

THE ACTIONS OF THE PANCREATIC HORMONES
ON ISOLATED CHICKEN HEPATOCYTES

by

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This thesis was composed by myself and the results therein are
the product of my own work.

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<u>CONTENTS</u>	<u>Page No.</u>
ABBREVIATIONS	i
ACKNOWLEDGEMENTS	ii
SUMMARY	iii
CHAPTER 1: GENERAL INTRODUCTION	
Introduction to avian metabolism and its hormone control	1
Early experiments using the endocrine pancreas and its secreted hormones	4
Insulin	5
Glucagon	8
Avian Pancreatic Polypeptide	10
Hormone action at the cellular level	11
CHAPTER 2: METHODS	
Determination of adenosine phosphates in cell extracts	18
Cyclic nucleotide determination (cAMP, cGMP)	20
Adenylate cyclase assay	23
Phosphodiesterase assay	23
Iodination and purification of hormones	24
Methods of radio-ligand assessment	28
Cell incubations	29
Glucose/glycogen determination	30
Lipogenesis	31
DNA and protein determination	32
Dry weights	32
Oxygen consumption	32
Electron microscopy	33
Scintillant solutions	33
Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of collagenase	34
Materials	34

CHAPTER 3: PREPARATION AND PROPERTIES OF ISOLATED CHICKEN HEPATOCYTES

Introduction - historical development of cell isolation	35
Preparation of hepatocytes	40
Method I	40
Method II	42
Assessment of hepatocyte viability	45

CHAPTER 4: HORMONE RECEPTOR INTERACTIONS

Introduction	50
The insulin receptor	51
The glucagon receptor	59
The APP receptor	64
Experimental approach	64
¹²⁵ I-Insulin - Receptor interactions	65
¹²⁵ I-Glucagon - Receptor interactions	69
¹²⁵ I-APP - Receptor interactions	72
Discussion	73
Insulin binding	74
Insulin - receptor dissociation	77
Glucagon binding	78
APP binding	80

CHAPTER 5: 'SECOND MESSENGERS' OF HORMONE ACTION

Introduction	82
Methods	87
Results	88
Cyclic AMP	88
Cyclic GMP	93
Adenylate cyclase activity	94
Phosphodiesterase activity	95
Discussion	96
Summary	102

CHAPTER 6: HORMONE CONTROL OF CARBOHYDRATE AND LIPID METABOLISM

Introduction	104
Methods	108
Results	109
Glycogenolysis	109
Gluconeogenesis	113
Lipogenesis	114
Effects of caffeine and theophylline	115
Discussion	115
Summary	121
CHAPTER 7: CONCLUDING DISCUSSION	123
References	132

Abbreviations

The conventions of the Biochemical Journal have been used throughout. Most abbreviations are indicated in the text, however, those more frequently used are also listed below.

APP	- Avian Pancreatic Polypeptide
ATP	- Adenosine 5'-triphosphate
ADP	- Adenosine 5'-diphosphate
AMP	- Adenosine 5'-monophosphate
cAMP	- Adenosine 3'5'-monophosphate, cyclic
Bt ₂ cAMP	- N ⁶ ,2'-O-Dibutyryl-adenosine-3':5'-monophosphate, cyclic
GTP	- Guanosine 5'-triphosphate
cGMP	- Guanosine 3':5'-monophosphate, cyclic
Bt ₂ cGMP	- N ² ,2'-O-Dibutyryl-guanosine-3':5'-monophosphate, cyclic
BSA	- Bovine serum albumin
KRB	- Krebs Ringer Bicarbonate
DNA	- Deoxyribonucleic acid
PCA	- Perchloric acid
TCA	- Trichloroacetic acid
TRIS	- 2-amino-2-(hydroxymethyl)propane-1,3-diol
RIA	- Radioimmunoassay

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SUMMARY

A recirculating type perfusion method was developed for the isolation of hepatocytes from chicken liver. The method adopted resulted in relatively high yields of metabolically active hepatocytes capable of responding to physiological concentrations of hormones. However, hormonal sensitivity was found to depend on the batch of collagenase used to prepare the cells.

Hepatocytes were used to investigate the binding characteristics and intracellular metabolic effects of the pancreatic hormones, insulin, glucagon and Avian Pancreatic Polypeptide (APP).

Although insulin was found to have a high affinity receptor present on the cell membrane (K_d 3×10^{-10} M; 2000 sites/cell) the hormone failed to induce any physiologically significant effect on the intracellular processes studied. Physiological concentrations of insulin failed to alter basal or glucagon stimulated cyclic AMP levels in the cell. Cyclic GMP levels were also unaffected. Other negative responses were found when directly examining adenylate cyclase or phosphodiesterase activities in cell homogenates. Consistent with these findings, no effect of insulin was found on glycogenolysis, glycogen synthesis, gluconeogenesis or lipogenesis.

Glucagon was also found to bind to a high affinity receptor site on the cell surface (K_d 9×10^{-9} M; 13,000 sites/cell), and to stimulate adenylate cyclase (ED_{50} 12.9×10^{-9} M) resulting in up to 60 fold increases in intracellular cAMP. In the presence of glucagon the cyclic nucleotide rapidly accumulated in the cell cytosol. After two to five minutes, production rapidly stopped, resulting in a gradual reduction of the cyclic nucleotide concentration due to hydrolysis by phosphodiesterase and escape of the nucleotide to the external medium.

Metabolic profiles in the hepatocyte were highly sensitive to glucagon with up to 200% stimulation of glycogenolysis ($ED_{50} 1.1 \times 10^{-9} M$), 30% stimulation of gluconeogenesis ($ED_{50} 4.8 \times 10^{-10} M$) and 90% inhibition of lipogenesis ($ED_{50} 1.0 \times 10^{-9} M$). Isolated cells were found to vary in their sensitivity to glucagon depending on the batch of collagenase used during tissue digestion. One particular collagenase preparation greatly increased the apparent affinity of glucagon in stimulating cellular glycogenolysis ($ED_{50} 1.7 \times 10^{-10} M$).

Adrenaline was also capable of stimulating cAMP levels in the hepatocytes ($ED_{50} 1.7 \times 10^{-6} M$) to a maximum of 30% that found with glucagon. Despite the lower maximum stimulation of cAMP levels, adrenaline increased glycogenolysis ($ED_{50} 1.36 \times 10^{-7} M$) and gluconeogenesis ($ED_{50} 2.1 \times 10^{-8} M$) and inhibited lipogenesis ($ED_{50} 6.9 \times 10^{-8} M$) to the same extent as found with glucagon. Values found for the affinity of adrenaline for the various metabolic effects were also dependent on the batch of collagenase used to prepare the cells.

No high affinity receptor was found on the cell membrane for APP; this was consistent with the lack of effects of the hormone on cellular cAMP and cGMP levels and also its inability to directly alter adenylate cyclase or phosphodiesterase activities. Although APP slightly potentiated glycogenolysis in the presence of sub-saturating concentrations of glucagon and also showed a small inhibition of glycogen breakdown it is questionable whether the effects were of any physiological importance.

The methylxanthines theophylline and caffeine were found to be effective in inhibiting cyclic nucleotide phosphodiesterase and were also capable of inhibiting basal and hormone stimulated effects on glycogenolysis, gluconeogenesis and lipogenesis. The site of action of theophylline and caffeine on these metabolic pathways may well be related to cellular respiration, as both a decrease in cellular oxygen consumption and ATP content were also found in the presence of the methylxanthines.

CHAPTER 1

GENERAL INTRODUCTION

Introduction to avian metabolism and its hormonal control

The metabolic actions of the endocrine pancreatic hormones on hepatic tissue have been well investigated over recent years, especially since the discovery of glucagon's 'second messenger', adenosine 3':5'-monophosphate (cAMP), by Sutherland and co-workers in 1957 (Rall et al. 1957). The majority of experiments have been carried out using the mammalian liver as the experimental model, with the rat as the predominant laboratory species, and there is a considerable volume of information about the regulation of metabolism by these hormones. During this time however, very little interest has been shown in birds, and thus investigations using avian liver as the model system have been relatively scarce. Consequently many aspects of the hormonal control of metabolism in birds have not kept pace with those of their mammalian counterparts.

The actions of insulin and glucagon on mammalian metabolism have been the subject of numerous review articles covering work in vivo and in vitro (DeBodo et al. 1963; Robison et al. 1971; Assan 1972; Exton and Park 1972; Exton et al. 1972; Nuttal 1972; Park and Exton 1972; Samols et al. 1972; Unger and Lefebvre 1972; Pilkis et al. 1978). Aspects of regulation of avian carbohydrate and lipid metabolism have mostly been restricted to whole body investigations in vivo and, as yet, little information is available on pancreatic hormone action at the cellular level. Reviews on the control of avian carbohydrate and lipid metabolism are not so common (Langslow and Hales 1971; Hazelwood 1972; Hazelwood 1976). From those investigations already reported, using mainly domestic fowl, pigeon, duck and goose as the experimental species, all metabolic pathways common to the mammalian liver are present in the avian hepatic tissue, although the actual flux through particular pathways is often different.

In chicken, the liver shows a remarkable lipogenic activity compared to adipose tissue and in fact is the major site of lipid synthesis

(Goodridge and Ball 1967; Goodridge 1968a; Goodridge 1968b; O'Hea and Leveille 1969a). The liver accounts for at least 90% of the total de novo fatty acid synthesis whereas in mammals the main lipogenic activity is in adipose tissue (Favarger 1965; Leveille 1967; O'Hea and Leveille 1969b). Surprisingly however, the activity of the pentose phosphate pathway, which supplies the NADPH necessary for lipogenesis, is very low (O'Hea and Leveille 1969a) and thus the reduced cofactor must be supplied from other sources, mainly from malic enzyme activity (Goodridge 1969). Avian adipose tissue therefore only serves as a lipid store with lipolysis in this tissue being activated predominantly by glucagon (Carlson et al. 1964; Goodridge 1964; Goodridge and Ball 1965; Grande and Prigge 1970) and not the catecholamines as in most mammals (DePlaen and Galansino 1966; Sokal et al. 1966; Burns and Langley 1968). Lipid metabolism in general seems to play a much more significant role in birds than in mammals, perhaps due to weight conservation in flight, especially in migrating birds (Blem 1976), and also as a high calorific energy supply for embryo development within the confines of the egg. Both the site of lipogenesis in the liver, and the control of lipolysis by glucagon may be of paramount importance in the integration and control of these important pathways with that of carbohydrate metabolism.

Of the other biochemical dissimilarities between birds and mammals, the most obvious must be the apparent diabetic-like blood glucose concentrations of 12-15 mM which are two to three times higher than in mammals. This immediately raises questions about the blood insulin: glucagon balance, which has been shown to be important in the regulation of blood sugar levels in mammals (Gliemann 1967; Exton and Park 1968; Mackrell and Sokal 1969; Unger 1972a,b; Seitz et al. 1977; Felig et al. 1979). In chickens, starvation for up to 7 days produces little decrease in total blood sugar (Hazelwood and Lorenz 1959) although age

and sex of the bird along with housing temperature can vary the response (Langslow et al. 1970). Glycogen stores, which are generally lower in birds (Langslow and Hales 1971), are quickly depleted but then after a few days partially replenished. Increasing levels of non-protein nitrogen (Hazelwood and Lorenz 1959) and increasing loss of body weight (Langslow et al. 1970) suggest that gluconeogenesis is responsible for this carbohydrate homeostasis.

These differences in metabolic profile, although interesting in their own right, may help to resolve the finer points of hormonal control which have been difficult, if not impossible to determine when using mammalian models. The effect of the pancreatic hormones, insulin and glucagon, in the control of carbohydrate and lipid metabolism, obviously must allow for these distinctive avian characteristics.

Another possible metabolic regulator, which could explain these dissimilarities, came to light after the discovery of a third hormone, avian pancreatic polypeptide (APP) by Kimmel and coworkers during the routine isolation of chicken insulin (Kimmel et al. 1968). The hormone was later isolated and the amino acid sequence derived (Kimmel et al. 1971; Kimmel et al. 1975). This new hormone has been shown to be present in the pancreases of eight different species of bird (Langslow et al. 1973) and has its site of origin in cells which are cytologically distinct from either the A, B or D cells of the pancreas (Larsson et al. 1974). Similar proteins have been found in mammalian pancreases (Lin and Chance 1972; Lin et al. 1973; Lin and Chance 1974) and some of their biological actions investigated (Lin ^{T.M.,} et al. 1977). Extracts of reptilian pancreas but not that of amphibians nor mammals showed cross-reactivity with antisera raised against APP (Langslow et al. 1973). As yet the main role, if any, of this new pancreatic hormone has not been found, although many effects have been noted (see review by Floyd et al. 1977).

The avian pancreas and its internally secreted hormones, insulin, glucagon and perhaps APP, therefore allow a different carbohydrate and lipid homeostasis from that of mammals. For a clearer understanding of the actions of the avian pancreatic hormones, the historical background of recent developments requires review. The information obtained and the hypotheses and concepts developed are of great importance when trying to establish the physiological significance of results in vitro at the cellular level.

Early experiments using the endocrine pancreas and its secreted hormones

The first insight into the role of the endocrine pancreas was made in 1889 by Minkowski and von Mehring, who repeated the inconclusive experiments in pancreatectomy carried out by Bernard in 1877. The excision of the canine pancreas induced conditions of severe diabetes mellitus indicated by a subsequent rapid and large rise in blood glucose levels, glucosuria and eventually convulsions and death. Similar experiments on ducks and geese by Minkowski in 1893 failed to produce a noticeable 'diabetic' effect on the already high blood glucose levels. This supported the earlier work of Langendorff (1879), who ligated the pancreatic ducts of pigeon, but failed to induce glucosuria even after the ingestion of large amounts of sugar. The birds subsequently died 6-12 days later through apparent inanition. Experiments by Weintraud (1894) on pancreatectomy and partial enterectomy of nineteen ducks showed four cases of glucosuria while Kausch (1896) found no glucosuria in over eighty operated birds, but noted a transient hyperglycaemia which soon returned to normal. Similar results to those of Kausch were reported by other investigators using ducks (Paton 1905; Fleming 1919; Seitz and Ivy 1929; Sprague and Ivy 1936; Mirsky et al. 1941; Mirsky and Gitelson 1957). Subsequent work using fowls also showed no apparent effect on carbohydrate homeostasis apart from an initial transient hyperglycaemia

sometimes reaching levels of 50-70 mM before returning to normal (Giaja 1912; Koppanyi et al. 1926; Hazelwood 1958; Lepkovsky et al. 1964; Koike et al. 1964).

Insulin

For many years the search for this hypoglycaemia factor, which seemed to be much more effective in mammals than birds, had proved unsuccessful. Various workers had shown that pancreatic extracts could, when injected intravenously, decrease blood glucose and excreted glucose in diabetic animals (Murlin and Kramer 1913; Kleiner 1919) but it was not until it was realized that the factor itself was degraded by pancreatic enzymes that the first stable extract of insulin was achieved (Banting and Best 1922). Four years later insulin was isolated in crystalline form (Abel 1926) which then allowed numerous physical and biochemical investigations into its properties.

The chemical properties of insulin have been reviewed (Humbel et al. 1972). Insulin, molecular weight 5734 (bovine hormone) was the first protein to be sequenced (Ryle et al. 1955) and was found to comprise two polypeptide chains of twenty-one and thirty amino acids linked by two disulphide bridges. Except for the guinea pig there are only slight variations in amino acid sequence between different mammalian insulins with resulting minor changes in biological activity (Humbel et al. 1972). The amino acid sequence of chicken insulin varies in six positions from that of the bovine hormone (Smith 1966) and has been reported as being more potent than the mammalian hormones (Humbel et al. 1972) especially when administered to fowls (Hazelwood et al. 1968). Turkey insulin has the same amino acid sequence as chicken (Weitzel et al. 1972) but the duck hormone shows variation in three positions (Markusson and Sundby 1973).

Insulin was found to be present in much lower concentrations in chicken pancreas (1-2 mg/100 g tissue) than in mammals (10-15 mg/100 g tissue-bovine) (Kimmel et al. 1968). Plasma levels of insulin are similar between birds and humans (Sands et al. 1968; Langslow and Hales 1971; Goodner and Porte 1972) but are more stable in chickens than mammals with very little change in concentration even after prolonged starvation (Langslow et al. 1970). Concentrations can vary from 0.5 to 10 ng/ml in peripheral blood although higher concentrations must be present in the hepatic portal vein.

The early experiments of pancreatectomy indicated a major difference in the pancreatic regulation of carbohydrate metabolism between mammals and birds. The extent of the pancreatectomy in these experiments was first questioned by Mialhe (1958) and then later by Mirsky et al. (1964). Due to the diffuse nature of the endocrine pancreas, complete pancreatectomy is very difficult. Complete pancreatectomy of ducks, carried out by Mialhe (1958) resulted in hypoglycaemia which could be antagonized by intravenous injection of glucagon or glucose. Mialhe concluded that in previous experiments, a small inaccessible portion of the splenic lobe of the pancreas was not removed. The avian pancreas has a very high glucagon content (Vuyksteke and DeDuve 1953) which is secreted by the α -cells. These cells are only found in the third and splenic lobes of the fowl pancreas whereas the β -cells (insulin secretory) are found throughout the gland (Mikami and Ono 1962). Removal of the dorsal and ventral lobes of the pancreas, which contain few or no α -cells results in a transient hyperglycaemia while removal of the third and splenic lobes is followed by the development of a pronounced hypoglycaemia in chicken (Mikami and Ono 1962) and in goose (Sitbon 1967). Mialhe suggested on the basis of these experiments, that insulin plays a relatively insignificant role compared with glucagon in the maintenance of blood

glucose levels. However the avian need for insulin was shown by Mirsky et al. (1964) who observed an immediate hyperglycaemia in normal and supposedly depancreatized ducks, after the administration of antiinsulin serum. Blood glucose levels returned to basal levels after three to four hours in normal and after five hours in the depancreatized birds. Data from depancreatized ducks indicated the presence of a remnant of the splenic lobe of the pancreas or some extra pancreatic source of insulin. Similar results have been reported in chickens (Bondareva 1970). Various other experiments have indicated the possibility of another source of insulin apart from the recognised pancreatic β cell (Redenbaugh et al. 1926; Hazelwood 1958; Colca and Hazelwood 1976).

Insulin therefore appears to be essential for the control of avian metabolism as it is with mammals, although its effects may not be as widespread nor as intense. Injections of large doses of mammalian insulins into fowls, ducks and pigeons failed to induce the hypoglycaemic coma and convulsions seen with mammals (Chen et al. 1945). Chickens showed an apparent resistance to insulin, surviving up to one thousand times that to induce convulsions in rabbits. In other studies the extent of hypoglycaemia induced by varying intravenous mammalian insulin loads, was less intense in birds than in mammals (Hazelwood and Lorenz 1959; Heald et al. 1965; Lepkovsky et al. 1967; Langslow et al. 1970). This difference in the effect of insulin cannot be explained by insensitivity of chicken tissues to low concentrations of mammalian insulins. Adipose tissue and muscle isolated from both rats and chickens showed the same sensitivity to bovine or chicken insulins (Goodridge 1968; Hazelwood et al. 1968; Langslow 1969; Langslow and Hales 1969). At lower concentrations, intravenous injections of bovine or chicken insulins induce similar effects on plasma glucose levels in chickens although the chicken hormone was potent over a longer period (Hazelwood

et al. 1968). Depancreatized birds also showed the same insensitivity to high doses of insulin suggesting that pancreatic glucagon was not antagonizing the hypoglycaemic action of insulin. The extent of the reported pancreatectomy however is obviously very important. As a result of earlier studies the adrenal catecholamines were not expected to effect the response as preinjection of reserpine which depletes catecholamine stores did not alter the apparent resistance to insulin (Heald et al. 1965). Later work by Pittman and Hazelwood (1973), however showed an increase of four to eight fold in the plasma concentration of adrenaline after insulin administration. Besides a possible antagonistic effect on insulin by the catecholamines plasma binding factors have also been described in an attempt to explain the apparent insulin insensitivity (Chen et al. 1945; Hazelwood et al. 1971). Increased insulin uptake by the liver with subsequent biliary excretion was not responsible (Turner and Hazelwood 1974) and the half life of both chicken and bovine insulins in chicken plasma of 4.5 minutes (Langslow 1976) was similar to that in man (Cesari and Luft 1969; Orskov and Christensen 1969).

