
Lane F: P-100 + 1μM cyclic AMP
Lane G: S-100
Lane H: S-100 + 1μM cyclic AMP

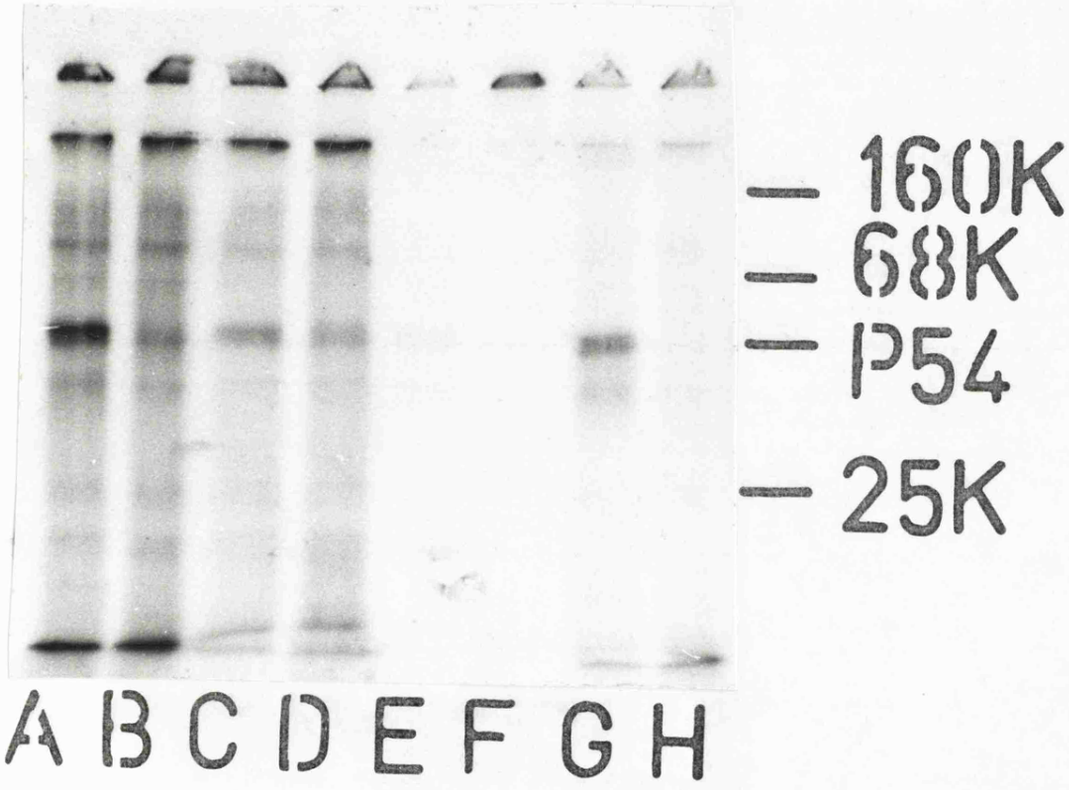
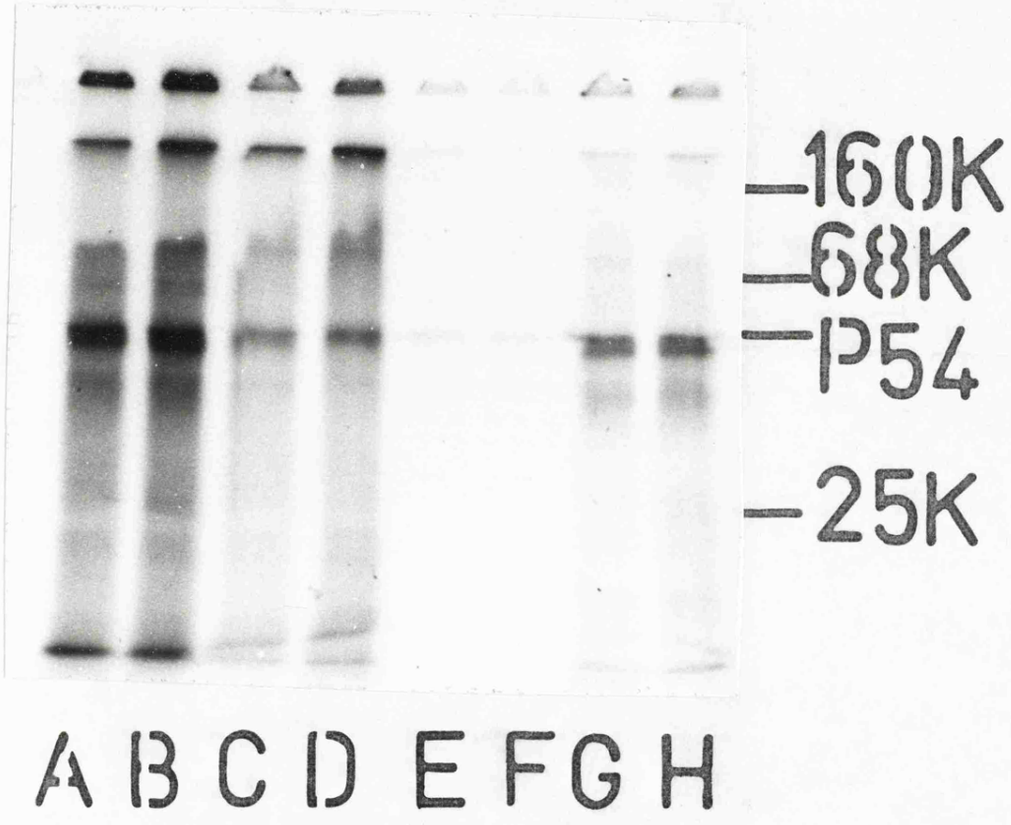

160K
68K
P54
25K
A B C D E F G H

Figure 5.13. Method A: Subcellular Localisation of Phosphorylated Islet (non-nuclear) Polypeptides. Effect of Calcium.

^{32}P labelled islet S-0.6 polypeptides were fractionated by differential centrifugation and then seperated by S.D.S. PAGE. ^{32}P incorporation into the S-0.6, S-100, P-100 and P-20 polypeptides was determined by autoradiography in the absence and presence of 5mM Calcium, after an incubation of 60 min, as described in detail in the text.

Lane A: S-0.6
Lane B: S-0.6 + 5mM Calcium
Lane C: P-20
Lane D: P-20 + 5mM Calcium
Lane E: P-100
Lane F: P-100 + 5mM Calcium
Lane G: S-100
Lane H: S-100 + 5mM Calcium


160K
68K
P54
25K
A B C D E F G H

Discussion

The islet S-0.6 ^{32}P incorporation patterns obtained with Method A and B, using 1 μM (γ-^{32}P)-ATP and 5 min incubation, were very different. With Method A, ^{32}P incorporation into three islet polypeptides (approx. molecular weight 76,000, 41,000 and 17,000 daltons) was observed. Cyclic AMP was found to have no effect on the ^{32}P incorporation pattern of Method A. However, using incubation times of 1 hour, cyclic AMP inhibition of the islet S-100 P54 polypeptide was observed using Method A and 1 μM (γ-^{32}P)-ATP. Thus the phosphorylation pattern of islet non-nuclear proteins was found to be dependent upon the pH and (γ-^{32}P)-ATP concentration (specific activity) present in the reaction mix.

Subcellular Localisation of Phosphorylated Islet Non-Nuclear Proteins

In this study the subcellular distribution of the phosphorylated islet polypeptides was determined after the completion of the phosphorylation reaction. This enabled the detection of proteins whose phosphorylation was catalysed by protein kinases located in a different subcellular fraction. In contrast to the study on the subcellular distribution of islet cell endogenous protein phosphorylation, a significant amount of the total islet cell endogenous phosphorylation was located in the P-20 subcellular fraction (containing secretory granules and mitochondria). Thus it is possible that the phosphorylation of secretory granule proteins and/or mitochondrial proteins by protein kinases present in the cytosol may occur in the islet cell.

As in the previous study, the islet P54 polypeptide, whose phosphorylation was inhibited by cyclic AMP, was

located in the S-100 subcellular fraction. It is worthy of note that an islet P54 polypeptide was also located in the P-20 subcellular fraction. The phosphorylation state of the islet P-20 P54 polypeptide was unaffected by cyclic AMP.

Calcium (5 mM) had no effect on the ^{32}P incorporation into islet non-nuclear polypeptides. This result was in direct contrast to the observation by Schubert et al. (1980) of a insulinoma cell polypeptide (approx. molecular weight 98,000 dalton) whose phosphorylation was stimulated by Calcium. This discrepancy may have been either due to the difference between the islet cell and the insulinoma cell line or due to insufficient E.G.T.A. (Ca^{2+} ion chelater) being present in the reaction mix of this study.

It is worthy of note that two major phosphorylated islet polypeptides (Figure 5.13) with approx. molecular weights 54,000 and 51,000 daltons were located in the S-100 subcellular fraction. Hence it is possible that there are two islet S-100 polypeptides (P54 and P51) whose phosphorylation is inhibited by cyclic AMP.

Phosphorylation of Ribosomal Proteins

In this study, the possibility that the low molecular weight phosphorylated islet proteins were ribosomal proteins was examined. Guinea-pig liver ribosomal proteins were used as exogenous substrates as it is reported, that within a species, ribosomal proteins of different tissues are very nearly identical (Sherton and Wool, 1974).

Method

Preparation of Guinea-pig Liver Ribosomal Proteins (Based on the Method Described by McConkey, 1974; Schubart et al., 1977)

3 g of Guinea-pig liver was homogenised in 30 ml of 10 mM Tris/HCl, pH 7.4, containing 10 mM KCl, 1 mM $MgCl_2$ and 1 mM D.T.T. 3 ml of 3M KCl containing 20 mM $MgCl_2$ was slowly added to the homogenate, which was then centrifuged at 15,000 g (MSE 65, 8 x 35 ml rotor, 15,000 r.p.m.) for 15 min. The supernatant was adjusted to 0.5% Brij 58 (Polyoxyethylene 20 Cetyl Ether) and 0.5% Sodium deoxycholate and centrifuged at 100,000 g (MSE 65, 8 x 35 ml rotor, 40,000 r.p.m.) for 18 hours through a cushion buffer consisting of 50 mM Tris/HCl, pH 7.4, containing 100 mM KCl, 1 mM $MgCl_2$, 1 mM D.T.T. and 1.75M Sucrose. The pellets were resuspended in 3 ml of 10 mM Tris/HCl, pH 7.4, 0.3 ml of 1M $MgCl_2$ and 6.6 ml of Glacial acetic acid was then added to the suspension. The suspension was stirred at 4°C for one hour and then centrifuged at 27,000 g (MSE 65, 8 x 35 ml rotor, 22,000 r.p.m.) for 15 min. The supernatant was dialysed against 2 litres of 0.5% Acetic acid for 18 hours with two changes of buffer, 3 ml aliquots (containing 180 µg protein) were freeze dried.

^{32}P Incorporation into Ribosomal Proteins

Guinea-pig islet S-0.6 subcellular fractions were prepared in Buffer A. 100 µl of 1 µM (γ-^{32}P)-ATP containing plus and minus cyclic AMP (1 µM) was either added to 100 µl of islet tissue sample in the absence and presence of 36 µg of Guinea-pig liver ribosomal proteins or added to 100 µl of buffer blank containing 36 µg of Guinea-pig ribosomal proteins. The reaction was incubated at 37°C for one hour. The reaction was terminated by the addition of 30 µl of 20% S.D.S. and the samples were processed for S.D.S. PAGE. S.D.S. PAGE was performed using 17% Acrylamide in the running gel and 4% Acrylamide in the stacking gel. Autoradiographs of the dried gels were prepared.

Results

^{32}P incorporation into two islet polypeptides P46 and P44 (approx. molecular weight 46,000 daltons and 44,000 daltons), was observed (Figure 5.14, Lanes A and B). ^{32}P incorporation was greater in the presence of Guinea-pig liver ribosomal proteins and one polypeptide (P46) comigrated with that observed in the absence of the liver ribosomal proteins. ^{32}P incorporation into five polypeptides (P46, P40, P32, P26 and P18) was observed in the presence of the liver ribosomal proteins but not in their absence (Lanes C and D). No significant ^{32}P incorporation into the liver ribosomal proteins occurred in the absence of the islet S-0.6 subcellular fraction (Lanes E and F).

Discussion

Cyclic AMP had no effect on the phosphorylation of Guinea-pig liver ribosomal proteins. The comigration of the islet P46 polypeptide with that of a liver ribosomal protein

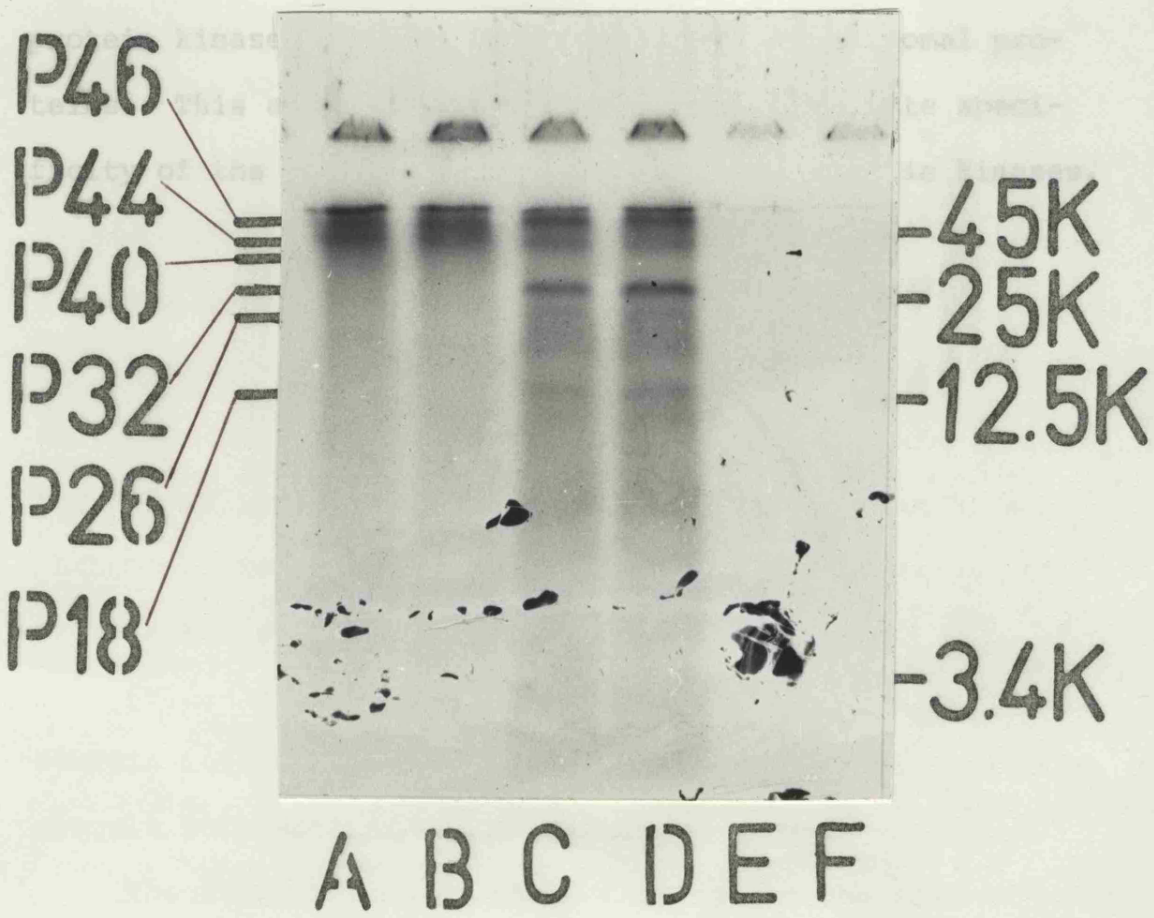
Figure 5.14. Phosphorylation of Ribosomal Proteins.