Intravenous injection of insulin in fowls and ducks leads to an increase in plasma free fatty acids (FFA) (Heald et al. 1965; Lepkovsky 1967; Langslow et al. 1970). Similar injections in mammals lead to decreased FFA levels (Dole 1956; Gordon and Cherkas 1956; Bierman et al. 1957; Shoemaker et al. 1962) due to a decreased rate of release from adipose tissue (Bierman and Dole 1957). The avian response to insulin injection is an increased glucagon secretion (Sands et al. 1969; Hazelwood and Langslow 1978) since there is no change in plasma FFA concentration in depancreatized birds (Mialhe 1969).

Glucagon

Glucagon, the second pancreatic hormone to be discovered, was isolated in crystalline form by Staub, Sinn and Behrens (1953 and 1955)

long after the hyperglycaemic factor was found in waste pancreatic extracts during the extraction of insulin (Murlin et al. 1923; Kimball and Murlin 1923). The relatively low concentration of the hormone in mammalian pancreas (10 µg/g tissue) compared to insulin made extraction and isolation difficult. Because of the excitement of the isolation of insulin and its association with the recognised disease state of diabetes mellitus little interest was shown in this hyperglycaemic factor, and progress made into the isolation and study of its biological actions was slow (Burger and Brandt 1935). Much later, when the pancreatic cells became well characterised, glucagon was recognised as a hormone and its importance in carbohydrate metabolism acknowledged. Glucagons isolated from various mammalian species consist of a single polypeptide chain of twenty-nine amino acids which are identical in sequence and hence have the same molecular weight of 3485 (Bromer et al. 1971; Thompson et al. 1972). Details of the chemistry of the hormone has been reviewed (Bromer 1972a,b). Chicken or turkey glucagons have an amino acid structure identical to that of porcine, bovine or human glucagons except for serine replacing asparagine as position 28 of the amino acid sequence (Markussen et al. 1972; Hazelwood 1976). As well as serine replacing asparagine, duck glucagon also differs at position 16 where threonine replaces a serine residue (Sundby et al. 1972; Hazelwood 1976). Despite such a small change in amino acid composition chicken glucagon is reported to be more active in avian and mammalian tissues than the mammalian hormone (Hazelwood 1976). Glucagon's action on avian metabolism is not entirely consistent with that of mammals. Intravenous glucagon in chickens raises plasma glucose, free fatty acid and plasma insulin concentrations (Langslow and Hales 1971; Hazelwood and Langslow 1978). In mammals intravenous injection is accompanied by an increase in plasma glucose with a decrease in free fatty acids. The latter effect is due to

the increase in plasma insulin levels stimulated by the administration of glucagon (Samols et al. 1965) which inhibits fatty acid release from mammalian adipose tissue (Mahler et al. 1964; Froesch et al. 1965; Fain et al. 1966; Chlouverakis 1967). The absence of a similar effect in chicken reflects the imbalance between the two hormones.

The avian pancreatic content of glucagon is some four to eight times higher than in mammals and peripheral plasma levels in ducks starved overnight can reach 1.5 ng/ml, some six times that found in Man (Samols et al. 1969). Hazelwood (1976) also reports circulating levels of glucagon of three to four times that of mammals. Although it is generally accepted that high levels of glucagon are present in avian plasma, accurate quantitation of pancreatic glucagon is difficult due to interactions of antibodies with the glucagon-like proteins (GLI) from the gut. For a review see Maier and Pfeiffer (1978). Due to this difficulty various ranges in plasma glucagon have been reported using various assay methods (Lawrence 1966; Unger 1969; Langslow and Hales 1970). The high sensitivity of chicken tissues to this hormone and its high levels in blood indicate that it may play a dominant role compared with insulin in the regulation of lipid and carbohydrate metabolism.

Avian Pancreatic Polypeptide

Avian pancreatic polypeptide (APP), the most recent hormone to be discovered comprises a single chain of thirty six amino acids with a molecular weight of 4258 (Kimmel et al. 1971; Kimmel and Pollock 1973). The amino acid sequence has been determined (Kimmel et al. 1975). Similar polypeptides have since been isolated from bovine (BPP), porcine (PPP), ovine (OPP) and human (HPP) pancreases by Lin and Chance (1974). The avian hormone exists in very high concentrations in the pancreas (8 to 9 mg/100 g tissue) and in blood where peripheral concentrations vary from 4 to 12 ng/ml (Kimmel et al. 1971). During fasting these levels are markedly reduced but are quickly restored to normal on re-feeding.

Intravenous injections of APP at physiological levels (1 to 25 $\mu\text{g}/\text{Kg}$ body weight) show a gastrin-like action on chicken proventriculus with increases in volume, free acid, pepsin and total protein content of the fluid secreted (Hazelwood et al. 1973; Hazelwood and Langslow 1978). At much higher doses (100 $\mu\text{g}/\text{Kg}$ body weight) the polypeptide stimulates hepatic glycogenolysis but with no apparent hyperglycaemia, and decreases blood glycerol levels. APP also lowers plasma free fatty acids despite stimulating the release of glucagon (Hazelwood and Langslow 1978).

Hormone Action at the Cellular Level

Early experiments into the action of the peptide hormones employed mostly experiments in vivo. It was soon clear that the precise mode of action of the hormones could not be determined from these experiments alone due to the complexity of the interacting systems of the whole animal. Investigations thus turned increasingly to experiments in vitro utilising the techniques of perfused organs, tissue slices, broken and eventually intact cell preparations to study the more direct effects of various hormones. At the same time however the use of birds as experimental models for work in vitro seemed to lose favour and thus studies into the role of hormones at the cellular level were carried out almost entirely using tissues isolated from mammals, mainly the rat.

Early investigations into the actions of adrenaline and glucagon on glycogen phosphorylase activation in liver by Sutherland and co-workers was the first major step towards elucidation of hormone action (Rall et al. 1956; Rall et al. 1957). The discovery of adenylate cyclase and adenosine 3'5'-phosphate (cAMP) as the mediator of hormone action in a variety of target tissues opened up numerous new corridors of investigation. Very quickly it was apparent that cAMP was implicated in the action of many other non-related hormones which stimulated the membrane bound adenylate cyclase. Cyclic AMP was then recognised as a general

second messenger for hormone action. The mode of action of the hormones acting on adenylate cyclase and cAMP has been extensively reviewed (Robison et al. 1970; Robison et al. 1971; Pohl 1972; Rodbell 1972; Catt and Dufau 1976; Helmreich 1976; Helmreich et al. 1978). Glucagon has been shown to bind specifically to receptor proteins in particulate fractions of rat liver membranes (Pohl et al. 1971; Birnbaumer et al. 1971; Rodbell et al. 1971a,b). Bound glucagon could be exchanged with free glucagon but not with biologically inactive glucagons, secretin, adrenocorticotrophic hormone (ACTH) nor insulin. The dissociation constant for the hormone receptor protein complex was in the nanomolar range which was similar to that found for adenylate cyclase activation. The cAMP produced by hormonal stimulation was found to activate a cytosolic enzyme capable of phosphorylating various proteins (Krebs 1972; Krebs 1973; Langan 1973). Activation of this protein kinase resulted in the phosphorylation of various key regulatory enzymes of metabolism with concomitant alterations in activity. Substrates for this protein kinase have only been shown in a few cAMP sensitive tissues and include liver phosphorylase kinase and glycogen synthetase and adipocyte triglyceride lipase (Soderling et al. 1970; Corbin et al. 1970; Khoo et al. 1973). Pyruvate kinase has also recently been shown to be regulated by a phosphorylation-dephosphorylation mechanism which is sensitive to a cAMP dependent protein kinase (Ljungström and Ekman 1977; Riou et al. 1978) and fructose diphosphatase has also been suggested as a substrate (Riou et al. 1977). As yet, it is not known if this cAMP dependent protein kinase is involved in all the actions of glucagon on the liver, which include stimulating glycogenolysis and gluconeogenesis, induction of potassium and calcium efflux, amino acid uptake and urea synthesis (Menahan and Wieland 1967; Exton and Park 1968; Friedmann and Park 1968; Mallette et al. 1969; Chambers and Bass 1970).

The action of adrenaline, although inducing similar effects to that of glucagon is not so clear cut. There are at least two types of catecholamine receptor (α and β) on liver cell membranes. Only occupancy of the β receptor induces the classical stimulation of cAMP by activation of adenylate cyclase. Catecholamines exert their action on the liver through both α and β receptors with occupancy of the former not affecting cAMP or cAMP dependent protein kinase (Sherline et al. 1972; Cherrington 1976; Keppens et al. 1977; Birnbaum and Fain 1977). Stimulation of gluconeogenesis in liver by catecholamines has been inhibited by α blocking agents such as phentolamine and dihydroergotamine but not by the β blocker propranolol (Tolbert et al. 1973; Tolbert and Fain 1974). Isoproterenol, a pure β agonist, has also been shown to bind to its receptor and elevate intracellular cAMP but with no resulting effect on gluconeogenesis. Hutson et al. 1976 report that adrenergic activation of hepatic glucose output is regulated mainly through the α receptor. Others have indicated that adrenergic activation of glucose output is independent of cAMP and acts by the α mechanism which is mediated by alterations in calcium flux (Sherline et al. 1972; Cherrington et al. 1976; Assimacopoulos-Jeannet et al. 1977; Chan and Exton 1977; Keppens et al. 1977; Pariza et al. 1977; Van de Werve et al. 1977; Le Cam and Freychet 1978). Both α , cAMP-independent, and β , cAMP dependent, actions of the catecholamines can be inhibited by insulin (Claus and Pilkis 1976). Insulin is thought to suppress β activation by lowering the elevated cAMP levels, but it is not known how insulin antagonises the α adrenergic response.

The investigation of the cellular action of insulin has not progressed as rapidly as that of glucagon or adrenaline. The actions of insulin were mainly investigated in tissues other than liver, including adipose tissue and muscle. Early studies showed insulin could bind to a variety of tissues (reviewed by Cuatrecasas and Hollenberg 1976). Insulin can

regulate the transport of many solutes, including glucose, some amino acids and a number of ions, across the cell membrane (Battaglia and Randle 1960; Crofford and Renold 1965; Margoulies 1968; Park et al. 1968; Wool 1968) and also antagonise hormone stimulated lipolysis in adipose tissue (Rodbell and Jones 1966; Fain 1967). Insulin was also shown to decrease catecholamine or glucagon stimulated cAMP levels with a subsequent reduction in the expected metabolic effect (Exton et al. 1966; Exton and Park 1968; Lewis et al. 1970).

Cuatrecasas (1969) provided the first strong evidence that the insulin receptor was present on the external surface of the cell membrane, but the validity of his experiments using Sepharose-bound insulin was questioned by others (Katzen and Vlahakes 1973). However many other workers using different experimental techniques have also found that insulin binds to specific receptor sites on the cell surface (Pastan et al. 1966; Kono 1969; Freychet et al. 1971; Bennet and Cuatrecasas 1973; Jarret and Smith 1975). Since no definite intracellular second messenger has been found for insulin other workers have investigated and reported specific insulin binding sites within the cell which may be responsible for the intracellular and long term effects on metabolism with the cell plasma membrane receptors only influencing transport processes in and out of the cell (Goldfine and Smith 1976; Goldfine et al. 1977). It has been suggested that insulin binding sites present in the Golgi fraction of a cellular homogenate are present only to maintain receptor density after exocytosis of various intracellular substances from the cell where the golgi vesicles are continually adding to the plasmalemma (Bergeron et al. 1973; Bergeron et al. 1978; Posner et al. 1978). This model system could also be used to explain the method of receptor turnover.

Iodinated insulin bound to liver membranes was shown to dissociate rapidly in the presence of excess cold hormone (Freychet et al. 1971). Insulin analogues with a wide range of biological potencies inhibited the binding of iodinated hormone to liver membranes in direct proportion to their ability to stimulate glucose oxidation in fat cells. Similar results were found for the insulin receptor of liver and fat cells (Cuatrecasas 1972a). A receptor protein has been isolated from the external surface of the plasma membrane by using the nonionic detergents Triton X-100 and Lubrol WX and found to be a glycoprotein with molecular weight of about 300,000 (Cuatrecasas 1972b,c). The receptor shows a high affinity for insulin (K_d 10^{-10} - 10^{-9} M) which is in the same range as circulating insulin levels in blood (Cuatrecasas 1972b,c; Kahn et al. 1974). How the signal of insulin binding is transmitted to and manifested in the cell cytosol is still unclear. Under various conditions insulin was reported to reduce hormone elevated intracellular levels of cAMP (Exton et al. 1971; Exton and Park 1972; Pilkis et al. 1975; Claus and Pilkis 1976). An elevated content of cellular cAMP was found in diabetic rats or normal rats injected with anti-insulin serum indicating that insulin plays a role in the maintenance of basal cAMP levels as well as reducing those stimulated by glucagon or adrenaline (Jefferson et al. 1968). A direct inhibition of adenylate cyclase by insulin has been reported (Illiano and Cuatrecasas 1972; Flawia and Torres 1973; Renner et al. 1974) but this has not been confirmed by others (Rosselin and Freychet 1973; Thompson et al. 1973; Pilkis et al. 1974). An insulin-sensitive cAMP phosphodiesterase has also been reported (Loten and Sneyd 1970; House et al. 1972; Manganiello and Vaughan 1973; Kono et al. 1975; Loten et al. 1978) although others have failed to note any effect (Thompson et al. 1973; Menahan et al. 1969; Pilkis et al. 1975). Although an insulin sensitive guanylate cyclase has been reported in rat

fat cells (Illiano et al. 1973; Fain and Butcher 1976) and liver cells (Pointer et al. 1976) very little additional evidence for cGMP as the possible second messenger has been shown. Fain and Butcher (1976) and Pointer et al. (1976) have both shown that catecholamines, carbachol, insulin, calcium ionophore A23187 and glucagon all increase intracellular levels of cGMP with resulting extremes of effects on metabolism. Insulin also has marked effects on the fluxes of several ions such as potassium, calcium and phosphate (Zietler 1966; Hepp et al. 1968; Williams et al. 1971). The possibility that one of these cations, particularly calcium, could be a possible second messenger for insulin is continually being suggested. The role of calcium in the hormonal, control of cellular processes has been reviewed (Rasmussen et al. 1972; Berridge 1975). It appears that intracellular calcium concentrations are as important as those of cAMP in enzyme regulation, particularly in tissues which respond to hormones by contraction or secretion.

Despite the fact that no definite intracellular second messenger for insulin has been found insulin is capable of stimulating glycogen synthesis (Nuttal 1972), protein synthesis (Pilkis and Park 1974), lipogenesis (Avruch et al. 1972) and inhibiting gluconeogenesis (Claus and Pilkis 1976), glucose release (Jefferson et al. 1968) and lipolysis (Avruch et al. 1972).

The study of the effects in vitro of insulin, glucagon and adrenaline on chicken tissues has been confined mostly to actions on adipose tissue (for review see Langslow and Hales, 1971). There have been very few studies to date on hormone action on the isolated chicken hepatocyte. Goodridge and co-workers have studied the effects of glucagon and insulin on lipogenesis in hepatocytes isolated from neonatal chicks (Goodridge 1973; Goodridge et al. 1974; Goodridge 1975; Goodridge and Adelman 1976) but only a few reports have been made on hormonal effects

in hepatocytes isolated from older chickens (Anderson and Langslow 1975; Dickson 1977; Dickson and Langslow 1978; Dickson et al. 1978).

While this summarises the background to the present work a more detailed account of recent studies on the actions of the hormones will be given in the following chapters.

The mode of action of glucagon, and perhaps to a lesser extent insulin, in mammalian hepatic metabolism is reasonably well quantified. It is now possible to estimate with considerable accuracy the affinity of hepatic receptors for either of the two hormones and gauge the extent of the metabolic response to a particular hormone at a given concentration. This preliminary investigation allows a more complete understanding of individual molecular interactions which take place between hormone and receptor, receptor and adenylate cyclase, cAMP and protein kinase, ion and ion binding protein etc. The finer details of hormone action and metabolic regulation are now under study. Although brief reports have been made about some metabolic actions of insulin and glucagon on chicken hepatocytes no complete investigation into the mode of action of these hormones or that of APP has been carried out. In the present work the actions of the pancreatic hormones and to a lesser extent that of adrenaline, have been investigated at the level of hormone receptor, second messenger and the ultimate physiological response using the chicken hepatocyte as the model system. A direct quantitative comparison can then be made between the mammalian and avian systems; it is hoped that this will help to explain the apparent differences in metabolic profile that have been discussed above.

CHAPTER 2

METHODS

1. Determination of adenosine phosphates in cell extracts

The intracellular concentration of all three adenosine phosphates was determined by a bioluminescent assay using the enzyme luciferase. Extracts of firefly lanterns are capable of emitting light when ATP, oxygen, a divalent cation and a fluorescent compound called luciferin are added (McElroy and Strehler 1949). Under certain conditions the light emitted is proportional to the amount of ATP added. Using this system the concentration of ATP in biological extracts can be determined (Strehler 1969; Bihler and Jeanrenaud 1970). Preincubating extracts, under appropriate conditions, with pyruvate kinase (PK) and myokinase (MK) converts ADP and AMP to ATP and therefore mono and diphosphorylated derivatives can also be estimated (Kimmich et al. 1975).

A simple modification in the basic spectrophotometric assay of ADP and AMP (Adam 1965; Jaworek et al. 1974) was used to quantitatively convert these two adenylates to ATP with their subsequent determination by the luciferase system.

Extraction of adenylates Cell suspension (1 ml) was added to ice cold 6% PCA (1 ml), shaken vigorously and left at 0°C for 10 minutes.

The protein precipitate was pelleted by centrifugation (2750 g; 30 s) and the supernatant neutralised by 3M potassium carbonate (K_2CO_3) and left at 0°C for a further 15 minutes to allow maximum precipitation of perchlorate. Samples were re-centrifuged (2750 g; 30 s) and supernatants stored at -20°C for adenylate determination by the luciferase assay.

Preparation of lantern extract Firefly lantern extract (FLE-50) was obtained from the Sigma Chemical Company and kept dessicated at 4°C until use. One vial containing 50 mg of extract was suspended in 1 ml of distilled water containing a trace of apyrase (grade 1, Sigma Chemical Company), 5 mg of dithiothreitol (DTT) and 50 mg of bovine serum albumin (BSA). The vial was incubated with constant agitation at 30°C for

15 minutes and then added to 200 ml of sodium arsenate buffer, 50 mM pH 7.5, containing 20 mM magnesium sulphate. The medium was stirred for a further 5 minutes and finally filtered through glass wool. Apyrase which removes any endogenous ATP in the lantern extract is inhibited by arsenate and therefore does not effect the response to subsequent additions of ATP. The medium was kept at room temperature for 30 minutes before use to allow activity of extract to stabilise. No advantage was obtained in keeping the extract on ice but stability throughout the duration of the assay was much improved by the addition of the DTT and BSA (Fig. 2.1).

Preparation of adenylate samples In order to estimate the concentrations of ADP and AMP it was first necessary to enzymatically convert them to ATP. The conversion was carried out by adding 0.4 ml of unknown extract or suitably diluted standard to 0.3 ml of a tris-HCl buffered reaction medium consisting of 50 mM tris-HCl pH 7.4, 2.5 mM phosphoenolpyruvate, 4.6 mM magnesium sulphate 387 mM potassium chloride and 2.75 mM ethylenediamine tetraacetic acid (EDTA). Unknowns were assayed in triplicate. The first tube contained no additions to the above mixture and was a direct measure of ATP. The second tube contained pyruvate kinase (15 $\mu\text{g/ml}$) to convert ADP to ATP while the third contained myokinase (10 $\mu\text{g/ml}$) as well as pyruvate kinase for the conversion of all adenylates to ATP. Stock solutions (10 μM) of the adenylate standards, ATP, ADP and a ATP/AMP mixture (1:1), was each diluted to give four or five different concentrations for the assay. Myokinase and/or pyruvate kinase was added as necessary to the standard tubes. In the AMP standard, ATP is required to initiate the myokinase reaction. Tubes were shaken and then incubated at 30^oC for 60 minutes. Reaction was then stopped by placing tubes on ice and adding 0.7 ml of 6% PCA. Samples were neutralised with 3M K_2CO_3 , tubes centrifuged (2750 g; 30 s) and supernatant sampled for luciferase assay.