^{32}P incorporation into islet S-0.6 polypeptides seperated by S.D.S. PAGE (17% Acrylamide), in the absence and presence of guinea-pig liver ribosomal proteins (180µg/ml) and 1µM cyclic AMP, after an incubation of 60 min, was determined by autoradiography as described in detail in the text.

Lane A: Islet S-0.6 polypeptides
Lane B: Islet S-0.6 polypeptides + 1µM cyclic AMP

Lane C: Islet S-0.6 polypeptides + Liver ribosomal proteins (180µg/ml)
Lane D: Islet S-0.6 polypeptides + Liver ribosomal proteins (180µg/ml) + 1µM cyclic AMP

Lane E: Liver ribosomal proteins (180µg/ml)
Lane F: Liver ribosomal proteins (180µg/ml) + 1µM cyclic AMP


P46
P44
P40
P32
P26
P18
-45K
-25K
-12.5K
-3.4K
A B C D E F

suggested the possibility that the islet P46 polypeptide may be a ribosomal protein. No major ^{32}P incorporation into islet polypeptides with molecular weights below 40,000 daltons was observed. Islet S-0.6 protein kinases were able to catalyse the phosphorylation of several liver ribosomal proteins. Hence islet cells probably contain protein kinases capable of phosphorylating ribosomal proteins. This study demonstrated the wide substrate specificity of the islet cyclic AMP-independent protein kinases.

Summary

The phosphorylation of islet cell proteins by islet cell protein kinases, using a cell free system, was found to be dependent upon the pH and (γ-^{32}P)-ATP concentration. In the presence of Walsh protein (a specific inhibitor of cyclic AMP-dependent protein kinase) cyclic AMP mediated phosphorylation of four polypeptides was observed at pH 6.0, using 100 μM (γ-^{32}P)-ATP. At pH 7.0, using 1 μM (γ-^{32}P)-ATP, cyclic AMP inhibited the phosphorylation of an islet S-100 54,000 dalton (P54) polypeptide. Cyclic AMP did not stimulate the dephosphorylation of the P54 polypeptide.

Nonidet-P40 inhibited the phosphorylation of an islet 88,000 dalton (P88) polypeptide at pH 7.0. It was thought the P88 polypeptide may be an ATPase enzyme or an ATPase phosphoenzyme intermediate. Nonidet-P40 'activation' of protein kinase activity did not occur at pH 7.0.

Evidence to suggest the possibility that islet S-100 protein kinases phosphorylated mitochondrial and secretory granule polypeptides, was presented.

The significance of these findings in the role of cyclic AMP in insulin secretion will be discussed in detail in Chapter VI.

CHAPTER VI

Role of Cyclic AMP in the Regulation of Insulin Secretion

General Discussion

Cyclic AMP and Insulin Secretion

It has been proposed that glucose (the primary stimulator of insulin secretion) can elevate total islet cyclic AMP concentration (Grill and Cerasi, 1974; Hellman et al., 1974). Previous studies have indicated that glucose-induced cyclic AMP elevation may be casually related to insulin secretion because a) cyclic AMP elevation occurred within seconds of stimulation, parallel with secretion (Hellman et al., 1974); b) after the glucose stimulation was removed, both cyclic AMP and secretion returned to control values with similiar kinetics (Zawalich et al., 1975); c) dose-response curves for cyclic AMP and insulin secretion were identical throughout the physiological range of glucose concentrations (Charles et al., 1975) and d) the α anomer of glucose enhanced both cyclic AMP elevation and secretion whereas the β anomer was less effective for both events (Grill and Cerasi, 1975).

In contrast to these observations it has been reported that glucose, in concentrations which stimulate insulin release does not cause any change in the cyclic AMP content of islet tissue (Montague and Cook, 1971; Cooper et al., 1973). Glucose does not alter islet adenyl cyclase (Davis and Lazarus, 1972; Howell and Montague, 1973) or phosphodiesterase activity (Ashcroft et al., 1972a; Bowen and Lazarus, 1973). Thus it appears that if glucose-induced elevation of islet intracellular concentrations of cyclic

AMP does occur, it is not due to changes in adenyl cyclase or cyclic AMP phosphodiesterase activities or due to the elevation of cyclic AMP efflux from the β cell (Grill and Cerasi, 1974). The possibility that a glucose metabolite may enhance islet cyclic AMP levels is supported by evidence that D-glyceraldehyde increases cyclic AMP levels and insulin secretion even during alloxan-inhibited glucose stimulation (Zawalich et al., 1975a).

If cyclic AMP elevation does occur during glucose-induced insulin release, does it have a funtional role? Glucose-induced insulin release does not produce increases in protein kinase activity, although glucagon, theophylline and tolbutamide stimulate, while adrenaline inhibits islet protein kinase activity (Montague and Howell, 1973). Hence islet protein kinase activity is sensitive to changes in islet cyclic AMP concentration, although insensitive to glucose-induced changes in cyclic AMP concentration. This suggests the possibility that different pools of cyclic AMP and protein kinase activity may exist in the islet cell. Glucose may elevate intracellular cyclic AMP concentration in a subcellular compartment which does not contain a cyclic AMP-dependent protein kinase, while glucagon may elevate intracellular cyclic AMP concentration in a separate subcellular compartment which does contain a protein kinase sensitive to changes in cyclic AMP levels. Evidence to suggest cyclic AMP compartmentalisation does occur within a cell has been demonstrated using Leydig cells (Dufau et al., 1978) and lymphocytes (Wednor et al., 1972).

Glucose may therefore either induce a futile elevation of cyclic AMP in a compartment of the islet cell, such as

that observed in rabbit perfused hearts (Hayes et al., 1980), or glucose-induced elevation of cyclic AMP may alter the activity of an enzyme, such as lipid phosphorylase (Jolles et al., 1980), rather than a protein kinase. Identification of islet cyclic AMP binding proteins which are not the regulatory subunits of cyclic AMP-dependent protein kinases would support the latter hypothesis.

Most authors conclude that cyclic AMP plays a minor role in the initiation of insulin secretion, hence the glucose-induced elevations of cyclic AMP may either be futile in respect of protein kinase activation or relate to processes occurring concurrently with increased insulin secretion. The latter correlates with reports that theophylline, I.B.M.X. and tolbutamide, when added alone or with low levels of glucose (< 5 mM, a concentration approaching the threshold for glucose-induced cyclic AMP elevation and insulin secretion) elevate islet cyclic AMP concentration much more than maximal concentrations of glucose, yet only transient or minimal insulin secretion ensues (Hellman et al., 1974). At levels of glucose greater than 5 mM, the same agents augment glucose-induced secretion, yet the elevated cyclic AMP concentration is only minimally increased compared with the concentration observed with the phosphodiesterase inhibitors alone.

In conclusion, cyclic AMP plays a minor or secondary role in glucose-induced insulin secretion. The observed glucose-induced elevation of islet cyclic AMP concentrations is not due to a direct activation of islet adenyl cyclase activity. Glucose is thought to initiate insulin secretion by elevating intracellular Ca^{2+} ion concentrations. Thus

it is possible glucose may activate an islet calcium binding protein such as calmodulin (Sugden et al., 1979a) which activates the mechanism for insulin secretion. Secondary elevations of islet cyclic AMP concentration could occur in the initiaion of insulin secretion by Ca^{2+} ions. Such rises in islet cyclic AMP concentration could occur in compartments of the islet cell separated from the islet cyclic AMP-dependent protein kinases. Further experiments in which changes in the subcellular distribution of cyclic AMP are determined in the intact islet cell under basal and stimulatory conditions (glucose (8 mM and 20 mM) and glucose 8 mM plus 0.2 mM I.B.M.X.), would lead to a greater understanding of the role of cyclic AMP in insulin secretion.

In elucidating the role of cyclic AMP in insulin secretion an understanding of the characteristics and properties of islet cyclic AMP-dependent protein kinases is required.

Cyclic AMP-dependent Protein Kinases and Insulin Secretion

Cyclic AMP is thought to play a major role in the regulation of insulin secretion by glucagon and other hormones and in the long term effect of pregnancy on the β cell (Green et al., 1973). Islet cells contain adenyl cyclase, cyclic AMP-dependent protein kinase and cyclic AMP phosphodiesterase activities and it appears that most actions of cyclic nucleotides are mediated by protein kinases. It is worthy of note that cyclic AMP-protein kinases have been identified in a variety of mammalian tissues, numerous phyla including Protozoa, Ceolenterata, Nematoda, Annelida, Mollusca, Arthopoda and Chordata. Its occurrence is so widespread that a report of its absence in any particular tissue or species would be thought unusual, although it does

appear that higher plant tissues lack cyclic AMP-stimulated protein kinase activity (Kuo and Greengard, 1969). Thus the appearance of the 'cyclic AMP enzymes' does not necessarily indicate that a cyclic AMP mediated process occurs, although it is extremely like it does.

Glucagon, I.B.M.X., caffeine, theophylline, glibenclamide and tolbutamide (agents known to potentiate insulin secretion) have been shown to increase cyclic AMP-dependent protein kinase activities in the presence of 6mM glucose (Montague and Howell, 1973). Somatostatin which inhibits insulin secretion (Gerich et al., 1975) and islet adenyl cyclase activity (Oliver et al., 1978) has been shown to inhibit both glucagon and theophylline induced increases in islet protein kinase activities (Oliver and Kemp, 1980). In the long term regulation of insulin secretion such as in the late stages of pregnancy, insulin release is increased (Green and Taylor, 1972) and pregnant rats have been shown to have higher basal and cyclic AMP-dependent levels of protein kinase activity than control rats (Lipson and Sharp, 1978). During fasting, insulin release is decreased (Malaisse et al., 1967a) although no apparent change in protein kinase activity is observed (Lipson et al., 1979). Thus changes in insulin release in some instances correlate with changes in islet protein kinase activities. It is worthy of note that cyclic AMP-mediated activation of protein kinase can occur during the extraction procedures typically used for measurement of protein kinase activity (Palmer et al., 1980). Thus the possibility arises that the observed increases in protein kinase activities may not be entirely due to intracellular cyclic AMP activation.

If cyclic AMP activation of protein kinase does occur in the islet cell, which molecular form of cyclic AMP-dependent protein kinase mediates the cyclic AMP response? In this study, using guinea-pig islets, the predominant molecular form was found to be Type II isozyme, in studies on rat islets the predominant molecular form was also found to be the Type II isozyme (Sugden et al., 1979; Oliver and Kemp, 1980). As in this study, the Type II isozyme was found to exhibit a greater dependency upon cyclic AMP, although Oliver and Kemp (1980) did find in their preparation that the Type I isozyme was completely dissociated. This suggests the possibility that the Type II isozyme may be the molecular form that mediates the cyclic AMP response. The different molecular forms of cyclic AMP-dependent protein kinases have similar catalytic subunits but different regulatory subunits and are thus modulated by two different mechanisms. Type II enzyme undergoes autophosphorylation in the presence of ATP (Erlichman et al., 1974) which increases the sensitivity of the enzyme towards cyclic AMP (Hofmann et al., 1975). Type I enzyme is also modulated by ATP, but via a different mechanism. The presence of ATP reduces the affinity for cyclic AMP, hence higher concentrations of the second messenger are needed for dissociation and activation of protein kinase I. Furthermore, ATP promotes the reassociation of the Type I enzyme in the absence of cyclic AMP. As ATP is in the cell at rather high concentrations, this process raises the concentration of cyclic AMP necessary for kinase activation under physiological conditions, to the micromolar range, whereas *in vitro*, in the absence of ATP, nanomolar concentrations of cyclic AMP were found to be sufficient for

kinase dissociation (Beavo et al., 1975; Hoppe et al., 1977). However, Type II protein kinase subunits interact more strongly with each other than the Type I subunit, hence the Type II isozyme has a smaller dissociation constant. This explains the observations that cyclic AMP binds less strongly to Type II than to Type I. The binding of cyclic AMP to the Type II kinase shows negative co-operativity while the binding of cyclic AMP to the Type I kinase shows positive co-operativity (Hoppe and Wagner, 1979). Autophosphorylation of the Type II isozyme enhances protein dissociation (Rangel-Aldao and Rosen, 1976) this results in a stronger cyclic AMP binding and the negativity co-operative binding becomes non-co-operative.

The binding of ATP to the Type I isozyme is also dependent upon the protein concentration and protein dissociation constant. ATP binding is non-co-operative under conditions where protein association is favoured (high enzyme concentration and low ionic strength) and becomes positively co-operative at increasing ionic strength which promotes protein dissociation. This effect is generated by the underlying positive co-operativity of the protein association (Hoppe and Wagner, 1979). Extrapolating from the <u>in vitro</u> results one might expect the following difference in behaviour <u>in vivo</u>: Type I enzymes are more sensitive towards a rise in the cyclic AMP concentration than Type II enzymes; the latter isozymes, however, reassociate faster than Type I after withdrawal of cyclic AMP due to the tighter R-C contacts (Corbin and Keely, 1977). Reassociation of Type II subunits could be further accelerated by removal of the phosphates from P_2 through a protein phosphatase (Chou et al.,

1979). Therefore Type I isozyme would be activated faster than Type II, following stimulation of adenyl cyclase and accumulation of cyclic AMP. This therefore would suggest that the islet cell Type I cyclic AMP-dependent protein kinase would be the mediator of the cyclic AMP action on insulin secretion (Oliver and Kemp, 1980) even though the Type II enzyme is the predominant molecular form.