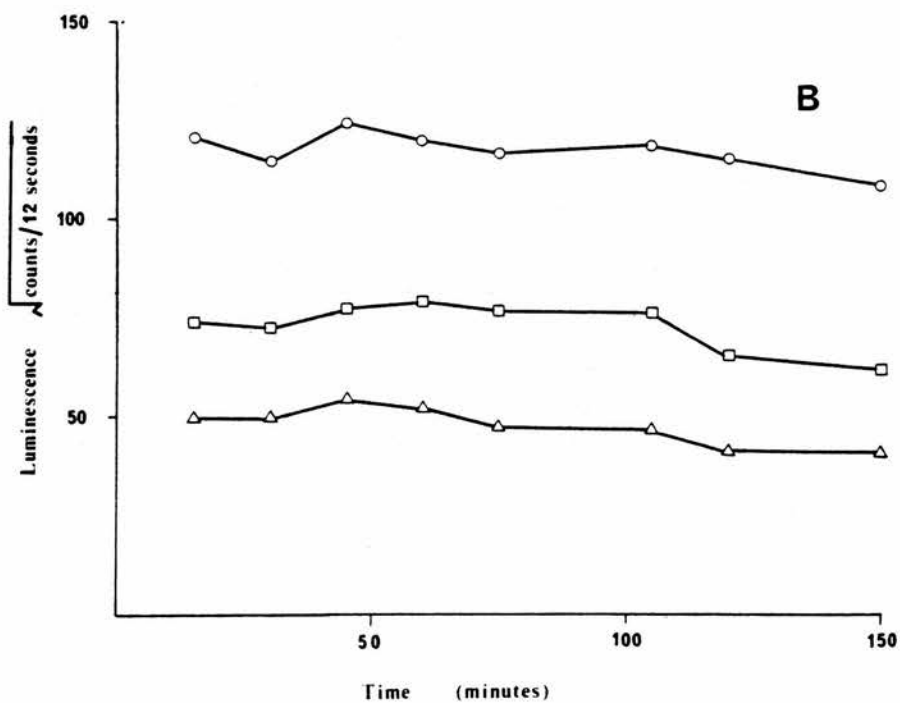
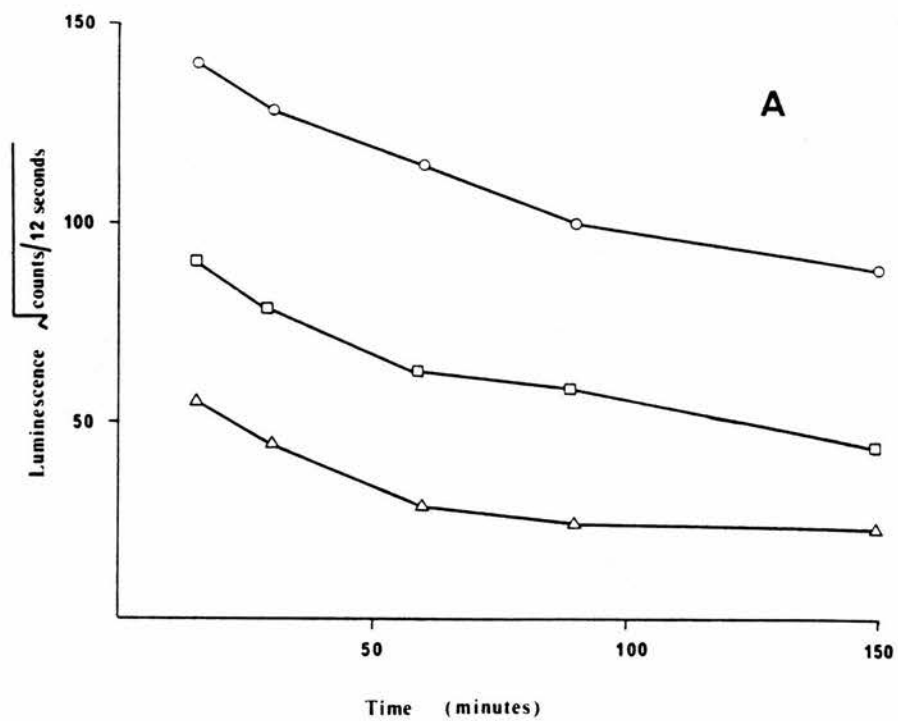


Fig. 2.1 Luminescence recorded on addition of 40 (Δ - Δ), 60 (\square - \square) and 100 (O-O) pmols ATP to Luciferin-luciferase extract incubated at room temperature for the time shown.

A. - Normal luciferin medium.

B. Luciferin medium containing Dithiothreitol and BSA.

Luciferase assay Aliquots (2 ml) of luciferase extract were added to 4.5 x 1.2 cm vials supported in 5.8 x 2.5 cm scintillation vials. Samples (10-30 μ l) of processed unknowns and standards were added to the extract and immediately counted for 12 seconds in a Tracerlab scintillation counter. Since the square root of the resulting count is directly proportional to the amount of ATP added, the concentration of the adenylate in each sample was determined. An equimolar concentration of ATP to AMP was needed in the standards to initiate the myokinase reaction under these experimental conditions (Fig. 2.2). The ATP plus ADP concentrations in the unknown extracts were always much higher than the AMP concentration. Adding an excess amount of ATP to the unknowns did not increase the AMP converted. Approximately 95% of ADP and 87% of AMP is converted to ATP by this method (Fig. 2.3) which compares favourably with other workers (Kimmich et al. 1975). The ratios of ATP:ADP:AMP in each unknown was found by the difference in the square ^{roots} of the counts between triplicate tubes and the subsequent determination of the nucleotide concentration by using the appropriate adenylate standard graph.

2. Cyclic nucleotide determination (cAMP, cGMP)

The determination of both nucleotides was carried out using the radioimmunoassay method of Steiner et al. (1969) as modified by Siddle et al. (1973). Specific antibodies to cAMP and cGMP were raised in rabbits by injections of 2'-O-succinyl-cyclic AMP or 2'-O-succinyl cyclic GMP coupled to human albumin. Antisera for cAMP and cGMP were kind gifts from Dr. K. Siddle, Department of Medical Biochemistry, Addenbrooke's Hospital, Cambridge.

Radioimmunoassay method. The assay was carried out in polyethylene microfuge tubes (Sarstedt 46/6 or Beckman EET23). An antibody-tracer solution was made up by the addition of 20-30 μ l antiserum and 1-2 μ l tracer ($[^3\text{H}]$ -cAMP or $[^3\text{H}]$ -cGMP) to 2 ml of 100 mM potassium phosphate buffer pH 7.0.

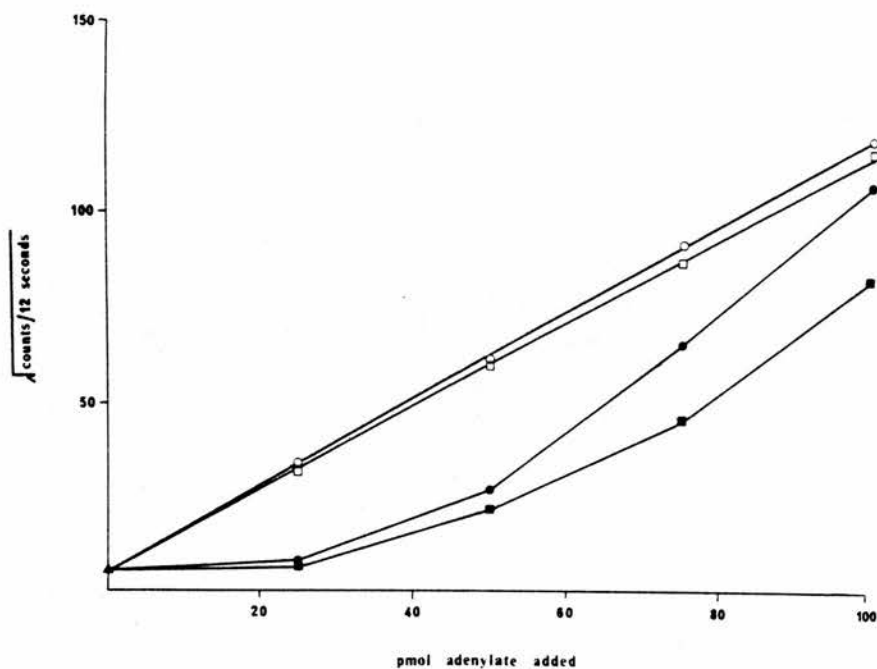


Fig. 2.2 Luminescence recorded after conversion of a range of AMP concentrations to ATP by myokinase and pyruvate kinase. AMP:ATP ratios of 20:1 ■-■, 10:1 ●-●, 2:1 □-□, 1:1 ○-○.

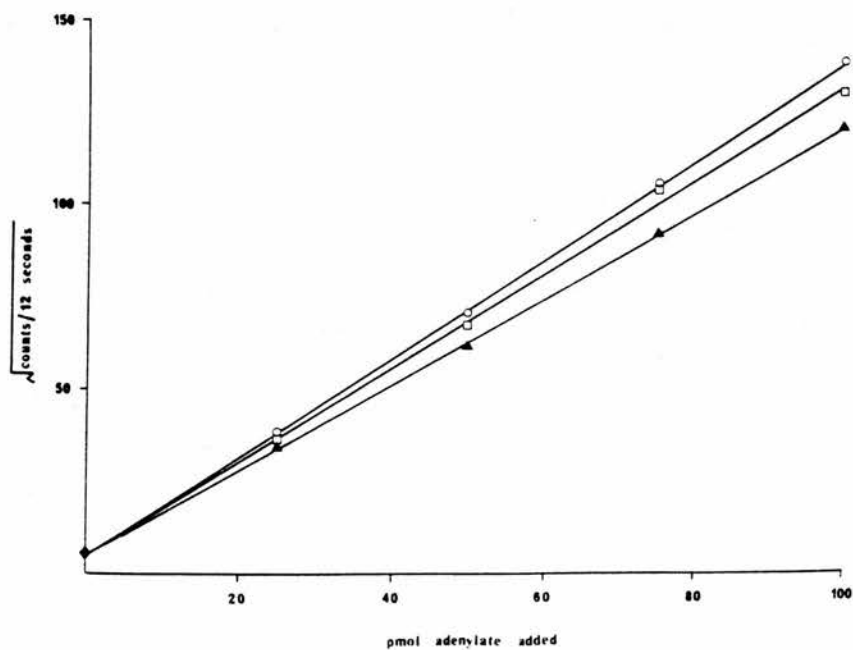


Fig. 2.3 Luminescence recorded from various concentrations of ATP (○-○); also ADP (□-□) and AMP (▲-▲) converted to ATP by myokinase and/or pyruvate kinase.

An aliquot (20 μ l) of this medium was added to each microfuge tube - 20 μ l samples were taken before the addition of the antiserum to estimate non-specific binding. Standards or samples (40 μ l) were then added and mixed thoroughly giving a final assay volume of 60 μ l containing sample or standard (0.1 to 16 pmol); tracer (approximately 0.66 pmol or 15,000 cpm for cAMP; 0.5 pmol or 7000 cpm for cGMP) and antiserum at final dilution of 1:200. Samples were then incubated overnight on ice. Bound tracer was separated by adsorption onto Millipore filters (HAWP - 0.45 μ m pore size-25 mm²) under suction. Microfuge tubes were washed out once with ice cold phosphate buffer and applied to filters. Filters were further washed with 5 ml of ice cold buffer, placed in 5.8 x 2.5 cm scintillation vials and dissolved in 1.5 ml of 2 methoxyethanol or 2 ethoxyethanol (approx. 1 hr). Scintillant (see method 14) (10 ml) was then added and radioactivity determined - Tracerlab scintillation counter.

Cell extracts Cell suspensions were deproteinised by adding an equal volume of 6% PCA and neutralised with 3M K₂CO₃ as with adenylates or an equal volume of 10% TCA and neutralised by washing with 4 x 5 ml water saturated ether. When assaying extracted cAMP both of these procedures reduced the sensitivity of the assay. The shape of the standard binding curve was found to be less consistent when using TCA in preference to PCA. Incomplete removal of TCA from the extract was indicated by a greatly reduced sensitivity of the assay (Fig. 2.4). At least four vigorous extractions in water saturated ether are necessary for maximal removal of TCA (Fig. 2.5). Sensitivity was less affected by PCA extraction which, unlike that of TCA, gave consistently reproducible curves (Fig. 2.6). Antibody-tracer binding was affected when PCA or TCA was incompletely removed. There was a linear fall in tracer binding when adding PCA directly to the assay mixture. Similar additions of TCA show a large drop in tracer binding above 15 mM (Fig. 2.7).

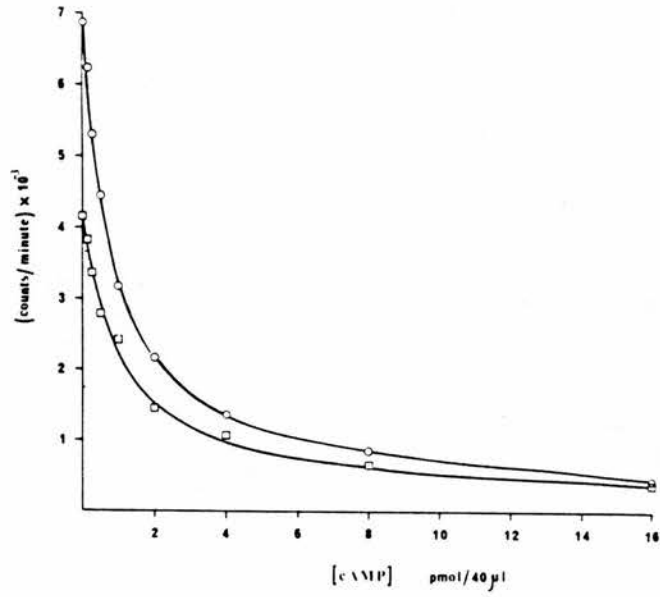


Fig. 2.4 cAMP radioimmunoassay binding curve. Normal standards (O-O), or standards pre-treated with TCA and neutralised (□-□).

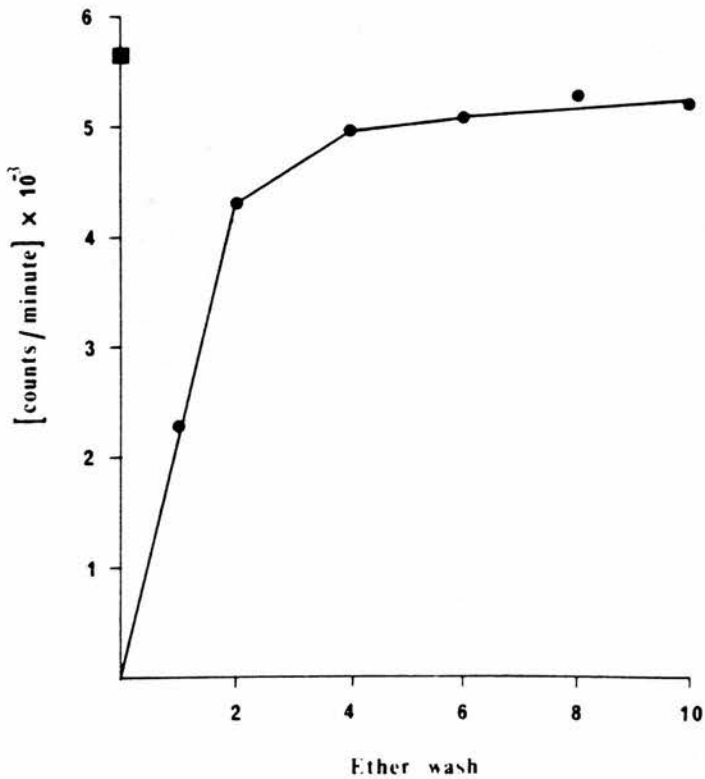


Fig. 2.5 Radioactive tracer binding to antibody: Effect of ether washing a TCA extracted cAMP solution (0.5 pmol/40 μl) (●-●). ■ - counts bound in the absence of TCA extraction.

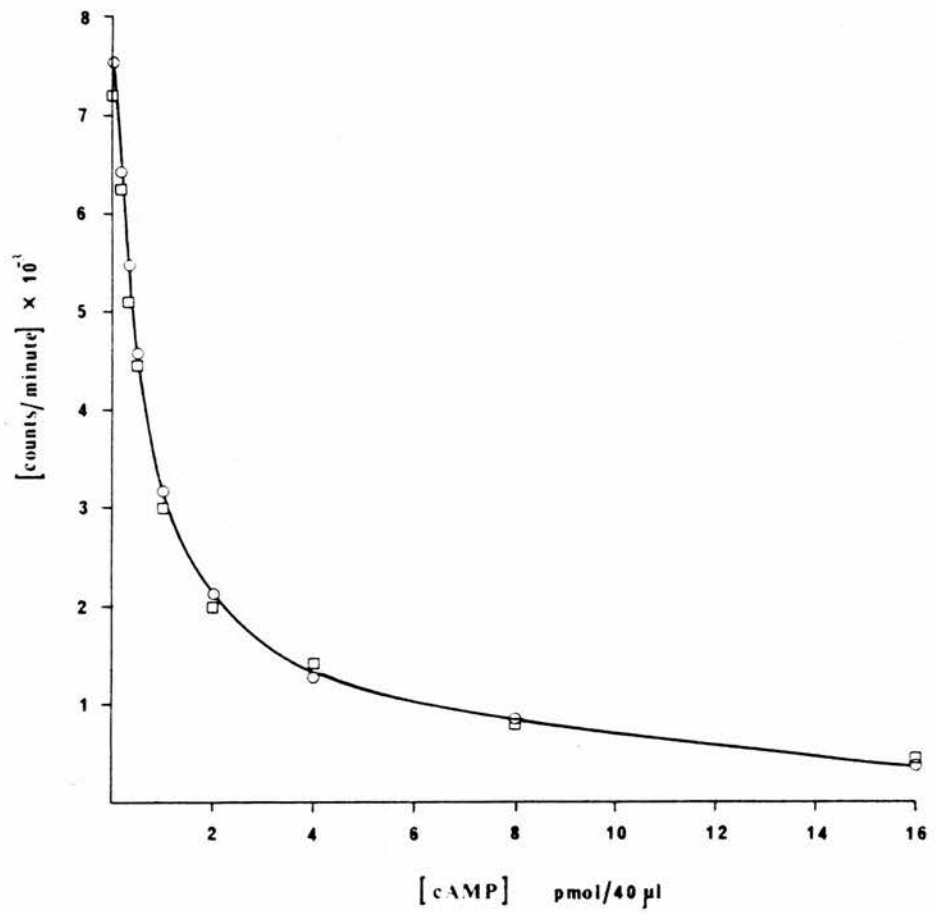


Fig. 2.6 cAMP radioimmunoassay binding curve.
 O-O Normal standards.
 □-□ Standards previously extracted with 6% PCA.

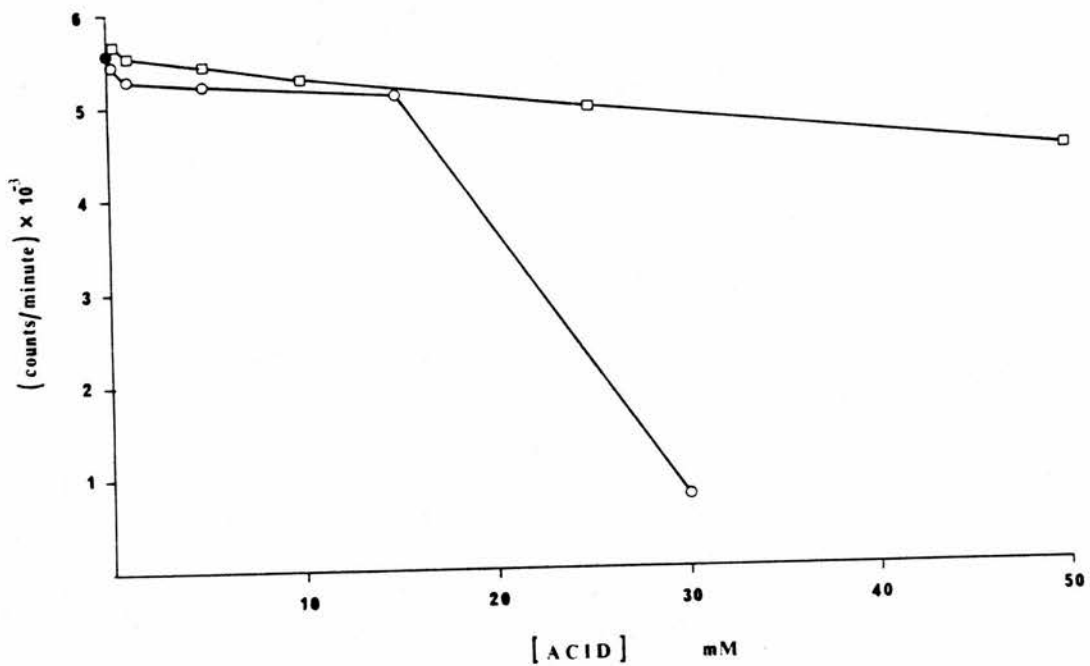


Fig. 2.7 Effect of adding TCA (O-O) or PCA (□-□) directly to the radioimmunoassay. Counts bound in the presence of 0.5 pmol cAMP/40 μ l.