The observed futile activation of protein kinases in perfused rat hearts by PGE_1 (Hayes et al., 1979; Brunton et al., 1979) was not found to be related to the predominance of either protein kinase isozyme. Isoproterenol and PGE_1 caused similar effects in both rat (> 80% Type I) and guinea-pig heart (> 90% Type II) (Hayes et al., 1980). This suggests the isozyme profile does not reflect the mechanism of cyclic AMP modulated events. This correlates with the wide species variation of isozyme profile for a specific tissue.

The broad tissue and species specificity of cyclic AMP-dependent protein kinase isozymes (Corbin and Keely, 1977) suggests the possibility that human islets of Langerhans may have a very different isozyme profile to that found in the rat and guinea-pig islets, although the kinetics and dynamics of insulin secretion in all three species are very similar.

It is now thought that the subcellular distribution of cyclic AMP-dependent protein kinases is a more important criterion in determining the functional protein kinase type. In the rabbit heart it was observed that the binding of cyclic AMP to the particulate regulatory subunits resulted in the translocation of catalytic subunits to the soluble fraction (Corbin et al., 1977). On comparing the differential

action of isoproterenol and PGE_1 on the perfused rabbit heart it was found that isoproterenol caused the accumulation of cyclic AMP in both soluble and particulate compartments, the accumulation in the particulate compartment being associated with translocation of the protein kinase catalytic activity to the soluble fraction. By contrast, PGE_1 activated only the soluble pool of protein kinase and had no effect on the particulate cyclic AMP or particulate protein kinase, causing none of the responses generally associated with elevated intracellular cyclic AMP. (Hayes et al., 1980). This data indicates that the responses of cardiac tissue and probably other tissues to increased intracellular cyclic AMP are more complex than simple responses in proportion to the cellular concentration of cyclic AMP or to the activation state of cyclic AMP-dependent protein kinase. Hayes et al. (1980) propose the presence of multiple receptor-adenyl cyclase populations which are capable of generating cyclic AMP into specific intracellular spaces. This in turn, would lead to the activation of sub-populations of protein kinases (isozymes, soluble and particulate fractions) and phosphorylation of proteins adjacent to the kinase. A portion of the catalytic subunit of the protein kinase may also translocate following activation, perhaps migrating to distinct intracellular localisations.

By analogy, glucose may activate islet cell adenyl cyclase, increase intracellular cyclic AMP and activate a protein kinase in a futile process. In contrast, glucagon activation of adenyl cyclase could result in a separate increase of intracellular cyclic AMP and protein kinase activation in a subcompartment of the cell, thus leading to

the phosphorylation of an islet protein and potentiation of the secretory process. Glucose is thought to initiate insulin secretion by elevating islet intracellular Ca^{2+} ion concentrations, the biochemical mechanism of the glucose-induced elevation of Ca^{2+} ions is still undetermined. The observation that glucose-induced insulin secretion has to occur before glucagon potentiation, suggests the possibility that the intracellular movement of Ca^{2+} ions may control the cyclic AMP mediated events. Hence the activation of a discrete subpopulation of protein kinases may also be controlled by calcium and/or calcium binding proteins.

The findings of this study have shown that a small amount of cyclic AMP-dependent protein kinase activity was located in the islet cell P-100 (particulate) fraction. The ratio of islet cell S-100 to P-100 cyclic AMP-dependent protein kinase activity compares very well to that found in the bovine heart and liver (Uno et al., 1976). Thus although problems were encountered in detecting the islet cell P-100 cyclic AMP-dependent protein kinases (due to the small amounts of tissue available), these may therefore still play an important role (similar to that in the rabbit heart) in the cyclic AMP modulation of insulin secretion. This correlates with the suggestion that the S-100 Type I isozyme may have originated from the particulate fraction. Further experiments to demonstrate selective changes in the protein kinase activity of islet subcellular fractions during stimulary conditions (as defined in 'cylcic AMP and insulin secretion') would support the hypothesis of compartmentalisation of cyclic AMP activation of islet protein kinase activity. The localisation and identification of the substrate(s) of

islet cyclic AMP-dependent protein kinases might indicate which kinase is the mediator in the cyclic AMP regulation of insulin secretion.

Protein Phosphorylation and Insulin Secretion

Although a great deal of interest has been shown in islet cyclic AMP-dependent protein kinases, very little data on the cyclic AMP induced phosphorylation of specific islet proteins has been published. In other secretory systems such as the mast cell (Theoharides et al., 1980), neurohypophysis (Trieman et al., 1980) and Leydig cells (Cooke et al., 1977) phosphorylation of individual proteins has been demonstrated and it has been suggested that the phosphorylation of these proteins is somehow connected to the cyclic AMP regulation of the stimulation-secretion process.

In this and previous studies (Sugden et al., 1979; Müller et al., 1976) it was found that cyclic AMP had no effect on total islet endogenous protein phosphorylation, in a cell free system. This observation has led previous authors to conclude that the majority of the islet cell endogenous protein phosphorylation was catalysed by protein kinases other than cyclic AMP-dependent protein kinases. Although this may be the case in the islet cell, the possibility that in the cell free system the disruption of subcellular compartments may affect the cyclic AMP response cannot be ignored.

The finding that Nonidet-P40 treatment of the islet cell S-100 subcellular fractions resulted in cyclic AMP stimulation of islet cell endogenous phosphorylation indicated that cyclic AMP-dependent protein kinases capable of phosphorylating S-100 islet cell proteins were present.

Although no direct effect of cyclic AMP on phosphorylated islet cell proteins separated by S.D.S.-P.A.G.E. was observed at pH 6.0. It was found that Walsh protein (a specific inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase) inhibited the phosphorylation of four islet cell polypeptides (approx. molecular weight, 110,000, 50,000, 37,000 and 26,000 daltons). This indicated that the phosphorylation of these islet cell polypeptides was catalysed by dissociated cyclic AMP-dependent protein kinases at pH 6.0.

However, at pH 7.0, using 1 μM (γ-^{32}P)-ATP and a reaction time of 5 min, cyclic AMP inhibited the phosphorylation of an islet cell P54 polypeptide (approx. molecular weight 54,000 daltons). A phosphorylated polypeptide (approx. molecular weight 56,000 daltons) which showed decreased levels of phosphorylation in the presence of cyclic AMP has been identified in rat synaptic membranes (Thérien and Mushynski, 1979). As in this study, the decrease in phosphorylation was found to be due to inhibition of phosphorylation rather than an increase in the rate of dephosphorylation.

The possibility that the islet cell S-100 P54 polypeptide may be the regulatory subunit of a cyclic AMP-dependent protein kinase (Ueda and Greengard, 1977) cannot entirely be dismissed. The dephosphorylation of the regulatory subunit (approx. molecular weight 54,000 daltons) may not be due to a direct interaction of cyclic AMP with the phosphatase but may result from an impeded interaction of the phosphatase with other substrate proteins following dissociation of the cyclic AMP-dependent protein kinase (Thérien and Mushynski,

1979).