For the reasons mentioned above plus the greater ease in handling large numbers of samples the PCA extraction procedure was used throughout when measuring cAMP. Standard cAMP samples were also extracted before each radioimmunoassay. Cross reactivity between cAMP antiserum and other nucleotides in the cell was very low (Table 2.1). Therefore the antibody was suitable for use in deproteinised extracts without further purification. The cAMP content in extracts of unstimulated cells could be decreased by 90% and that in stimulated cells by nearly 100% after incubation with commercial phosphodiesterase. However when the intracellular level of cGMP was estimated the cAMP which was also present in the acid extracts was at such a concentration to effectively crossreact with the antiserum for cGMP. Therefore after acid precipitation of protein the cGMP was further purified from the other cell nucleotides and then concentrated by freeze drying in order to estimate the low intracellular levels present. The separation of the nucleotides was carried out using an anion exchange column of Bio-Rad AG1 X2 200-400 mesh, chloride form and necessitated the use of the TCA extraction procedure. Procedure was basically that of Siddle et al. (1976).

Preparation of columns Anion exchange resin was washed in alternate acid and alkali solutions (0.5M HCl: 0.5M NaOH). After the final acid wash the resin was further washed in distilled water until the pH of the effluent was greater than 5. The resin was then suspended in a 1:1 slurry (vol/vol) with 20 mM NaCl containing 0.01% sodium azide as a preservative. Columns were prepared by adding 1 ml slurry to a 1 ml syringe with a Whatman No. 1 filter disc in the bottom. Columns were then washed with 1.5 ml 50 mM HCl and then with 6 ml H₂O. A 5 ml syringe connected by a microfuge tube with excised tip was used as a reservoir for each column.

Purification procedure After four vigorous ether washes the sample (1 to 2 ml) was neutralised to pH 7 by addition of 50 to 100 μ l of 0.1M tris

Table 2.1: Cross-reactivity of cAMP anti-serum with other nucleotides.

All values are averages of duplicate observations.

Nucleotide	Concentration pmol/40 μ l	$\{^3\text{H}\}$ cAMP bound counts/ min	Nucleotide	Concentration nmol/40 μ l	$\{^3\text{H}\}$ cAMP bound counts/ min
-	-	5373			
cAMP	0.125	4281	ATP	0.1	5201
	0.25	3840		1.0	5266
	0.5	2931		5.0	5240
	1.0	2163		10.0	5076
	2.0	1399	ADP	0.1	5105
	4.0	868		1.0	5056
	8.0	564		5.0	5237
	16.0	325		10.0	5306
cGMP	1	5076	AMP	0.5	5350
	5	5240		1.0	5364
	10	5266		5.0	5267
	20	5281		10.0	5051
Bt ₂ cAMP	1	3699			
	10	1977			
	50	732			
	100	453			
	1000	199			
Bt ₂ cGMP	62.5	4796			
	125	4540			
	250	4099			
	500	3461			
	1000	2884			
GTP	62.5	5251			
	125	5290			
	250	5473			
	500	5300			
	1000	5328			

base and applied to the column. The column was washed sequentially with 3 ml distilled water, 3 ml 5 mM HCl, 4 ml 50 mM HCl and finally 3 ml 300 mM HCl. Cyclic AMP was eluted in the 50 mM HCl wash with the cGMP collected by the 300 mM HCl wash. Table 2.2 shows the elution pattern of [^3H]-cAMP and [^3H]-cGMP from this type of column.

If isolation of cAMP is not required then the column can be washed directly with 4 ml of 50 mM HCl before eluting cGMP. The adenylate nucleotide was eluted into 1.5 x 4.0 cm plastic tubes, frozen to -40°C and freeze dried. The samples were resuspended in a small volume of phosphate buffer (0.1-0.2 ml) to concentrate the cGMP and were refrozen and thawed once more. This last step increases the recovery of cGMP from the tube (C.J. Davies, personal communication) (Fig. 2.8). Samples (40 μl) were then taken for radioimmunoassay as outlined previously. Standard cGMP standards put through the complete extraction and purification procedures showed nearly 100% recovery even at the lower concentrations (Fig. 2.8). It was therefore considered unnecessary to process standards through extraction procedures with each experiment.

3. Adenylate cyclase assay

Method used was as Luzio et al. 1976. The cAMP produced from cell extracts incubated with theophylline and an ATP regenerating system, was measured by radioimmunoassay as given under cAMP/cGMP determinations.

4. Phosphodiesterase assay

Phosphodiesterase activity in cell extracts was measured by the method of Arch and Newsholme (1976). The [^3H]-5'AMP produced from [^3H]-cAMP hydrolysis was converted to [^3H]-adenosine by the inclusion of 5'nucleotidase in the incubation medium. Unreacted [^3H]-cAMP was bound by the addition of anion exchange resin AG1 x 2 200-400 mesh chloride form (prepared as described above) leaving [^3H]-adenosine free in the supernatant. Samples (0.4-0.5 ml) were removed and added to scintillant (see method 14 for composition) for radioactive determination in the Tracerlab scintillation counter.

Table 2.2: Elution of $\{^3\text{H}\}$ cAMP and/or $\{^3\text{H}\}$ cGMP from anion exchange column.

<u>Application</u>	<u>Column No. - Total counts/min in eluate</u>		
	<u>2</u>	<u>1</u>	<u>3</u>
Sample (2 ml)	2140	40	1860
H ₂ O (3 ml)	6990	1260	2010
5 mM HCl (3 ml)	30	717	1110
50 mM HCl (2 ml)	740	58120	31500
50 mM HCl (2 ml)	60	8020	4620
50 mM HCl (2 ml)	600	500	120
300 mM HCl (3 ml)	45630	450	24030
300 mM HCl (2 ml)	540	10	20

<u>Column No.</u>	<u>Cyclic Nucleotide</u>	<u>counts/min applied</u>	<u>% total recovery</u>	<u>% 50 mM</u>	<u>% 300 mM</u>
1	cAMP	approx. 78,500	87	84	0.6
2	cGMP	approx. 57,500	99	2.4	80
3	(cAMP	39,250)	Ave 88		
	(+	+)			
	(cGMP	28,750)			
	()			

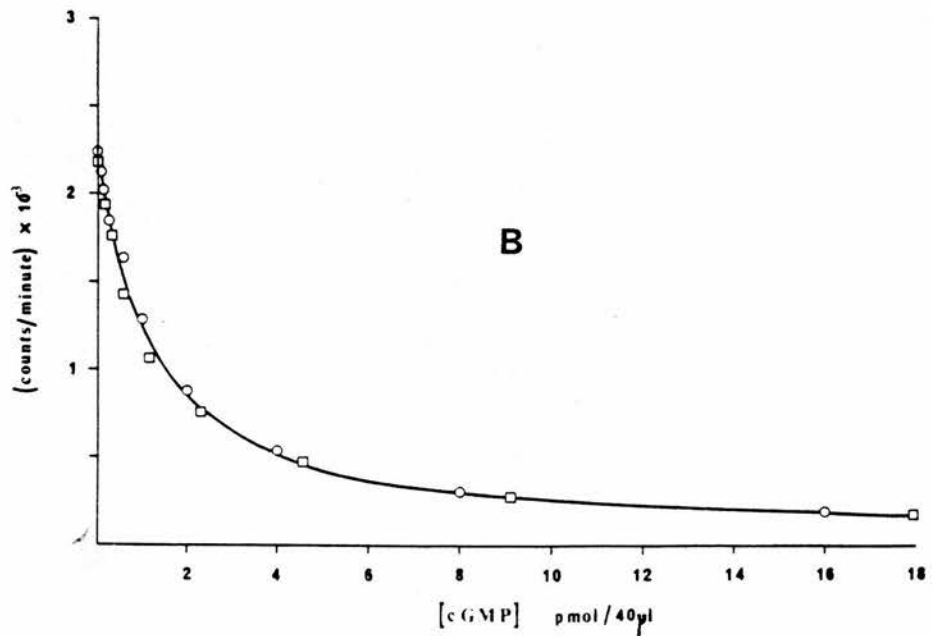
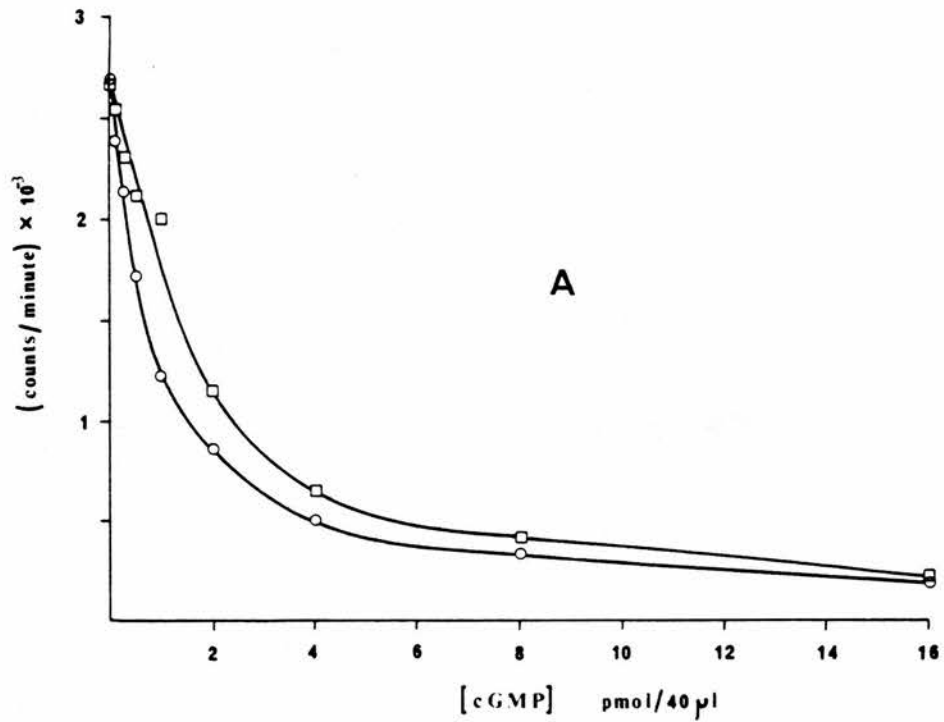


Fig. 2.8 cGMP radioimmunoassay standard curves.

O-O standard solutions added directly to immunoassay.

□-□ standards put through complete TCA extraction and purification procedures.

A. Freeze dried standards resuspended and used immediately.

B. Freeze dried standards resuspended and then refrozen and thawed before use.

5. Iodination and purification of hormones

For a review of most of the possible techniques available for iodination and purification of hormones see Cuatrecasas and Hollenberg 1976.

The chloramine T iodination procedure (Hunter and Greenwood 1962; Greenwood et al. 1963; Greenwood 1971) was used for all protein iodinations. Slight modifications to the original procedures were employed to reduce the extent of multiple iodination of the sample molecule (Freychet et al. 1972). All solutions were prepared fresh before each iodination. Hormone solutions were made up with 0.01M HCl giving the following concentrations:

Insulin	0.5 mg/ml	86 nmol/ml
Glucagon	2.2 or 0.22 mg/ml	630 or 63 nmol/ml
APP	0.33 mg/ml	79 nmol/l.

Iodination was carried out in a small glass tube in as small a volume as possible by adding 10 μ l of the hormone solution to 20 μ l of 0.25M sodium phosphate buffer pH 7.4 containing 1 mCi of radioactive iodide (Na^{125}I IMS.30; 100 μ Ci/ μ l-63 nmol/ml). Iodination was initiated by adding 10 μ l of chloramine T (2.5 mg/ml) and then after shaking gently terminated 15 seconds later by the further addition of 100 μ l sodium metabisulphite (0.5 mg/ml). Both the chloramine T and sodium metabisulphite solutions were made up with 50 mM phosphate buffer pH 7.4. Carrier protein was then introduced by the addition of 200 μ l of 50 mM sodium phosphate buffer pH 7.4 containing potassium iodide (5 mg/ml), bovine serum albumin-radioimmunoassay grade (2.5%) or gelatine (0.25%).

Under the above conditions approximately 80% of the total radioactive iodide was incorporated into protein as judged by chromatoelectrophoresis, PCA or TCA precipitation and dialysis (see later sections).

Purification procedures The following column purification methods were used to isolate the iodinated hormone.

a) ^{125}I -insulin

Gel filtration Sephadex G25 or G50 (fine grade) was swollen in 50 mM phosphate buffer pH 7.4 containing 0.25% gelatine, and packed in a 8.0 x 1.5 cm glass column. Columns were prewashed with 5 ml of buffer containing 250 mg BSA (RIA grade) and finally with 20 ml of original buffer. This helped minimise irreversible adsorption of iodinated hormone to the column. The iodination mixture was applied and eluted in 0.5 ml fractions with gelatine containing buffer. After recovery of the protein peak, buffer containing 2% BSA or 2% horse serum was passed through the column to facilitate rapid recovery of remaining radioactive iodide.

Adsorption columns Viable insulin binds in a specific manner to a number of inert supports including talc and pure cellulose powders (Cuatrecasas and Hollenberg 1976; Freychet 1977). Adsorption purification procedures utilise insulin's ability to bind to the column while other iodination products, including any damaged insulin, are eluted.

Talc or cellulose was soaked in 30 mM barbitone buffer pH 8.6 and after removal of fines poured to a height of 2.5 cm in a glass pasteur pipette plugged with non-absorbent glass wool. The iodination mixture was then applied and the column washed with 6 x 1.0 ml aliquots of barbitone buffer to elute unwanted reaction products. Viable ^{125}I -insulin was then eluted with 4 ml of 50 mM phosphate buffer pH 7.4 containing 24% BSA (RIA grade).

b) ^{125}I -Glucagon

Gel filtration Method of glucagon purification carried out exactly as with insulin.

Adsorption columns As with insulin, but with the following modifications and additions.

Talc was not used as an inert support. Pure cellulose columns were eluted with 2 ml barbitone buffer and 2 ml distilled water. ^{125}I -glucagon

was then collected with 2 ml 25 mM phosphate buffer pH 7.4 in 50% ethanol. Iodinated hormone was eluted into tubes containing 50 mM phosphate buffer pH 7.4 and 1% BSA (RIA grade) as carrier protein.

DEAE cellulose columns were also used to isolate the monoiodoglucagon product of the iodination (Nottey and Rosselin 1971). Iodinations were carried out using a glucagon to iodide ratio of 10:1 to prevent multiple incorporation of iodide into the same glucagon molecule. DEAE cellulose (Whatman DE52) was equilibrated with a 50 mM tris-HCl buffer containing 7M urea pH 9.3 at 4°C and packed in a 0.9 x 22 cm Pharmacia column under a flow rate of 36 ml/hr. The iodination mixture was added to 5 ml of running buffer and applied to the column. The column was eluted at 36 ml/hr. After a column volume (approx. 12 ml) of buffer had been collected, a 100 ml linear salt gradient up to 0.2M NaCl was applied and 2 ml fractions were collected. ^{125}I -Glucagon eluted from the column with the majority of the radioactivity in one peak (Fig. 2.9). The main fractions were pooled and urea was removed by gel chromatography using Sephadex G25 (fine grade) in a 2.0 x 22 cm column, using 50 mM tris HCl pH 7.4 containing 0.25% gelatine for elution.

DEAE cellulose chromatography of an iodination mixture using equimolar glucagon to iodide results in three peaks of eluted radioactivity (Fig. 2.10). The second and third peaks indicate an increase in negative charge on the protein thus requiring a higher ionic strength to elute from the column. An increased negative charge on the protein is indicative of multiple incorporation of iodine into tyrosine residues thus lowering the pK_a of the amino acid from 9.11 to 6.48 (Weast 1977). It therefore seems most likely that peaks 2 and 3 correspond to the di- and tri-iodinated species of the hormone. The relative ratios of the three iodinated species mono:di:tri are approximately 5:4:1 respectively. Assuming 80% of total iodide is incorporated into protein the ratio of

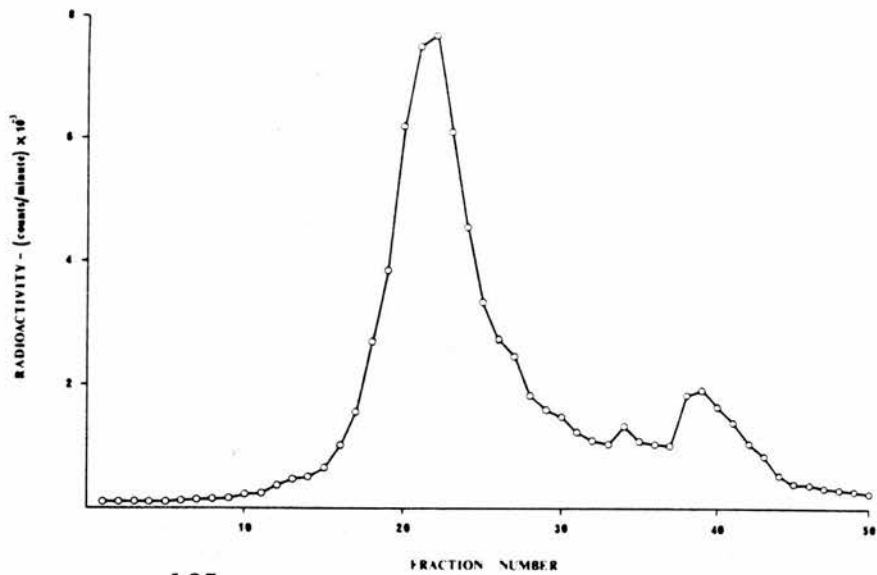


Fig. 2.9 Elution of ^{125}I -glucagon from DEAE cellulose. A 100 ml linear NaCl gradient up to 0.2M applied after fraction 6 and 2 ml fractions collected. Iodination reaction with glucagon:iodide ratio of 10:1.

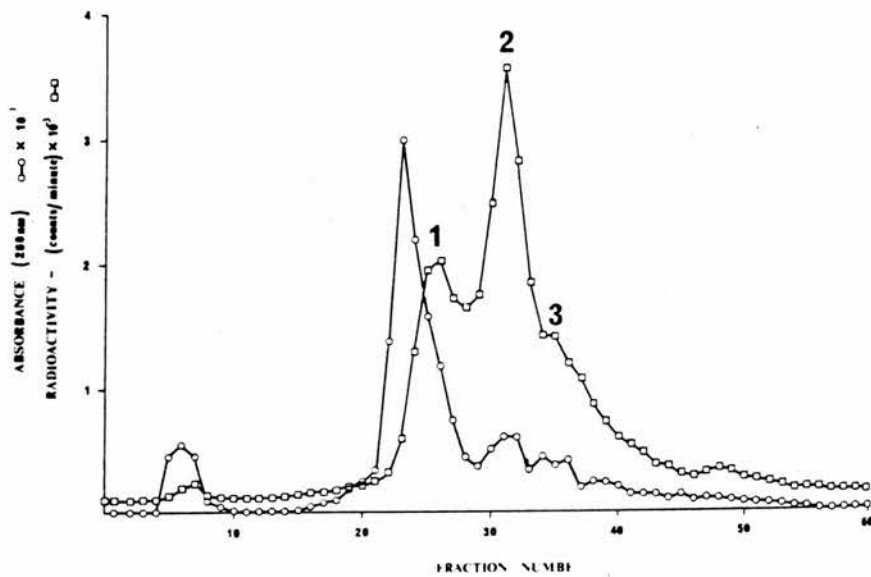


Fig. 2.10 Elution of glucagon and ^{125}I glucagon from DEAE cellulose. A 100 ml linear NaCl gradient up to 0.2M applied after fraction 10 and 2 ml fractions collected. A sample of iodination mixture - with glucagon:iodide ratio of 1:1 was added to a native glucagon solution and applied to the column. Radioactivity (\square - \square) and absorbance at 280 nm (O-O) shown.

iodinated to noniodinated hormone is approximately 1:1. The DEAE cellulose purification procedure gives poor separation of the individual glucagon species however and therefore when iodination reactions are carried out using a 10:1 ratio of glucagon to iodide there must be a substantial, but unknown amount of contamination of the monoiodoglucagon product by the non-iodinated species. The non-iodinated glucagon that is still present after removal of the urea on gel filtration columns thus lowers the final specific activity of the iodinated glucagon from the theoretical calculated value with one iodide atom per glucagon molecule. If high specific activities of iodinated hormone are required for investigation of very low levels of hormone binding then a much more effective separation of the individual species is necessary.

c) Avian Pancreatic Polypeptide (APP)

Gel filtration. Carried out using Sephadex G25 (fine grade).