Cyclic AMP stimulated dephosphorylation of specific polypeptides has been observed in rat adrenal glands in response to Adrenocorticotrophic Hormone (ACTH), using an intact cell system (Koroscil and Gallant, 1980). Hence it is possible in secretory systems such as the islets of Langerhans, cyclic AMP may act via a specific dephosphorylation mechanism. This might explain the finding that cyclic AMP does not stimulate total islet cell endogenous protein phosphorylation. Although cyclic AMP stimulated dephosphorylation of the islet S-100 P54 polypeptide was observed at the physiological pH, this observation may have been an artifact due to the cell free system and/or the unphysiological concentration of ATP. ATP concentration has a profound effect on the cyclic AMP-dependent protein kinase isozyme activity (see Cyclic AMP-dependent Protein Kinases and Insulin Secretion). Further experiments using intact cells are required before the physiological significance of the dephosphorylation of the islet cell S-100 P54 polypeptide in the regulation of insulin secretion by cyclic AMP can be determined.

Ca^{2+} stimulated protein phosphorylation (Schulman and Greengard, 1978; Sulakhe et al., 1980) may also play an important role in the regulation of insulin secretion. Although in this study, no Ca^{2+} stimulation of islet cell protein phosphorylation was observed. This may have been due to the concentration of E.G.T.A. in the reaction mix not being sufficient to chelate all of the available Ca^{2+} ions. Experiments in which the effects of Ca^{2+} ions and calmodulin on the phosphorylation of specific islet proteins are examined in a cell free or intact cell system, using the conditions

described by Schubart et al. (1980) might suggest the mechanism by which Ca^{2+} ions (and perhaps cyclic AMP) regulate insulin secretion in the islets of Langerhans.

SUMMARY

In conclusion the findings of this study have demonstrated the majority (70%) of islet cell cyclic AMP-dependent protein kinase activity is located in the S-100 fraction. The predominant molecular form in the S-100 fraction was found to be the Type II isozyme, although it is thought the Type I isozyme may be the mediator in the cyclic AMP regulation of islet cell insulin secretion.

The majority (42%) of islet cell cyclic AMP-independent protein kinase activity is located in the P-100 fraction. It is very probable that islet cell cyclic AMP- independent protein phosphorylation (such as that which may be involved in the regulation of insulin secretion by calcium) is catalysed by protein kinases present in this fraction.

The percentage of the total islet cell protein kinase activity which was cyclic AMP-dependent was found to vary with the exogenous protein substrate. Using histone as exogenous protein substrate, the majority (75%) of the total islet protein kinase activity was found to be cyclic AMP-dependent. However, when protamine was used as exogenous protein substrate 50% of the total islet protein kinase activity was found to be cyclic AMP- dependent.

Treatment of the islet cell membrane containing fractions with Nonidet-P40 (a non-ionic detergent) did not release significant amounts of protein kinase activity. This was not due to Nonidet-P40 inhibition of protein kinase activity, on the contrary it was found Nonidet-P40 stimulated islet cell S-100 protein kinase activity. This stimulation was not due to the observed Nonidet-P40 inhibition of islet cell ATPase activity.

Using a cell free system, it was found cyclic AMP

inhibited the phosphorylation of an islet S-100 54,000 dalton molecular weight polypeptide. Although the identity and significance of this polypeptide in the regulation of insulin secretion by cyclic AMP *in vivo* has yet to be determined, it is proposed that cyclic AMP may regulate insulin secretion by a specific dephosphorylation mechanism.

Nonidet-P40 inhibited the phosphorylation of an islet 88,000 dalton molecular weight polypeptide. It is thought that this polypeptide may be an islet ATPase or an ATPase phosphoenzyme intermediate.

Appendix

Buffer A

50 mM Sodium β glycerophosphate, pH 6.0, containing 20 mM NaF, 0.3 mM Ethyleneglycol-bis-(-amino ethyl ether) N,N^1-tetra acetic acid (E.G.T.A.), 0.1 mM Diaminoethanetetra-acetic acid (E.D.T.A.), 10 mM Magnesium acetate, 0.5 mM Isobutylmethylxanthine (I.B.M.X.), 10 mM Benzamidine, 1 mM Dithiothreitol (D.T.T.) and 250 mM Sucrose.

Buffer B

10 mM Hepes/NaOH, pH 7.5, containing 1 mM E.D.T.A., 1 mM D.T.T., 10 mM Benzamidine and 250 mM Sucrose.

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(VIP) at concentrations equivalent to 100ng/tube. The antiserum reacted weakly with a mouse gut extract enriched in glucagon-like immunoreactivity (GLI), corresponding to 3.2% cross-reaction at 250pg equivalents/tube.

The glucagon antiserum was administered without further purification as a single intraperitoneal injection at a dose of 1.5ml/100g (binding capacity 1.47μg/1.5ml) to 20-week-old fed *ob/ob* and +/+ mice of the Birmingham strain. The mean body weights of the two groups were 89.7±5.2g and 38.8±1.3g respectively. Blood samples were obtained from conscious mice immediately before and 180 and 360min after injection. Plasma glucose was determined by the glucose oxidase method (Stevens, 1971), and insulin was measured by radioimmunoassay (Albano *et al.*, 1972). Administration of an equivalent volume of normal rabbit serum to control mice did not significantly affect the parameters studied.

Administration of glucagon antiserum to fed *ob/ob* and +/+ mice at a dose sufficient to neutralize more than 1.5 times the total pancreatic glucagon content (Findlay *et al.*, 1973) caused a significant decrease ($P<0.001$) in the plasma glucose concentration of both groups of mice (Fig. 1). This effect was particularly pronounced in *ob/ob* mice, where the glucose concentrations fell progressively from hyperglycaemic values to attain values comparable with those of +/+ mice. Contrasting with such dramatic changes, relatively small alterations in plasma insulin concentrations were observed. In +/+ mice insulin concentrations were only slightly lowered after administration of glucagon antiserum. The marked hyperinsulinaemia of *ob/ob* mice, however, was significantly depressed ($P<0.05$) by this treatment.

In conclusion, the administration of specific antibodies with an affinity for glucagon considerably greater than that of the hepatic glucagon receptor (Rodbell *et al.*, 1971) resulted in hypoglycaemic reactions in *ob/ob* and +/+ mice. Although circulating glucagon seems to be of relatively minor importance for the maintenance of hyperinsulinaemia, it appears to contribute significantly to the diabetic hyperglycaemia of fed *ob/ob* mice.

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Subcellular Distribution of Protein Kinase Activities in Mammalian Islets of Langerhans

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Changes in the intracellular concentrations of cyclic AMP in the pancreatic β-cell produce changes in rates of insulin secretion, although little is known of the exact mechanism by which this nucleotide may influence the secretory process. Cyclic AMP-