Procedure the same as with insulin and glucagon.

Adsorption columns. Although this hormone did adsorb to inert supports (as do glucagon and insulin) there is no published method suggesting the use of this technique as a purification procedure. As no biological assay or radioimmunoassay was available to estimate the viability of the iodinated hormone, this type of purification procedure was avoided.

d) Storage

Purified iodinated hormones were apportioned into small tubes and kept at -20°C before use. Labelled insulin and APP retained the same behaviour when subjected to chromatoelectrophoresis, acid precipitation, dialysis and also gave similar binding responses to isolated cells, for up to one month of storage apart from the reduction in specific activity due to radioactive decay. However, by measurement of the same parameters, labelled glucagon showed signs of damage after two weeks of storage at -20°C . It is not known if this is due to some factor particular to the iodinated glucagon molecule itself or to the high ethanol content of the carrier buffer.

6. Methods of Radio-ligand Assessment

a) TCA or PCA precipitability for assessment of ^{125}I bound to protein. Aliquots of eluates from column purifications were diluted one hundred fold with 50 mM phosphate buffer containing 1% BSA and were added to an equal volume of 10% TCA or 6% PCA at 4°C . Samples were left for 15 min and centrifuged (2750 g, 30 s). The supernatant was sampled for radioactivity in order to assess the free iodide. This was routinely less than 10% of the total radioactivity. However, using the same buffer a variable percentage of a sample of $^{125}\text{I}^{-}$ was also shown to precipitate with PCA or TCA treatment. Sometimes as much as 20% of the radioactive free iodide could be precipitated with the carrier BSA, and less than half of this could be removed by extensive washing of the denatured pellet with 50 mM phosphate buffer pH 7.4.

b) Dialysis. Eluted fractions were again diluted one hundred fold by 50 mM phosphate buffer pH 7.4 containing 1% BSA. Samples were extensively dialysed at 4°C against several changes of 50 mM phosphate buffer. At least 95% of the radioactivity was retained by the dialysis tubing. If BSA was omitted a significant proportion (up to 40% after 48 hr) of the radioactivity adsorbed irreversibly to the dialysis membrane.

c) Binding to talc. The percentage of the iodinated protein which binds to talc gives an estimation of the viability of the tracer (Cuatrecasas and Hollenberg 1975). Talc (25 mg) was added to 1 ml of a suitably diluted protein sample. The bound radioactive count was proportional to the viability of the radioactive tracer, as judged by the other methods of assessment, but was also dependant on the concentration of carrier BSA in the sample. An increase in carrier BSA concentration reduced the total tracer bound to talc. Excessive amounts of talc (up to 0.5 g) partially overcame the effect of BSA. Up to 70% of radioactive tracer would bind to talc (25 mg) on the presence of 0.25% BSA in the diluent buffer.

d) Paper chromatoelectrophoresis - Whatman 3MM filter strips (2.5 x 35 cm) were soaked in 30 mM barbitone buffer pH 8.6 and samples (10 μ l) of iodinated hormones applied to a pencil line drawn 5 cm from one end. Strips were then placed in a Shandon chromatoelectrophoresis tank containing the same barbitone buffer and electrophoresis was carried out for 1 hour at 1.5 mAmp/strip (220-300 volts). Strips were then removed dried in an oven and cut into 1 cm segments for assessment of radioactivity in the Tracerlab gamma counter.

e) Radioimmunoassay of 125 I-insulin. Six different anti-sera for insulin, raised in guinea pigs, were obtained from Wellcome Laboratories. The double antibody technique (Yalow and Berson 1966) was used for dilution titration of antiserum and subsequent determination of maximum percentage binding of radioactive tracer.

Assay was carried out in Luckham LP3 tubes and using a 50 mM phosphate buffer pH 7.4 containing 0.5% BSA (RIA grade) and 0.1% NaN_3 as diluent. A suitably diluted iodinated insulin sample (0.2 ml) was added to an incubation medium containing guinea pig anti-insulin serum (AIS-GP dilution 1:5000) and normal guinea pig serum (NGPS - diluted 1:500) in a volume of 0.5 ml diluent buffer. Separate control tubes also contained unlabelled hormone (5 μ g/ml) to estimate non-specific binding. Tubes were shaken and incubated for 24 hours at 4 $^{\circ}$ C. Anti-guinea-pig serum (AGPS) (0.3 ml) diluted 1:12 or 1:16 with diluent buffer was then added and the tubes shaken and incubated for a further 18 hours at 4 $^{\circ}$ C. Antibody bound tracer was separated by filtration on Oxoid cellulose filters pore size 0.45 μ m. The percentage maximum binding of 125 I-insulin varied from 57 to 85% depending on which antibody was used.

7. Cell Incubations

Cell suspension prepared from liver perfusion (Chapter 3) was diluted to give 5 to 20 x 10 6 cells/ml in normal Krebs Ringer Bicarbonate buffer

(KRB). When using the hepatocytes for cAMP or cGMP determinations the cell suspension was preincubated for 15 minutes at 37°C with constant gassing with O₂/CO₂ in a circular shaking water bath. This allowed the elevated cyclic nucleotide levels due to the extraction procedure to stabilise. When the hepatocytes were used for all other parameters the cell suspension was used immediately.

Cell suspension (0.5 ml) was added to a 7.6 x 2.5 cm plastic incubation pot fitted with an air tight cap, containing 1 ml of KRB pre-equilibrated with 95% O₂/5% CO₂. If required, a concentrated hormone solution or metabolic substrate was also previously added in a small volume (10-50 µl). Pots were then gassed with O₂/CO₂, caps fitted and incubated at 37°C in a reciprocal shaking water bath for the desired time. After the incubation period the pots were removed and immediately placed on ice for further processing.

8. Glucose/Glycogen Determination

Cell suspension was centrifuged (2750 g, 30 s) and a sample of supernatant (0.2-0.5 ml) removed for the determination of glucose by the glucose oxidase method using 4-aminophenazone as the chromogenic oxygen acceptor in place of o-dianisidine (Hugget and Nixon 1957). A stock reagent mixture was made up containing 4-aminophenazone (0.3 mg/ml), phenol (1.0 mg/ml), glucose oxidase (9 mg/100 ml) and peroxidase (3 mg/100 ml) in 10 mM sodium phosphate buffer pH 7.4, which was stable for up to 1 month when stored at 4°C. Aliquots of this reagent (2 ml) were added to 0.2 to 0.5 ml of the unknown samples and a series of suitably diluted glucose standards (0-100 µg/ml), mixed thoroughly and incubated at 37°C for 40 minutes to allow the colour to develop. Absorption was read at 510 nm using a Cecil spectrophotometer. The absorbance was linearly proportional to glucose concentration over the range used. Deproteinisation of the unknown samples with TCA or PCA before glucose estimation was not necessary.

The glycogen content of the cells was determined by digesting the remaining cell pellet with 1 ml 30% KOH for 30 minutes at 100°C. Samples were allowed to cool to room temperature and 0.4 ml of 2% Na₂SO₄ followed by 2.8 ml 96% ethanol were added. Samples were left overnight to allow maximum precipitation of glycogen. The precipitate was washed twice in 70% ethanol, hydrolysed in 1 ml 1M H₂SO₄ for 3 hr at 100°C, allowed to cool and then neutralised by the addition of 1 ml 0.1M sodium phosphate buffer pH 7.4 and 1 ml 2M NaOH. Aliquots of the digest (0.4-0.8 ml) were used for glucose determination. The glycogen content of the cells was expressed in µg glucose equivalents per 10⁶ cells. Recovery of a series of standard glycogen samples subjected to the above procedure was nearly 100% and therefore freshly prepared glucose solutions were used as standards.

9. Lipogenesis

Cell suspension was incubated with various acetate concentrations containing a tracer amount of [¹⁴C]acetate. After incubation, cell suspension (1 ml) was removed and centrifuged (2750 g; 30 s) to sediment cells. Lipid extraction of the pellet was by the method of Folch et al. (1957). The pellet was resuspended in 5 ml 2:1 chloroform methanol (v/v) and vigorously shaken. Material was centrifuged once more (2750 g; 1 min), pellet discarded and 1 ml of a 0.04% CaCl₂ solution added to the supernatant. After vigorous shaking the upper aqueous layer was removed and the lower organic layer washed three times with 0.5 ml of "pure solvents upper phase" (see below), discarding the aqueous layer each time. The chloroform was then removed by evaporation and the triglyceride pellet dissolved in 10 ml of scintillant (see method 14) for radioactive determination of label incorporated. Lipogenic activity was then expressed in µmole acetate incorporated per 10⁶ cells.

'Pure solvents upper phase' (PSUP) was prepared as described by Folch et al. (1957). Chloroform, methanol and a 0.04% CaCl_2 solution in the proportions 8:4:3 respectively were mixed in a separating funnel by vigorous shaking. The mixture was left to separate into two layers and the upper layer removed for use in the assay.

10. DNA and Protein Determination

Cell suspension was centrifuged (250 g, 1 min) and pellet washed 3 times with, and finally resuspended to the original volume in isotonic NaCl. Samples were frozen at -20°C for DNA and protein determinations.

Protein was measured by the method of Lowry et al. (1951) and DNA determined by the diphenylamine reaction of Burton (1956).

11. Dry Weights

The dry weight of the cells was determined by pipetting triplicate 1 ml samples of cell suspension and of KRB buffer into tared vials. Samples were dried to constant weight and weight of dried cells determined by difference from the two groups of vials.

12. Oxygen Consumption

Oxygen consumption was measured at 37°C with a Clark type oxygen electrode giving a direct readout to a Servoscribe chart recorder. The recorder was calibrated with fully oxygenated KRB buffer and with buffer deoxygenated by addition of sodium dithionite. Cell suspension (0.5 ml) was added to 3.5 ml of gassed KRB buffer in the water jacketed vessel. Suspension was stirred continuously by a small magnetic stirrer and the oxygen content of the buffer monitored. At 37°C the maximum oxygen content of KRB gassed with 95% O_2 /5% CO_2 is 1.071 mM. This figure is calculated from the Bunsen coefficient (α), of 0.024 for a dilute salt solution at 37°C (Dawson et al. 1969). Due to oxygen leakage from the chamber a blank rate was also taken. Consumption of oxygen was expressed as $\mu\text{mol/hr}/10^6$ hepatocytes or $\mu\text{mol/hr/g}$ dry weight.

13. Electron Microscopy

Albumin was used as a carrier for fixing and embedding of the isolated cells (Farrant and McLean 1969).

A sample (5 ml) of concentrated cell suspension (20×10^6 cells/ml) was centrifuged (50 g; 1 min) and resuspended with 2 ml gassed KRB buffer containing 10% BSA. A sample of this suspension (0.5 ml) was placed on a microscope slide which had been previously silicone treated with "Repelcote", and 170 μ l of a 9% glutaraldehyde (v/v) solution in KRB added and mixed thoroughly. The sample was allowed to stand at room temperature for 5 minutes until gelling occurred and then cut into approximately 1 mm squares. The diced sample was transferred to a vial containing 3% glutaraldehyde (v/v) in KRB buffer and left for 1 hour at 4°C. The gel sections were then washed three times with ice cold 0.1M sodium cacodylate buffer pH 7.4 and resuspended in 1% OsO_4 (w/v) in 0.1M cacodylate buffer for 45 minutes at 4°C. The gel cubes were then washed in four or five changes of cacodylate buffer prior to dehydration in ethanol. Embedding, sectioning, lead citrate and uranyl acetate staining was carried out by Mr. I.D. Sneddon, Department of Anatomy, Royal (Dick) School of Veterinary Studies, Edinburgh.

14. Scintillant solutions

The following scintillant cocktails were universally used for all ^{14}C and ^3H determinations. The scintillant cocktail for the phosphodiesterase assay comprised a mixture of toluene (625 ml) and 2-methoxyethanol (375 ml) containing 4 g 2,5-diphenyloxazole (PPO) and 100 mg 1,4-Di 2-(5-phenyloxazolyl) benzene (POPOP). All other assays using scintillant used the same amounts of the fluors but in 1000 ml toluene plus 300 ml 2-methoxyethanol or 2-ethoxyethanol.

15. Sodium Dodecyl Sulphate (SDS) Polyacrylamide Gel Electrophoresis of Collagenase

SDS gel electrophoresis (Payne 1976 - review) was used to separate the constituents present in the different batches of crude collagenase obtained from Boehringer Ltd. 7.5% acrylamide 0.2% methylenebisacrylamide cylindrical gels containing 0.1% SDS were made up in 75 mM tris-glycine buffer pH 8.6. Gels were prerun in a Shandon electrophoresis tank for 1 hr at 2 mAmp/tube and then 20 μ l of the prepared collagenase solution containing 800 μ g was added. Gels were run for 3½ hours at 3 mAmp/tube and then stained in Coomassie Brilliant Blue. Gels were scanned using a Gilford gel scanner and the protein peaks recorded by Servoscribe recorder.

Sources of Materials Used

Except as indicated below, biochemicals were obtained from Sigma (London) Ltd., laboratory reagents were of AnalaR quality (B.D.H., Ltd.), enzymes were obtained from Boehringer Ltd. and radiochemicals from The Radiochemical Centre.

Insulin, glucagon and calcium ionophore A23187 were obtained from Eli Lilly & Co. Ltd., Indianapolis, U.S.A.

Avian pancreatic polypeptide was a kind gift from Dr. J.R. Kimmel, University of Kansas.

Boehringer collagenase - batch nos. used, 9, 13, 15, 17, 21, 25, 27, 29, 30.

Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex.

CHAPTER 3

PREPARATION AND PROPERTIES OF ISOLATED

CHICKEN HEPATOCYTES

Introduction - historical development of cell isolation

Early experimental techniques used to study the control of hepatic metabolism employed the use of isolated perfused livers (Mortimore 1961; Sokal et al. 1964; Hems et al. 1966; Ross et al. 1967; Exton and Park 1969; Exton et al. 1971b) or liver slices (Ashmore 1964; Krebs et al. 1966; Miller et al. 1974). Both systems remove the hepatic tissue from the complex interactions between the different organs of the body, thus allowing direct studies on liver metabolism to be made. Using the former technique it was found that many of the metabolic characteristics found in vivo were retained, including hormone sensitivity, adenylate and potassium contents, cell-cell interactions and retention of cytosolic enzymes. However using this method had several major disadvantages. Due to the size, density and the high respiratory rate, it was very difficult to keep the tissue adequately oxygenated and unless red blood cells were also incorporated into the perfusion medium the tissue very quickly became hypoxic, leading to cell deterioration induced by autolytic digestion. Another disadvantage of this type of system was the few experimental conditions which could be imposed on the liver during each experiment. In order to study a variety of related effects or conditions a large number of livers had to be isolated. Due to individual variations between animals it was often difficult to correlate the results using different livers. Liver slicing partially overcame the disadvantages of perfused whole liver in that many different conditions could be studied from tissue isolated from the same liver. Even allowing for slight variations in composition between slices this method theoretically allowed more accurate comparisons between effectors to be made. If slices were cut thin enough (theoretically one cell thick) adequate oxygenation could be supplied without the need for erythrocytes in the medium. However the various experimental difficulties encountered with this method

prevented its widescale use. Slicing the liver causes a great deal of damage to cells resulting in the release of cellular constituents, including the proteolytic enzymes. Slices could not be guaranteed of uniform thickness and if too thick often the cells towards the interior of the section became hypoxic and tissue degeneration quickly ensued. Metabolic rates were found to be lower than in whole liver perfusion due to the loss of essential cellular components on slicing (Ross et al. 1967; Krebs 1970). Although tissue slicing resulted in a more versatile method which allowed a more varied experimental approach, the quality of the tissue was poor and often resulted in inaccurate measurement of various parameters.

The difficulties found with liver slicing or whole liver perfusion induced many workers to investigate complete tissue dispersion resulting in the production of intact single cells. This type of system was much more versatile than that of liver slicing but again the major problem was in the difficulty of obtaining a technique which could assure the release of large numbers of metabolically viable cells. Early methods were based purely mechanical dispersion of cells by loose homogenizers (Potter and Elvehjem 1936; Berry 1962; Howard and Green 1965) which resulted in broken cell preparations. Pre-perfusion of the liver with calcium chelating agents, which reduced intercellular adhesion, helped cell recovery, but generally yields were very low with extensive damage to the cells (Anderson 1953; Jacob and Bhargava 1962; Takeda 1964). The digestion of the extracellular connective tissue by the enzyme collagenase was demonstrated in the first preparation of fat cells from rat adipose tissue by Rodbell (1964a,b). The isolated cells retained their metabolic integrity with identical or increased hormone sensitivity to that of the complete tissue. Enzymatic dispersion of the liver was first demonstrated using the two enzymes collagenase and hyaluronidase

in a calcium free buffer (Howard et al. 1967). After the initial perfusion of the liver with 10 ml ice cold enzyme solution tissue slices were then incubated in the enzyme solution for 1 hour at 37°C. The original method has since been modified resulting in improvements in cellular morphology and metabolic activity (Howard and Pesch 1968; Howard et al. 1973) but still necessitates slicing of the liver before complete digestion of the connective tissue. Although the damaged cells and other debris resulting from the slicing can be removed after incubation by low speed centrifugation procedures, the intact cells are still exposed for up to one hour at 37°C to the proteolytic enzymes released from the large numbers of damaged cells and thus not only is the cell yield low but the hormone sensitivity of the isolated hepatocytes may be affected due to receptor degradation.

The liver perfusion system of Mortimore (1961) was used by Berry and Friend (1969) for the first isolation of cells by a recirculating type perfusion which did not necessitate liver slicing. This method also reduced the length of time the liver was exposed to the digesting enzymes. Rat liver was continually perfused in situ with the collagenase-hyaluronidase enzyme solution and the perfusate was then returned to a reservoir for reoxygenation before being used again. As a result, high yields of metabolically active and hormonally sensitive cells were produced. This method has now been universally accepted and used by many other workers with only slight modifications to isolated hepatocytes from various sources (Capuzzi et al. 1971; Seglen 1973; Capuzzi et al. 1974; Cornell et al. 1974; Krebs et al. 1974; Arinze and Rowley 1975; Staichfield and Yagar 1978; Goldfarb et al. 1978).

The preparation of isolated hepatocytes has many advantages over the other methods used to study various aspects of liver metabolism especially those under hormonal control. The isolation procedure purifies the parenchymal cells from the erythrocytes and other sinusoidal cells (non-parenchymal cells) and thus a direct study on the hepatocytes can

be made. Other techniques have been developed from Berry and Friend's perfusion method to preferentially isolate the Kupffer and other sinusoidal cells such as endothelial cells, fat storing cells and pit cells, while digesting the parenchymal cells with pronase (Knook et al. 1977; Wisse and Knook 1977). Therefore different populations of cells within the liver are now accessible and can be purified from most other cell types.

Isolated hepatocytes can be easily oxygenated and supplied with metabolic precursors or hormones without the need for diffusion through surrounding tissue. The ease of handling allows for a large number of variables to be tested on the same preparation of cells and due to the homogeneity of the suspensions very accurate comparisons can be made between different conditions.

Viable hepatocytes can be kept in fortified media as non-proliferating monolayer cultures for up to several days (Bissell et al. 1973; Laishes and Williams 1976; Goodridge and Adelman 1976) although after several weeks in culture cells show transformed characteristics and columns of proliferating cells are formed (Borek 1972; Williams and Gunn 1974). Longer term experiments are therefore possible for up to a few days after isolation of the cells.

The main disadvantage in cell isolation is the possible subtle damage done to cell surface components which could lead to altered cell interactions with solutes in the external medium. Gross morphological damage to the cell is more easily recognised by various techniques including trypan blue staining of damaged cell nuclei, leakage of cytosolic enzymes, rates of endogenous oxygen consumption, ion and total adenylate content as well as examination of intracellular fine structure by electron microscopy. The extent of the more subtle damage to the cell surface is more difficult to determine. Hormonal sensitivity is one

possible method of estimating this type of damage and therefore gives a reasonable indication of the viability of the hepatocyte. If the isolated cells show the hormone sensitivity of the whole tissue to various metabolic responses then this is reasonable evidence for little damage to the external components of the plasma membrane and thus little damage to the cell as a whole. Various other possible disadvantages using this system are often overlooked. Hepatocytes isolated by this method are now free of cell-cell contact which may be important in the intercellular regulation of metabolism. The Kupffer and other liver sinusoidal cells have been greatly reduced in number if not completely removed from cell suspensions. It is unknown if they impose any regulatory effects on the surrounding hepatocytes in the liver. Digestion of the hepatic tissue with collagenase may select particular parenchymal cell populations within the liver. Thus the isolated cells may be uncharacteristic of the parenchymal cells as a whole. Certain specific cell receptors or carrier proteins which are present in the plasma membrane may be more sensitive to collagenase digestion than others, therefore imposing a regulatory imbalance on metabolism when compared to whole tissue. The metabolic shock suffered by the isolated hepatocytes by anoxia and loss of ion gradients due to the harsh isolation procedures may have marked effects on metabolism. Rat liver cells take up to 4 hours after isolation before various physical and metabolic parameters stabilise (Goldfarb et al. 1978). It is possible to prepare non-proliferating monolayer cultures of rat hepatocytes (Bissel et al. 1973; Laishes and Williams 1976; Goldfarb et al. 1978) and chicken hepatocytes (Tarlow et al. 1977) which allows longer term experiments of up to a few days to be carried out. However these cultures cannot be maintained indefinitely and the general metabolic functions of the cells are gradually lost. If and when it becomes possible to culture colonies of replicating cells which

do not display any transformed characteristics many of these disadvantages will not apply. The percentage of original cells isolated from the liver will become increasingly small and therefore any irreversibly modified characteristics induced by the tissue digestion will be diluted out. With the growth of monolayer cultures cell-cell contact is re-established and adequate oxygenation and accessibility to metabolites maintained. Various liver sinusoidal cells can also be added back onto the culture medium and their effects on parenchymal cell metabolism, if any, noted.

Until it becomes possible to use untransformed hepatocyte cell lines it is essential that the above mentioned factors are recognised and must be considered when interpreting any results.

Preparation of Hepatocytes

During the period of study two different procedures were used to isolate chicken hepatocytes. The initial method used (Method I) was based on the procedure of Howard et al. 1973 as modified for the chicken (Dickson and Langslow 1975; Dickson 1977; Dickson et al. 1978). This method was used for most of the experiments using caffeine and theophylline as outlined in Chapter 6. All other experiments employed the use of a recirculating type perfusion (Method II) modified from the original procedure of Berry and Friend (1969) for use with the chicken.

Method I

Isolation of hepatocytes was carried out using 'method B' reported by Dickson 1977. The chicken liver was perfused in situ with calcium free Krebs-Ringer bicarbonate (KRB) buffer (Krebs and Henseleit 1932) at 4°C using a reservoir of two 10 ml syringe bodies connected by a three way tap and tubing to a number 19G syringe needle. The reservoir was clamped at a height of 40 cm above the worktop and perfusion carried out under gravity. The KRB buffer was fully oxygenated by equilibrating

with 95% O₂/5% CO₂ till pH 7.4. Bovine serum albumin (BSA) 1% (w/v) was added to the buffer to protect the isolated cells by preventing clumping and denaturation on the walls of the preparation vessels and to bind any toxic substances released during the digest and purification procedures (Krebs et al. 1974).

The chicken was killed by cervical dislocation. The skin was then cut away at the inner top of both thighs and the legs dislocated and restrained. The skin was again cut near the pelvic region and stripped forward exposing the sternum and surrounding musculature. An incision was then made at the tip of the keel and the rib cage opened by cutting down both sides to the backbone. The rib cage was then pulled upwards to expose the liver. Holding the gall bladder with blunt forceps the bile ducts were eased free from the portal vein and then cut. Still holding the gall bladder the portal vein was eased up out of the body cavity free of the surrounding connective tissue and fat depots by using fine pointed forceps. The needle of the perfusion apparatus was inserted into the portal vein and the inferior vena cava cut. Immediately the liver was perfused in situ with 10 ml of ice cold Ca²⁺ free KRB. Due to the rapid clotting of chicken blood on some occasions portions of the liver did not blanch with this initial flush through of buffer. Gentle massaging of the liver sometimes succeeded in dispersing the blood clot. The three way tap on the reservoir was then turned to allow perfusion to continue by adding 50 ml of ice cold Ca²⁺ free KRB buffer containing collagenase (0.15-0.25 mg/ml) to the other syringe body. All perfusate was allowed to run to waste after passing through the liver.

The liver was then excised free of the body cavity, the connective tissue and liver capsule removed and then minced with scissors. The minced tissue was then incubated with 20 ml Ca²⁺ free KRB buffer for 5 minutes at 40°C in a circular shaking water bath (100 cycles/min) with

constant gassing with O_2/CO_2 . The digest was then filtered through coarse nylon mesh, returned to the shaking water bath for a further 2 min and refiltered through fine nylon mesh into 30 ml of ice cold Ca^{2+} free KRB. The cell suspension was then centrifuged at 335 g for 5 minutes, the pellet being resuspended in 20 ml of calcium free buffer by pasteur pipette and recentrifuged at 60 g for 2 min. This procedure removes nearly all of the non-parenchymal cells from the suspension but maintains a good yield of hepatocytes (Dickson 1977). The final pellet was then resuspended in the required volume of KRB buffer containing calcium. A sample of cell suspension (0.2 ml) was diluted with 0.1% trypan blue in isotonic KCl (0.8 ml) and cell number and viability estimated using a counting chamber. The remaining suspension was diluted accordingly and used for experiments.

Method II

By use of a water jacketed reservoir and organ chamber (fig. 3.1) all perfusion procedures were maintained at $37^{\circ}C$. All buffers used were pre-equilibrated to this temperature beforehand. Subsequent isolation and purification procedures were also carried out as close to $37^{\circ}C$ as possible although exposing the liver or liver digest to lower temperatures was not detrimental to the isolation procedure.

With the clamp on the perfusion apparatus closed the reservoir was filled with 60 ml of Ca^{2+} free KRB buffer and the O_2/CO_2 gassing adjusted to give a steady stream of bubbles. Overgassing resulted in frothing of the BSA containing buffer and was therefore avoided. Initial stages of the preparation were the same as with method I until the portal vein was eased up free of the body cavity. A 25 cm length of silk suture (Mersilis-Sutupak, Ethicon Ltd.) was placed under the portal vein ready to tie in the needle. The needle was then inserted into the vein and perfusion started immediately on cutting the inferior vena cava.

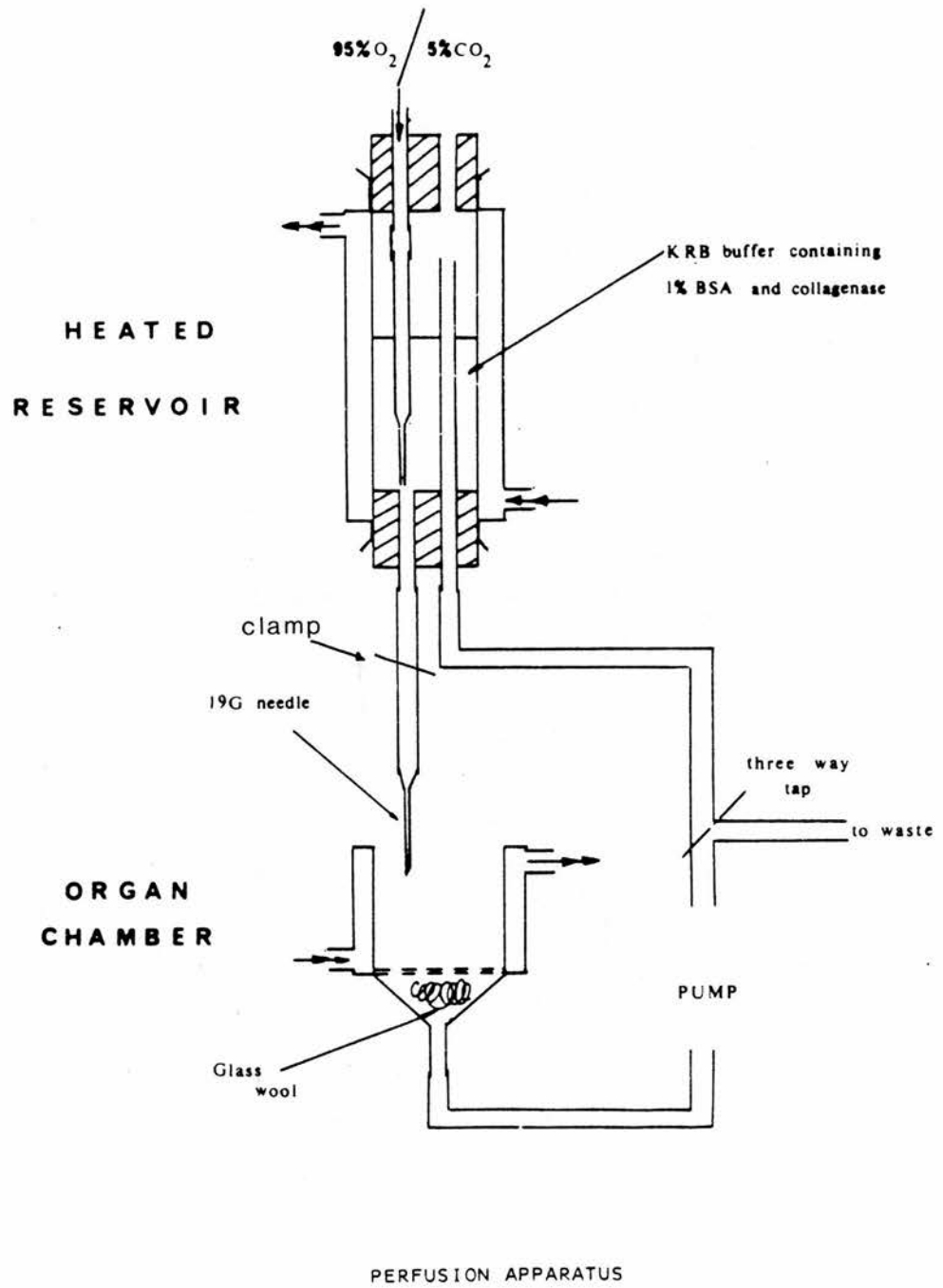


Fig. 3.1

As the liver was perfused in situ with 40 to 50 ml of the reservoir buffer the needle was tied firmly in place with the suture. A good perfusion was indicated by an even blanching of the liver as the blood was flushed out. Since the perfusing buffer was close to 37°C the rate of blood clotting was much quicker than with method I, but if the procedure was carried out quickly enough the liver blanched evenly. Perfusion was then stopped by closing the clamp on the perfusion apparatus. Due to the anatomical features of the chicken it was very difficult to adopt the recirculating perfusion method if the liver remained in situ and it was therefore removed from the body cavity. To facilitate easy removal of the liver a further excision was made through the backbone and skin allowing the head and wings of the bird to be removed from the lower half of the body. The liver, gall bladder and spleen were then carefully excised from the body cavity and placed in the organ chamber and perfusion restarted. Leakage of the perfusion buffer from the portal vein is usually sufficient to keep the exterior of the liver moist. If not a small amount of Ca^{2+} free KRB buffer was sprayed on the liver. After the remaining 10 to 15 ml of buffer had passed through the liver and allowed to run to waste, 50 ml of Ca^{2+} free KRB buffer containing collagenase (0.2-0.3 mg/ml) was added to the reservoir and the perfusate was then recycled to the reservoir by means of a rotary pump (Watson-Marlow Ltd.). The height of the reservoir above the organ chamber was adjusted to around 40 cm which resulted in flow rates in the order of 15 to 25 ml/min through the liver. The initial oxygen concentration of the gassed KRB buffer (1.071 mM) was reduced to less than 5% after passing through a medium sized liver of 8 g. After perfusion had been carried out for 10 minutes the oxygen content of the buffer in the reservoir was in the region of 65-80% of the original value. Increasing the height of the reservoir did not substantially increase the flow rate through the liver, which was found to depend mainly

on the success of the initial wash out of blood. The buffer was not pumped through the liver as the increase in pressure of the perfusate within the liver itself could cause damage. Slight hypoxia for 10-15 minutes does not appreciably effect the quality of isolated hepatocytes (Capuzzi et al. 1974). After 10 to 15 minutes of perfusion with collagenase containing buffer the liver goes limp in the oxygen chamber. Perfusion was then terminated and both spleen and gall bladder removed from the liver. The liver capsule was stripped off and the digested tissue was then broken up by gentle maceration with scissors and transferred to a weighed siliconised flask containing 20 ml Ca^{2+} free KRB. The flask was reweighed and incubated in the same procedure as method I except that the orbital shaking bath was set at 80 cycles/min, the pellet was finally washed with 80 ml Ca^{2+} free KRB and resuspension of pellets was carried out by continual transfer of KRB buffer between centrifuge tubes rather than by pasteur pipette.

For many preparations a Krebs Ringer bicarbonate buffer containing a high potassium, low sodium content was used where the concentrations of the two cations were interchanged. The normal high sodium buffer was then used after cell isolation for all experimental purposes. The high potassium medium inhibited the breakdown of intracellular glycogen in the hepatocytes (Dickson 1977) possibly by inhibiting phosphorylase activity (Hue et al. 1975). Preparing the cells using a high Na^+ medium greatly reduced the already low glycogen content of the cells thus giving a false representation of the fed state. This could not be overcome by using a glucose containing buffer during perfusion. The use of the high K^+ medium allowed studies to be carried out into the regulation of glycogen breakdown by measurement of glucose release from cells isolated from fed birds. The preparation of the cells in high K^+ medium did not alter any of the other parameters measured with the hepatocytes.

Hepatocytes could be isolated in 30 to 40 minutes ready for experimentation by both methods.

All chickens used were males of the Thornber 909 breed and were 3 to 5 weeks old. All embryonic metabolic characteristics have been lost but sexual development is still at a very early stage (Goodridge 1969). The success rate of perfusion using both methods was found to be around 80% although often certain areas of the liver failed to blanch. After collagenase treatment these unperfused areas were removed before isolation of the hepatocytes. In some cases, particularly when using method I and with livers from older chickens (5 to 6 weeks) low cell yields were obtained due to excision of large portions of unperfused liver. Livers of deep red colouration were more difficult to perfuse than those of lighter colouration. In general, the older the bird used, the deeper the colour of the liver and the lower the success rate of the perfusion. Later it was found that the success rate of the perfusion was dependent on breed of bird. Both the Shaver and Broiler breeds of chicken, of ages 3 to 8 weeks, showed 100% success rates during perfusion with complete blanching of the liver in all cases. The livers isolated from these birds were all of a light colouration and no blood clotting within the hepatic tissue was noted. The reasons for these variations among breeds is unknown.

Assessment of Hepatocyte Viability

Various metabolic and physical parameters were measured in order to assess the viability and stability of the hepatocytes isolated by the two methods (Table 3.1). Cells were initially examined under the light microscope and were found to consist of single cells or cells clumped in small groups of up to 5 or 6. Contamination of parenchymal cells by other cell types including red blood cells was always less than 5%. The exclusion of vital dyes such as trypan blue (Eoskins et al. ¹⁹⁵⁶) gives a

Table 3.1: Characteristics of hepatocytes isolated by the two perfusion methods.

A. Physical and metabolic characteristics

B. Adenylate content

*Results reported by Dickson(1977).

A.

Parameter	Method I		Method II	
	Average value	± S.D. (no. of individual preparations)	Average value	± S.D. (no. of individual preparations)
1. Percentage cells staining with trypan blue immediately after isolation	17.3 ± 7.5	(28)	4.9 ± 2.2	(148)
	*11.1 ± 13.4	(50)		
2. Cell yield				
a) 10 ⁶ cells/g wet weight tissue	56.7 ± 23.5	(22)	147 ± 55	(148)
	*74.5 ± 77.1	(50)		
b) mg dry weight/10 ⁶ cells	*0.34 ± 0.16	(13)	0.32 ± 0.06	(86)
3. Oxygen consumption μmol O ₂ /10 ⁶ cells/hr	*0.36 ± 0.14	(8)	0.268 ± 0.062	(100)
4. Protein content μg/10 ⁶ cells	*122.1 ± 59.5	(20)	109 ± 22.6	(8)
5. DNA content μg/10 ⁶ cells	*5.89 ± 1.83	(19)	5.28 ± 0.95	(6)

Table 3.1 contd.

B.

Parameter	Method I		Method II	
	Average value	+ S.D. (no. of individual preparations)	Average value	+ S.D. (no. of individual preparations)
Adenylylate content pmol/10 ⁶ cells				
a) Immediately after isolation				
ATP	*972	+ 150 (5)	1346	+ 294 (5)
ADP	*1458	+ 458 (5)	1422	+ 353 (5)
AMP	*1353	+ 407 (5)	1695	+ 142 (5)
<u>ATP</u>	*0.35	+ 0.056 (5)	0.43	+ 0.024 (5)
<u>ADP + AMP</u>	*3795	+ 988 (5)	4463	+ 789 (5)
Σ adenylates				
b) After 30 minutes incubation at 37°C				
ATP	-	-	2103	+ 132 (4)
ADP	-	-	1554	+ 323 (4)
AMP	-	-	1207	+ 284 (4)
<u>ATP</u>	-	-	0.76	+ 0.096 (4)
<u>ADP + AMP</u>	-	-	4864	+ 739 (4)
Σ adenylates				

conveniently rapid and reasonably quantitative measure of gross morphological damage to the cell membrane (Howard et al. 1967; Berry 1974). Fig. 3.2 shows the effect of incubation at 37°C on cell concentration and percentage trypan blue staining. However other workers have questioned the significance of trypan blue as a vital stain and suggest measurement of enzyme leakage as a more accurate method. Determination of lactate dehydrogenase activities are routinely used by some laboratories and may well give a more accurate indication of gross structural damage to the hepatocyte membrane. There is however reasonable correlation between measurement of leakage of various cytosolic enzymes and extent of trypan blue stained nuclei (Dickson 1977). A rapid test for intactness of the hepatocytes using the oxygen electrode has been reported by Letko (1978). Due to plasma membrane impermeability, the increase in respiration rate on addition of succinate is used to estimate cell viability. However such measures do not give any indication of other signs of damage to the cell such as the irreversible loss of ion and electrochemical gradients across the plasma membrane nor proteolytic damage to surface components such as hormone receptors which are only reflected in the metabolic competence of the cell. Both measurement of enzyme leakage and trypan blue staining allow direct comparisons of viability to be made between individual preparations of cells but they only serve as a rough indication of metabolic integrity.