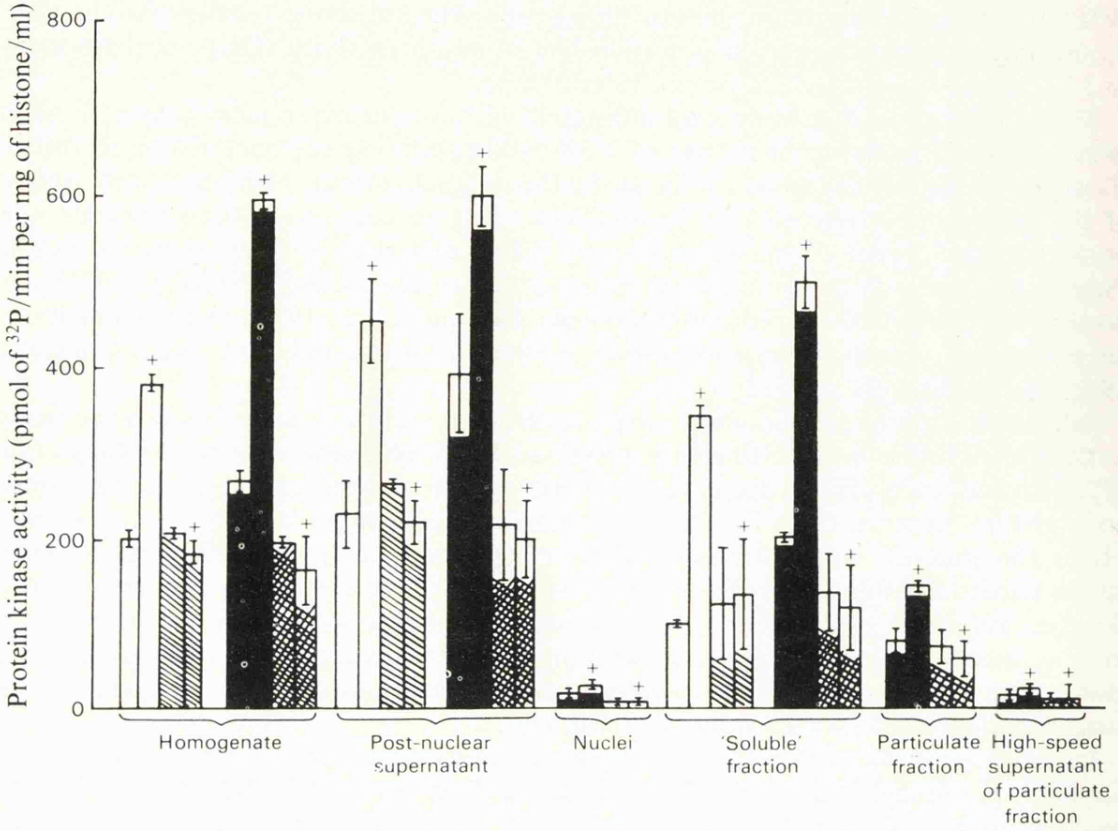

Fig. 1 *Subcellular distribution of protein kinase activities in guinea-pig islets of Langerhans*

Subcellular fractions of 3000 islets were prepared by differential centrifugation as described in the text. Protein kinase activity was assayed in duplicate in the absence and presence (+) of 1 μM-cyclic AMP, absence (□,▨) and presence (■,▩) of 0.5% Nonidet P40 and absence (□,■) and presence (▨,▩) of Walsh protein (>300 units/ml), with histone (2mg/ml) as the substrate. Incubation conditions were 30min at 37°C at pH6.0.

dependent protein kinase activity has been identified in the β-cell (Dods & Burdowski, 1973; Müller & Sharp, 1974) and was largely localized to the 'soluble' fraction (Montague & Howell, 1972). The present work was undertaken to investigate the possible occurrence of cyclic AMP-dependent protein kinase activity in the particulate subcellular fractions of the β-cell that might be released by the detergent Nonidet P40.

Nuclear (600*g* for 10min), particulate (100000*g* for 60min) and 'soluble' subcellular fractions of isolated islets of Langerhans were prepared by differential centrifugation. The particulate fraction, which contained secretory granules, mitochondria and microsomal fragments, was treated with Nonidet P40 (0.5%) and re-centrifuged at 100000*g* for 60min, and the resultant supernatant was assayed, along with the 'slouble' fraction, for cyclic AMP-dependent protein kinase activity, with histone as substrate. It was found that the supernatant from the Nonidet-P40 extracted particulate fraction had 7% of the cyclic AMP-dependent protein kinase activity of the 'soluble' fraction.

To investigate the possibility that Nonidet P40 inhibited cyclic AMP-dependent protein kinase activity, samples from the homogenate, post-nuclear supernatant and 'soluble' subcellular fractions were treated with 0.5% Nonidet P40, and the cyclic AMP-dependent protein kinase activities were compared with those of corresponding untreated fractions. The nuclear and particulate fractions were also treated with 0.5% Nonidet P40 and assayed for cyclic AMP-dependent protein kinase activity, with histone as substrate. The distribution of cyclic AMP-dependent protein kinase activities in the subcellular fractions and the effects of Nonidet P40 on the activities in the homogenate, post-nuclear supernatant and 'soluble' fractions are illustrated in Fig. 1. Treatment of

the homogenate, post-nuclear supernatant and 'soluble' fractions with 0.5% Nonidet P40 produced a 50% increase in activity, the distribution of total activity in the presence of Nonidet P40 being 80% in the 'soluble' fraction, 15% in the particulate fraction and 5% in the nuclear fraction. There was a substantial loss of activity when the Nonidet-P40-treated particulate fraction was re-centrifuged (100000*g* for 60min) and the resultant supernatant was assayed for cyclic AMP-dependent protein kinase activity. This may be due to the production of micelles by Nonidet P40 that are large enough to be sedimentated down (along with the contained protein) by centrifugation at 100000*g* for 60min.

The use of the Walsh protein (Ashby & Walsh, 1972), a specific inhibitor of cyclic AMP-dependent protein kinases, suggested that the release of protein kinase activity on treatment with Nonidet P40 was specifically due to that which was cyclic AMP-dependent (Fig. 1). The subcellular distribution of total cyclic AMP-independent protein kinase activity was found to be 60% in the 'soluble' fraction, 35% in the particulate fraction and 5% in the nuclear fraction. It is noteworthy that the proportion of total cyclic AMP-independent protein kinase activity found in the particulate fraction is double that of the porportion of total cyclic AMP-dependent activity in the islet homogenate.

These results suggest that the 'soluble' subcellular fraction of guinea-pig islets of Langerhans contains most of the total β-cell cyclic AMP-dependent protein kinase activity. Treatment of this subcellular fraction with Nonidet P40 (0.5%) produces a 50% increase in protein kinase activity that has been shown to be specifically cyclic AMP-dependent. The particulate subcellular fraction contains 15% of the total cyclic AMP-dependent protein kinase activity as compared with 35% of the total cyclic AMP-independent activity.

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Hormonal Activation of Bovine Luteal Adenylate Cyclase

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The 600*g* sediment of bovine corpus-luteum homogenate contains adenylate cyclase, which is activated *in vitro* by NaF and by guanosine 5′-[$\beta\gamma$-imido]triphosphate or GTP (Young & Stansfield, 1978*a*, *b*, *c*) but which has failed to respond significantly or consistently to treatment *in vitro* with lutropin or human choriogonadotropin (N. B. Lydon, J. L. Young & D. A. Stansfield, unpublished work). Marsh (1971) reported stimulation *in vitro* of luteal adenylate cyclase in whole homogenates of bovine tissue, but had difficulty at times in observing a stimulation (J. M. Marsh, personal communication). Menon & Kiburz (1974) reported activation *in vitro* by human choriogonadotropin of bovine luteal adenylate cyclase in a plasma-membrane preparation. However, their method has not, in our hands, given satisfactory yields of membrane; this has also been noted by Mintz *et al.* (1978).

A method of preparing rat luteal plasma membranes (Mintz *et al.*, 1978) has been used by us to prepare a membrane fraction from bovine corpora lutea with a yield of approx. 7.4mg of membrane protein/g of luteal tissue.