Although there were significant differences in the parameters measured between the two methods only slight variations in characteristics were noted when method II was routinely used. There was a correlation between the success of the initial flushing of red blood cells and cell yield and viability. Unlike that reported for method I (Dickson 1977) there was no increase in trypan blue stained cells nor decrease in cell yield when using livers from 24 hour starved birds. Different crude

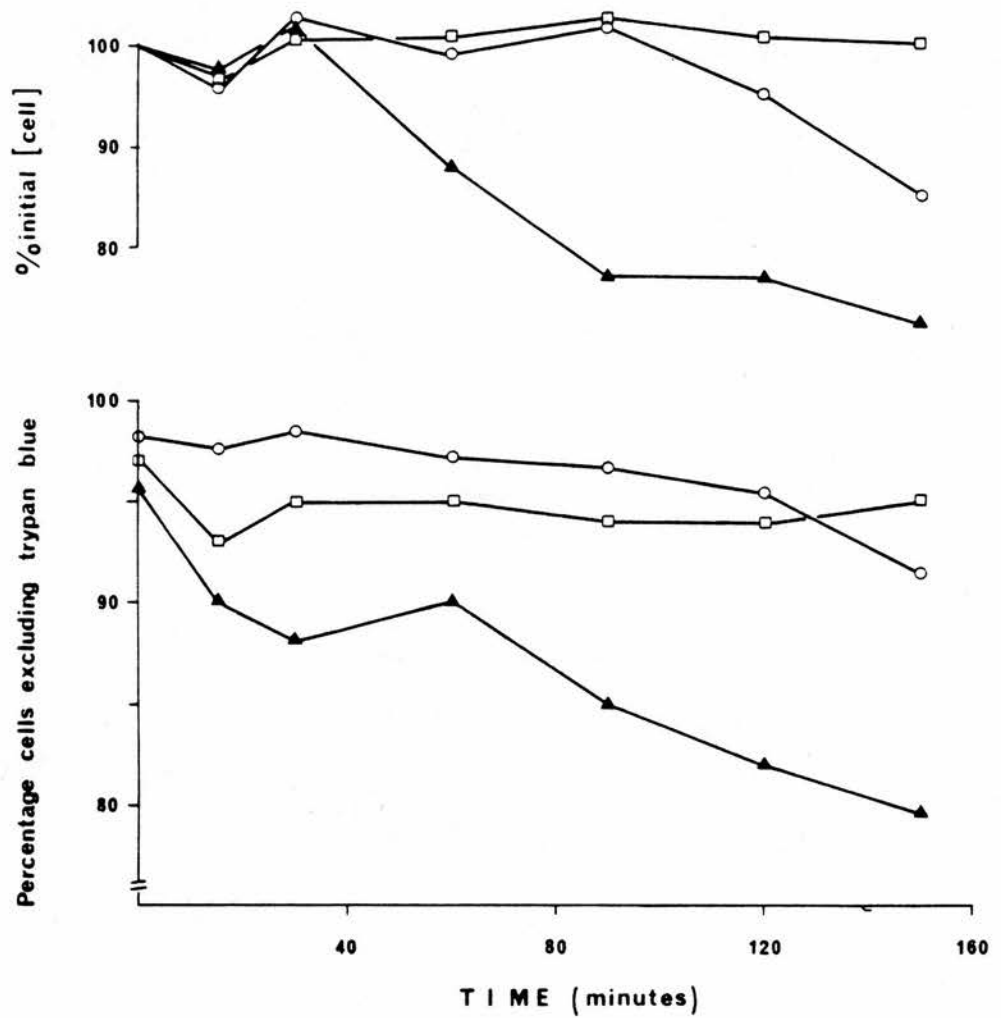


Fig. 3.2 Effect of incubation at 37°C on cell number (upper diagram) and cells staining with trypan blue (lower diagram) for three individual cell preparations ▲, □ and ○.

collagenase batches obtained from Boehringer Corporation Ltd. gave considerable variations in yield and percentage stained cells when using method I (Dickson 1977). No such comparison could be made when method II was adopted. It is not known if this is due to increased consistency in batch preparation by the suppliers or to the different methods used to isolate the cells. SDS polyacrylamide gel electrophoresis of different collagenase batches resulted in variations in the amounts of the individual protein components (fig. 3.3). In seven different batches of collagenase used throughout the study there were no major fluctuations in yield or percentage stained cells. However there were marked differences in hormone sensitivity when studying the stimulation of glucose release from the cells (Chapter 6). Cell sensitivity to glucagon varied between 0.1 and 1.0 ng/ml with similar variations in sensitivity to adrenaline. It is considered that hormonal sensitivity gives the most accurate measure of cell viability.

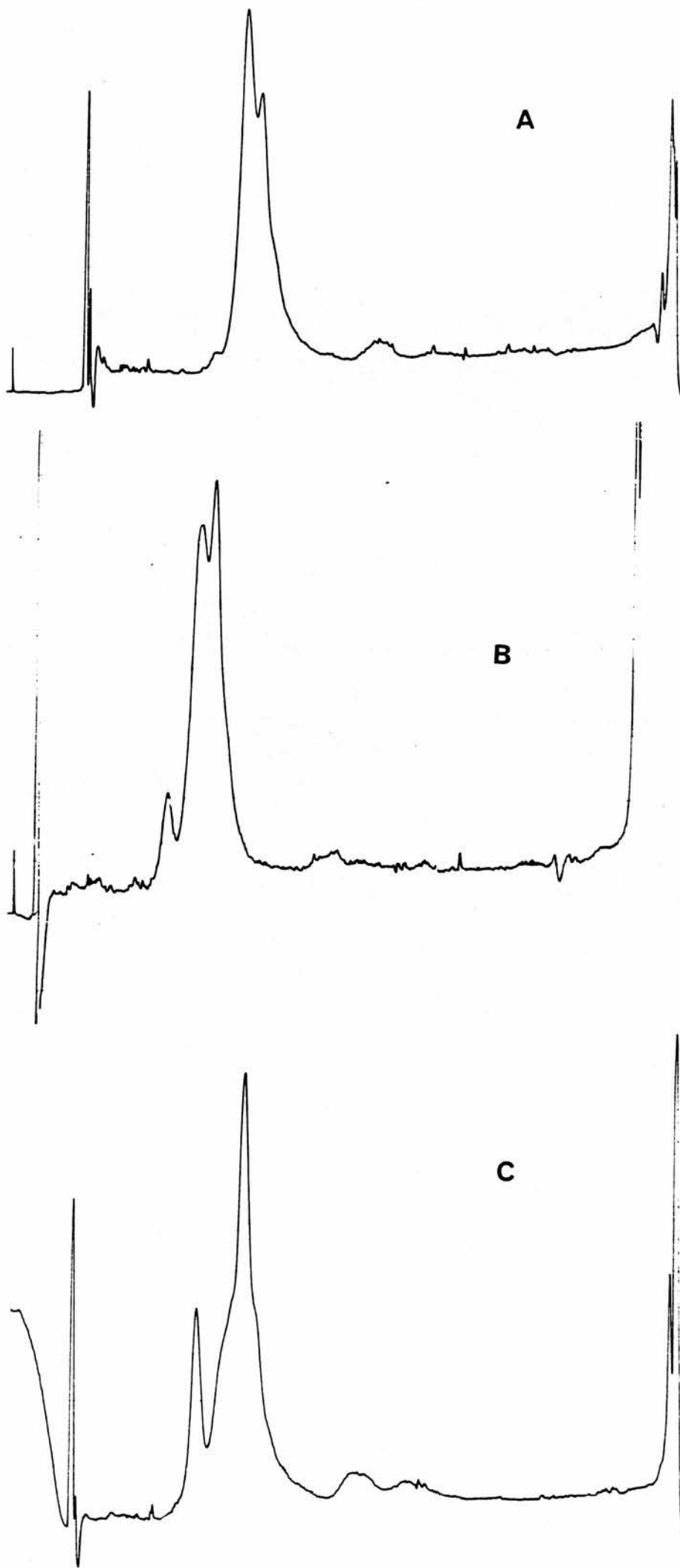
Changes in the batch of bovine serum albumin used (Fraction V Armour Pharmaceuticals) did not effect any of the basal nor hormone stimulated parameters measured.

Cell yield varied considerably depending on the initial success of perfusion. Yields ranged from 60 to 250×10^6 cells/g wet of liver. Using the conversion factor of wet to dry weights of 3.74 for chickens hepatocytes (Dickson 1977) - 3.7 for rat hepatocytes (Krebs et al. 1974) - this accounts for a 7 to 30% yield based on weight. Although this is much lower than the 50% yield reported for rat hepatocytes (Berry and Friend 1969; Krebs et al. 1974) it compares favourably with other workers isolating chicken hepatocytes (Capuzzi et al. ^{1974;} Dickson 1977).

Total adenylate contents for cells immediately after isolation by method II were higher than those reported for method I and also higher than the figures quoted for whole liver (Dickson 1977) (Table 3.1).

Fig. 3.3 SDS Polyacrylamide gel electrophoresis of different Boehringer collagenase batches.

A. Batch 17. B. Batch 25. C. Batch 29.



However on incubation hepatocytes isolated by method I also showed higher total adenylates than whole liver (Dickson 1977). If adequate oxygenation is not supplied during the isolation procedures the total adenylate content of the cells falls to values as low as 2000 pmol/10⁶ hepatocytes. The adenylate charge - the ratio of triphosphorylated adenylate to the sum of mono- and di-phosphorylated ($\frac{\text{ATP}}{\text{ADP} + \text{AMP}}$) in these conditions can approach zero despite the fact that the lower phosphorylated derivatives of adenosine escape more easily from the cell than ATP. With adequate oxygenation, adenylate content of the cells rises to around 5000 pmol/10⁶ cells. On incubation at 37°C there is a gradual increase in ATP content of the cells at the expense initially of AMP and then ADP (fig. 3.4). With incubation the adenylate charge increases continually if adequate oxygenation is given to the cell suspension. This was not found when incubating cells prepared by method I (Dickson 1977). No adenylate leakage could be demonstrated in cell suspension supernatants after incubation periods of up to one hour. Similar findings were reported for rat hepatocytes and perfused rat liver (Krebs et al. 1974).

Oxygen consumption rates of hepatocytes isolated by method II are intermediate in value to those reported for chicken hepatocytes by Badenoch-Jones and Buttery (1975) and Dickson (1977). The oxygen consumption of certain cell preparations isolated from fed chickens could be stimulated by adding sodium lactate to a concentration of 10 mM (fig. 3.5) but other preparations showed no effect. The reason for this is not known. The administration of caffeine or theophylline to cell suspensions at 10 mM reduced the oxygen consumption rate by 13-25%. The effects of these two methylxanthines is discussed in more detail in Chapter 6.

The morphological integrity of the hepatocytes was also examined by the use of electron microscopy. Hepatocytes isolated from the livers of both fed and 24 hour starved birds were processed. Hepatocytes from fed birds maintained their structural characteristics for up to 2 hours of

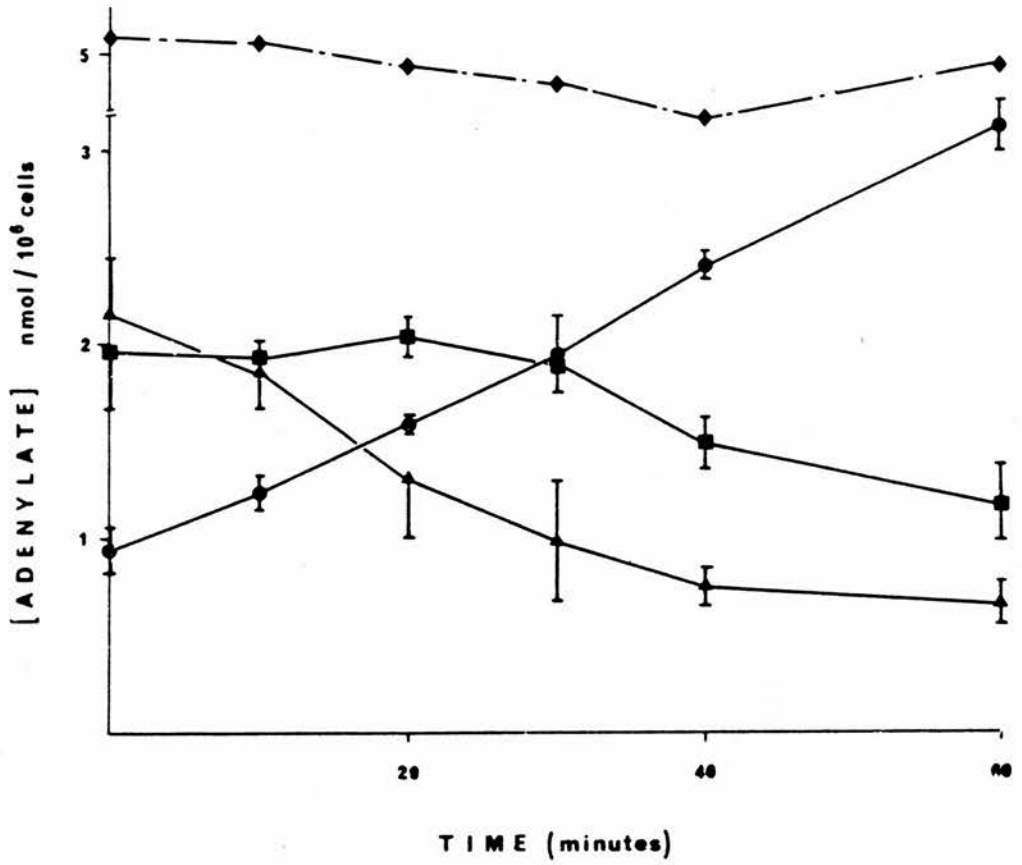


Fig. 3.4 Effects of incubation at 37°C on cellular ATP (●-●), ADP (■-■), AMP (▲-▲) and total adenylate contents (◆-◆). Cells prepared using method II.

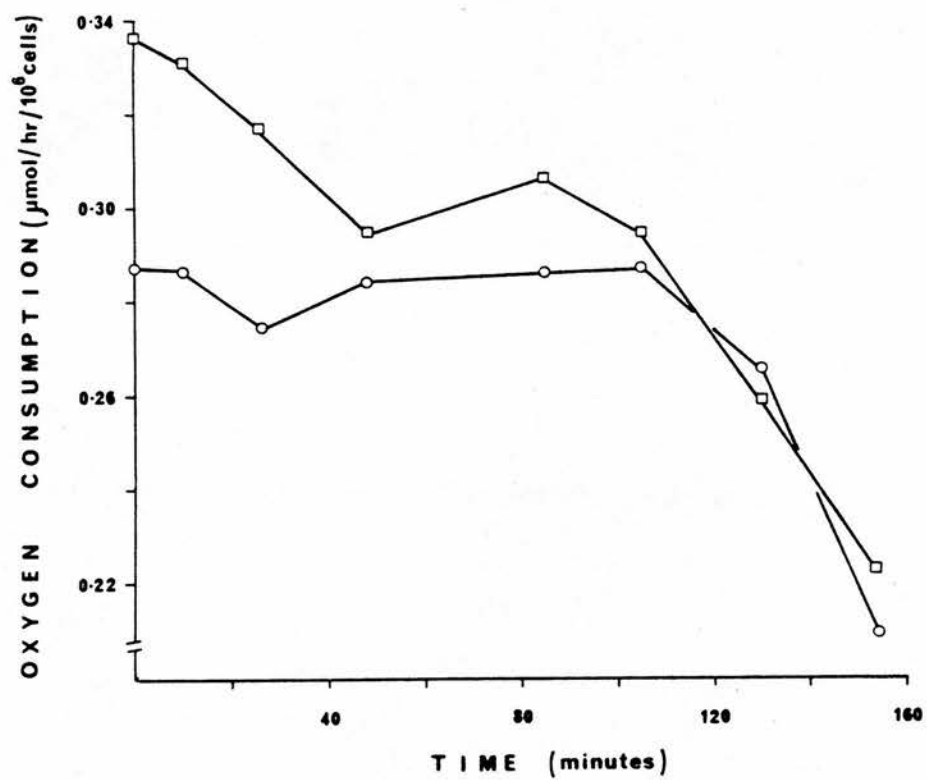


Fig. 3.5 Effect of incubation on respiration rate of cell suspensions.
 O-O Normal cell suspension.
 □-□ Cell suspension containing 10 mM lactate.

incubation (fig. 3.6 and 3.7). After this time, long processes of cytoplasm attached to the main cell by plasma membrane were obvious (fig. 3.8). A large proportion of the cells showed numerous vacuoles throughout the cytoplasm with some containing more vacuoles than others (fig. 3.9). These tended to merge together to form larger vacuoles when incubated for longer periods of time. Vacuoles were not apparent in hepatocytes isolated from 24 hour starved birds (fig. 3.10). It is not known if these vacuoles are a result of some artefact of the preparation procedures for the electron microscope or indeed if they are lipid droplets associated with cell metabolism. Other workers have also demonstrated the presence of large numbers of lipid droplets in isolated chicken hepatocytes (Tarlow et al. 1977). Which ever is the case it is obvious that there are certain definite populations of hepatocytes isolated from fed birds which gave this appearance in the electron microscope. Some parenchymal cells contain very few, if any vacuoles whereas in others they constitute the majority of the cytoplasm. It is not known if these are cells which didnot take up trypan blue but are damaged during preparation for electron microscopy or indeed are cells which have a high lipogenic activity or lipid storing capacity.

Although potassium contents and enzyme leakage from the cells were not investigated, the parameters tested compare favourably with those reported by others. It is therefore concluded that the recirculating type perfusion (method II) provides a rapid and convenient method to isolate large numbers of structurally and metabolically viable hepatocytes from the livers of starved or fed chickens. These hepatocytes were then used to examine the various aspects of chicken liver metabolism related to the pancreatic polypeptide hormones which are presented in the following chapters.

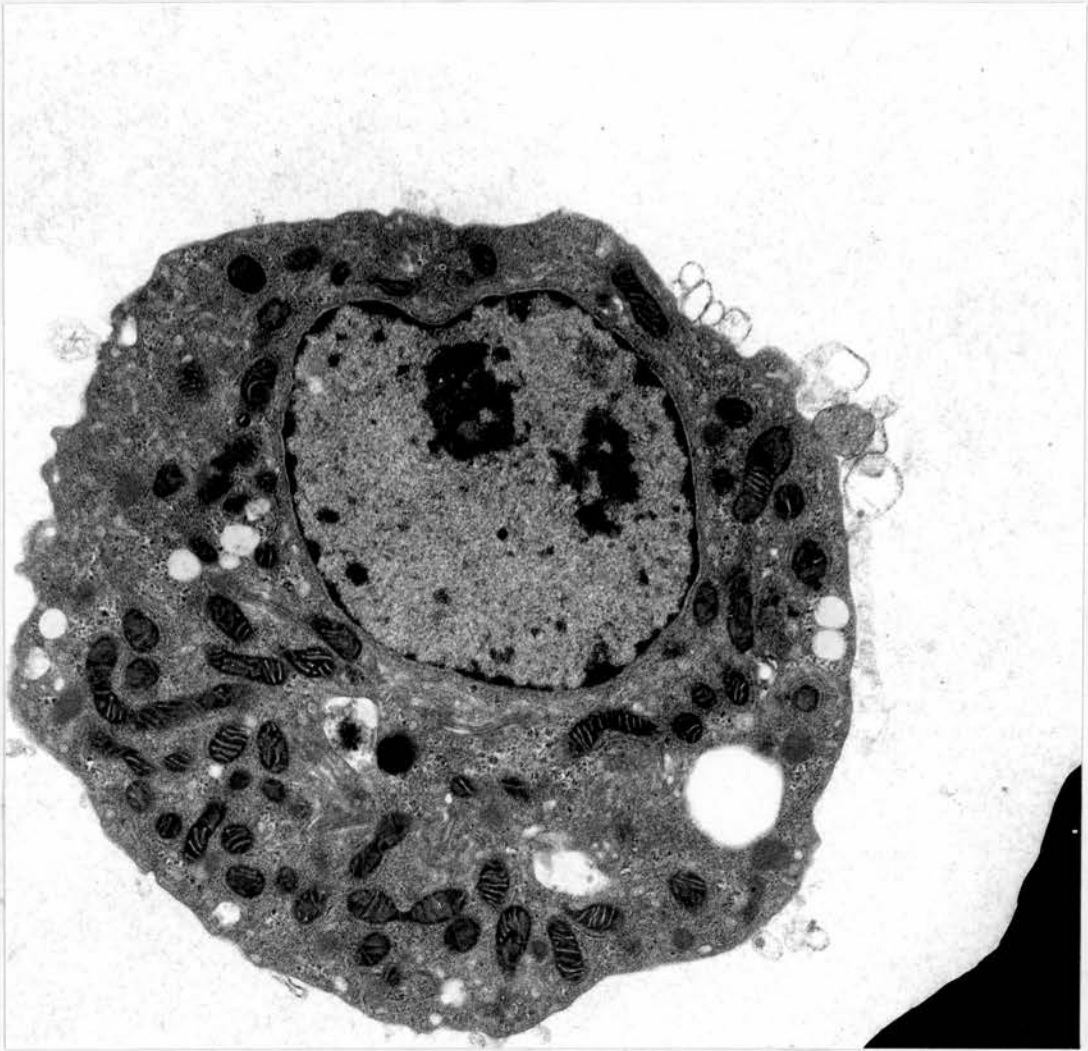


Fig. 3.6 Electron micrograph of hepatocyte - preincubated for 30 minutes at 37°C - isolated from a fed chicken. Magnification X 15,000.