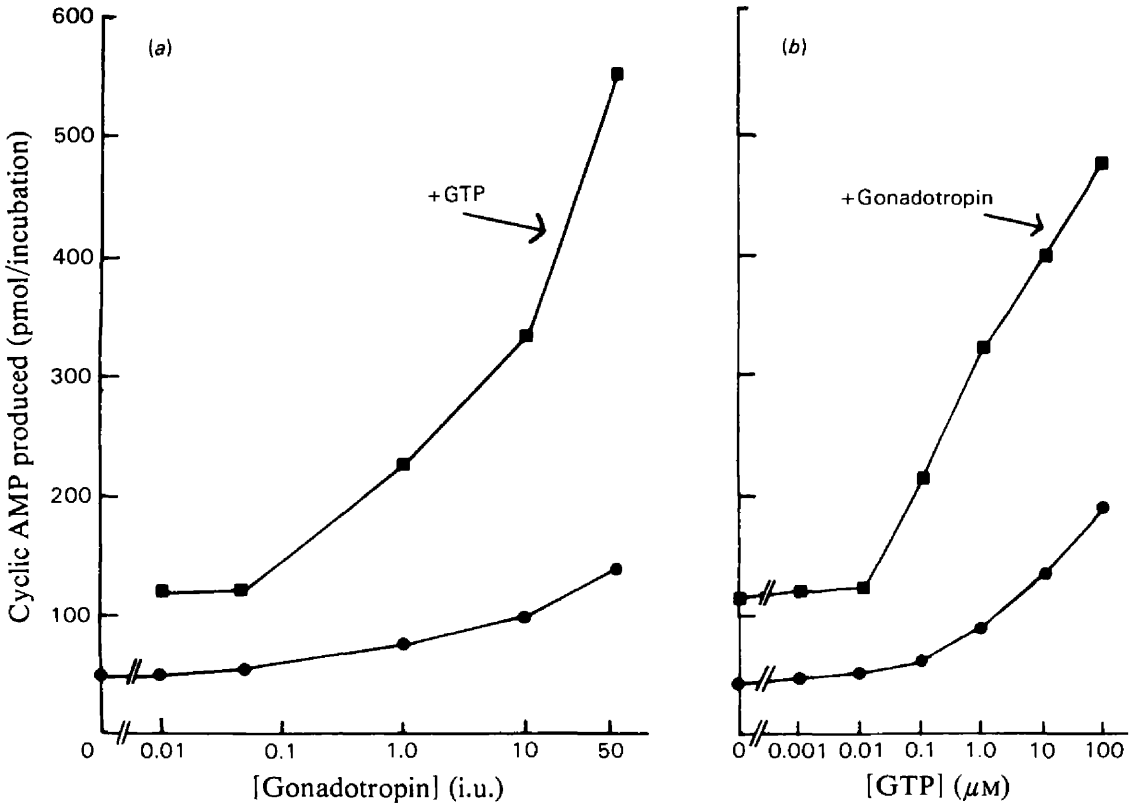

Fig. 1. *Dose–response curves of bovine luteal-membrane adenylate cyclase stimulated by human choriogonadotropin and GTP*

(*a*) Cyclic AMP produced/incubation at various doses of gonadotropin in the absence (●) or in the presence (■) of GTP (10μM); (*b*) cyclic AMP produced/incubation at various doses of GTP in the absence (●) or in the presence (■) of gonadotropin (50i.u.). The graphs in (*a*) and (*b*) were derived from experiments with two different batches of membranes, containing 0.7 and 1.3mg of protein per incubation respectively.

Adenylate cyclase incubation mixtures contained (in a volume of 520μl) 25mM-Tris/acetate buffer, pH7.6, 5mM-magnesium acetate, 0.5mM-ATP, 1mM-dithiothreitol, 0.1mM-3-isobutyl-1-methylxanthine, 5mM-phosphocreatine, 26 units of creatine phosphokinase and 0.052mg of bovine serum albumin. When present NaF was 10mM and, unless otherwise stated, human choriogonadotropin (Pregnyl, Organon; or Chorulon, Intervet; 2500i.u./mg) was 50i.u./incubation and GTP was 10μM. Incubation was for 15min at 30°C after addition of 0.5–1.5mg of membrane protein. The reaction was terminated by immersion of the tubes in boiling water for 3min. Cyclic AMP was assayed as described previously (Young & Stansfield, 1977) by competitive protein binding.

The dose-dependent stimulation of luteal adenylate cyclase by gonadotropin is shown in Fig. 1(*a*), which also shows the further stimulation achieved by including GTP (10μM) in the incubations. The maximum dose of gonadotropin used (50i.u.) gave stimulations of 2.7–3.5-fold over basal values in the absence of GTP, and 6.3–9.1-fold in the presence of GTP. Doses of 0.01–0.1i.u. of gonadotropin caused detectable increases in adenylate cyclase activity.

Fig. 1(*b*) shows that increasing doses of GTP stimulates luteal adenylate cyclase, the activation being approx. 4.3-fold over the basal value with 100μM-GTP; in the presence of gonadotropin (50i.u.) this was increased still further to approx. 11-fold. The response to gonadotropin appears to plateau above 50i.u./incubation, but more detailed study is required to confirm this.

The bovine luteal membrane adenylate cyclase was stimulated 6-18-fold by NaF (10mM).

STUDIES ON THE MODE OF ACTION OF CYCLIC AMP IN REGULATING THE RELEASE OF INSULIN FROM ISLETS OF LANGERHANS

Ph.D. thesis presented by Yvonne Soars nee Ducommun

ABSTRACT

The subcellular distribution of protein kinase activity in isolated islets of Langerhans was determined. The majority (70%) of cyclic AMP-dependent protein kinase activity was located in the S-100 (soluble) fraction, while the majority (42%) of cyclic AMP-independent activity was located in the solublised P-100 (containing mitochondria, secretory granules and microsomes) fraction. Partial characterisation of the islet cyclic AMP-dependent protein kinase activity revealed the presence of two isozymes designated Type I and Type II. Type II kinase was the predominant isozyme of the S-100 fraction and Type I was the predominant isozyme found in the solublised P-100 fraction.

Nonidet-P40 (a non-ionic detergent) was found to 'activate' the S-100 cyclic AMP-dependent protein kinase activity, although no significant increase in protein kinase activity was observed when the P-0.6 (containing nuclei and cellular debris) fraction and P-100 fraction were solublised with Nonidet-P40. This 'activation' was not directly due to the observed Nonidet-P40 inhibition of (γ-^{32}P)-ATP hydrolysis and is likely to be related to changes in the charged state of the holoenzyme induced by Nonidet-P40.

The phosphorylation of islet cell proteins by islet cell protein kinases has been studied, using a cell free system. The incorporation of ^{32}P from (γ-^{32}P)-ATP into islet non-nuclear proteins was found to vary with pH. In the presence of Walsh protein (a specific inhibitor of cyclic AMP-dependent protein kinases) cyclic AMP mediated phosphorylation of four polypeptides was demonstrated at pH 6.0. At pH 7.0, cyclic AMP inhibition of ^{32}P incorporation into a S-100 54,000 dalton polypeptide was observed. At pH 7.0, Nonidet-P40 inhibited ^{32}P incorporation into a 88,000 dalton polypeptide. This protein was tentatively identified as an ATPase whose activity was modulated by its phosphorylation state. The results of these studies are discussed in relation to the role of cyclic AMP in insulin secretion.