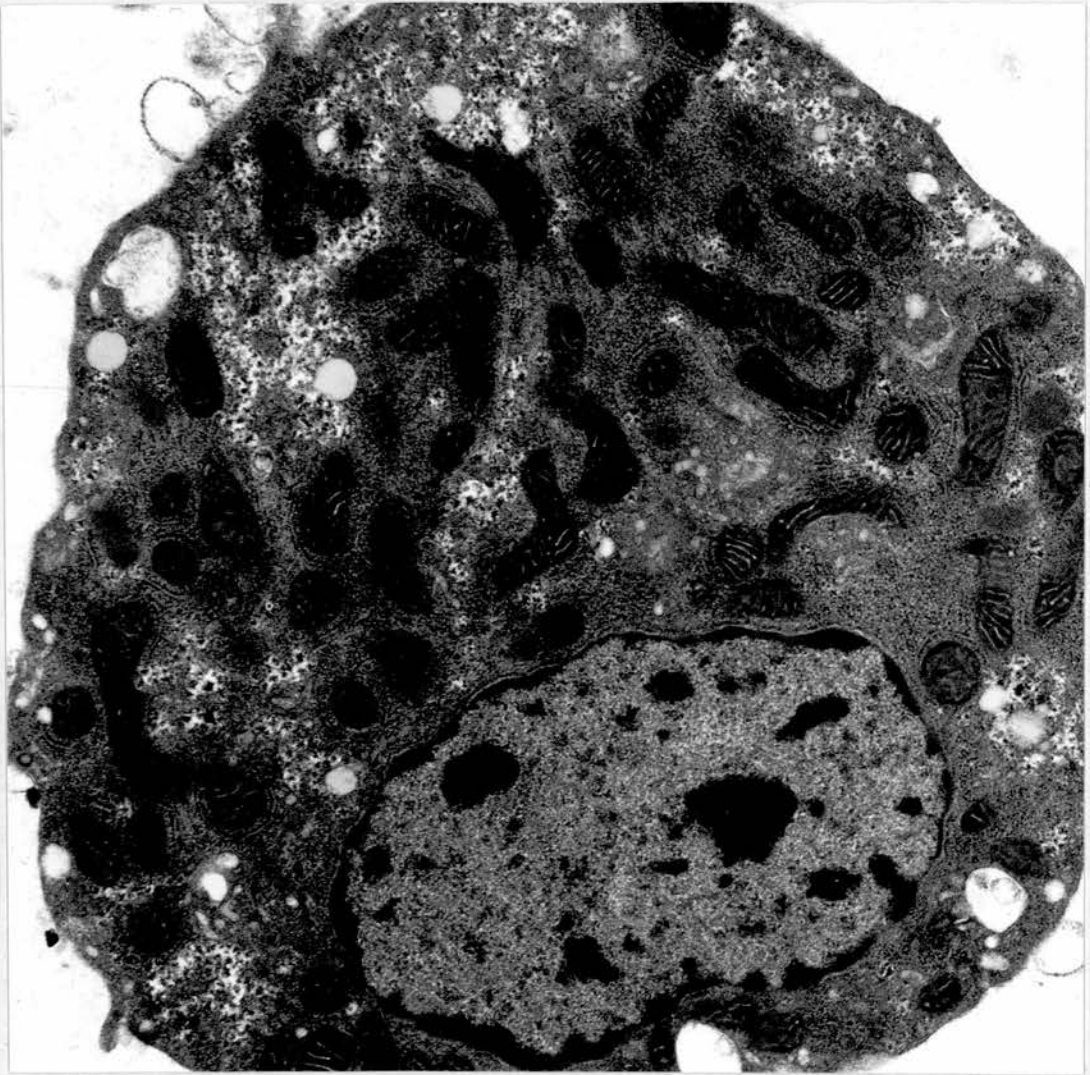


Fig. 3.7 Electron micrograph of hepatocyte - preincubated for 60 minutes at 37°C - isolated from a fed chicken. Magnification X20,000.

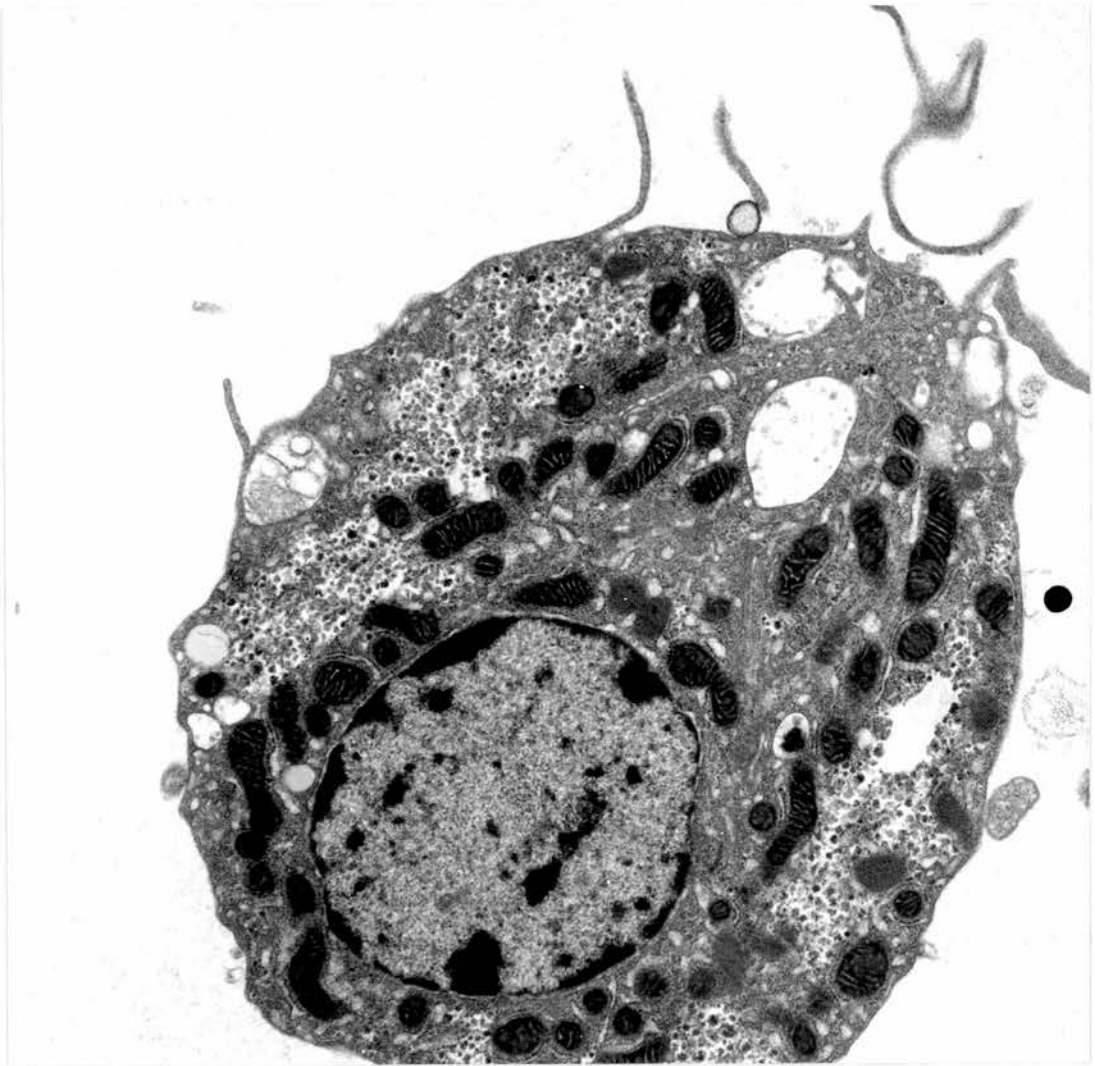


Fig. 3.8 Electron micrograph of hepatocyte - preincubated for 120 minutes at 37°C - isolated from a fed chicken. Magnification X15,000.

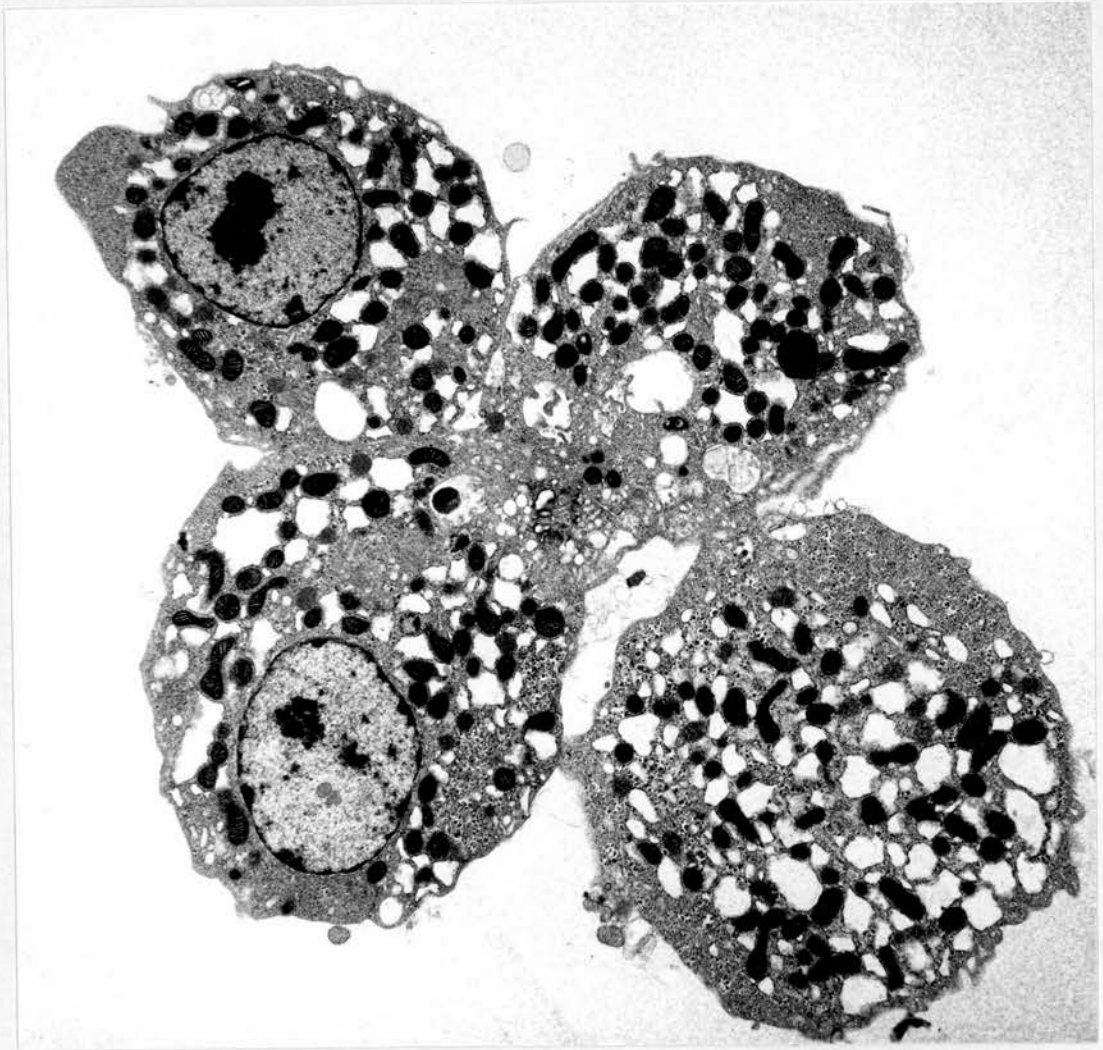


Fig. 3.9 Electron micrograph of hepatocytes - preincubated for 30 minutes at 37°C - isolated from a fed chicken. Magnification X10,000.

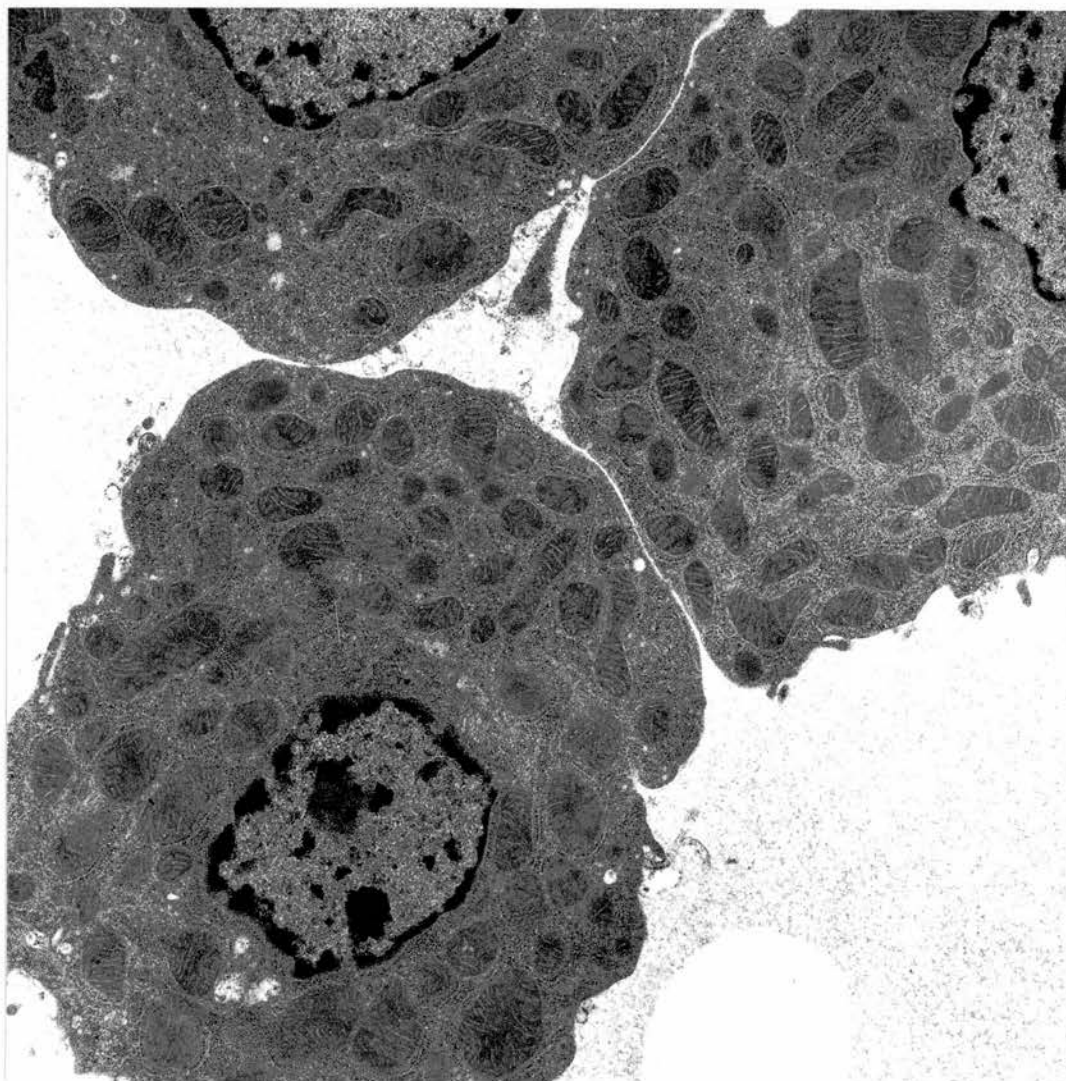


Fig. 3.10 Electron micrograph of hepatocytes - preincubated for 30 minutes at 37°C - isolated from a 24 hour starved chicken. Magnification X15,400.

CHAPTER 4

HORMONE RECEPTOR INTERACTIONS

Introduction

It is now well accepted that there are components of the liver cell plasma membrane (receptors) which specifically bind various polypeptide hormones. This is the primary action in a series of related events which lead to the alteration of various metabolic processes within the cell and is therefore an important point at which to control the extent of hormonal regulation. Metabolic rates within the liver cell are regulated by the rate of release of the pancreatic hormones from islet tissue and also by the hepatocytes' ability to recognise the presence of these hormone signals carried in the blood via their specific receptors. The affinity of the receptor for the hormone as well as receptor concentration on the cell surface are the main initial factors which influence the sensitivity of the cell to fluctuations in the circulating hormone concentration. These hormone receptor interactions have been extensively studied in a number of suitable preparations and many comprehensive reviews have been published (Roth 1973; Kahn 1975; Cuatrecasas and Hollenberg 1976; Freychet 1976; Kahn 1976).

Hormone-receptor interactions are monitored by incubating radioactively labelled hormone (^{125}I is the most universally used isotope) with a suitable receptor preparation and affinity constants are determined by kinetic or more often steady state binding experiments. Receptor bound hormone can be separated from free hormone by centrifugation, filtration or precipitation. Receptor affinity and specificity can be calculated by the addition of various amounts of unlabelled hormone or related agonists. Results are analysed by methods for competitive protein binding assays, and thus quantitative measures of affinity and saturability are obtained. Binding studies are mostly carried out at low temperatures (usually room temperature or below) to minimise hormone and receptor degradation which takes place during incubation. Methods and conditions

used to study hormone binding and the various mathematical analyses of results have been reviewed (Rodbard 1973; Kahn 1975; De Meyts 1976; Cuatrecasas and Hollenberg 1976).

Apart from hormone recognition the receptor must also be capable of transmitting the signal of hormone occupancy across the plasma membrane to another membrane constituent or directly to the cell cytosol or both. Many hormones, like glucagon act through their specific membrane receptor by stimulating adenylate cyclase which is situated on the inner surface of the plasma membrane (for a review see Helmreich et al. 1976) but the precise molecular mechanism by which this is carried out is unknown. The mode of signal transfer by the insulin receptor is even less understood although it is continually under investigation. Years of extensive search for a second messenger such as cAMP for glucagon have so far been unsuccessful, although a few candidates have been proposed. Many recent reports on insulin degradation indicate the strong possibility of receptor bound insulin "internalisation" into the cell which may explain effects on intracellular processes (Goldfine 1977; Steiner 1977; Bergeron et al. 1978, 1979).

Due to the relatively recent discovery of APP, very little is known about its metabolic actions on the liver. APP has been reported to have direct effects on adipose tissue (McCumbee and Hazelwood 1977) but it is not known whether it affects the liver directly. Effects of whole body carbohydrate and lipid metabolism have been reported (Hazelwood et al. 1973).

The Insulin Receptor

The interaction of insulin with a specific receptor has been investigated in a variety of tissues. The receptor has been isolated from fat and liver cell membranes (Cuatrecasas 1972a,b; 1974) as well as lymphocytes (Gavin et al. 1973) and avian erythrocytes (Ginsberg et al. 1976, 1977)



by treatment with nonionic detergents. Similar, if not identical, properties have been found for all preparations. The solubilised receptor was found to be a glycoprotein with molecular weight about 300,000 and a Stokes radius, when complexed with detergent molecules, of 70.7 \AA ; this preparation still retains the ability to bind insulin specifically. Insulin receptors from many other sources show identical kinetic parameters to those of the major target tissues, liver and adipose tissue, indicating that the receptor molecules are identical or very similar in all preparations studied (Kahn 1976).

Some investigators studying the binding of radio iodinated insulin have used small pieces of tissue, such as intact muscle, or tissue slices as the receptor preparations (Stadie et al. 1953; Newerly and Berson 1957; Garrett 1966; Wohltmann and Narahara 1966; Lambert 1972). Besides the obvious diffusion difficulties encountered, results were often difficult to interpret due to high non-specific binding and degradation of the hormone. The development of cell isolation techniques provided a more convenient receptor preparation since both the binding of hormone and biological effect could in many cases be directly compared, degradation rates could be controlled and non-specific adsorption of tracer was greatly reduced. Cells isolated from the major target organs - adipose tissue and liver - have been extensively used. Isolated fat cells have been the main candidates when studying insulin binding with numerous reports in the literature (Cuatrecasas 1971a,b; Cuatrecasas and Illiano 1971; Freychet et al. 1971; Kono and Barham 1971; Bennet and Cuatrecasas 1973; Gammeltoft and Gliemann 1973; Gliemann et al. 1975; Gliemann and Sonne 1978; Kahn and Baird 1978). Isolated rat hepatocytes have also been successfully used under conditions which limit the high rates of hormone degradation by this tissue (Kahn et al. 1973; Freychet et al. 1974; Olefsky et al. 1975; Terris and Steiner 1975; Gammeltoft

et al. 1978). Insulin binding to chicken hepatocytes isolated by the method of Capuzzi et al. (1974) has also been recently reported (Simon et al. 1977a). Other receptor preparations studied include purified plasma membrane fractions (Neville 1968) from rat (Freychet et al. 1971a,b; Freychet et al. 1972a,b; Goldfine et al. 1973; Kahn et al. 1973; Freychet 1974; Kahn et al. 1974; Kahn 1975) and chicken liver (Simon et al. 1977a). Plasma membrane preparations from rat adipocytes (Cuatrecasas 1971b; Freychet 1971b; Hammond et al. 1972; Jarett and Smith 1975) and rat and mouse cardiac muscle (Freychet and Forgue 1974; Forgue and Freychet 1975) have also been investigated. All preparations studied indicate the presence of a high affinity receptor for insulin with dissociation constants in the range 10^{-10} to 10^{-8} M. Cuatrecasas (1971a) reported the presence of a high affinity insulin receptor on the rat adipocyte membrane with a dissociation constant (K_d) of 6×10^{-11} M. This value compares well with the dose response curve of insulin stimulated lipogenesis in the same tissue (Gliemann 1967; Crofford 1968; Kono and Barham 1971) and thus binding and metabolic action curves are superimposable. This implies that total receptor occupancy is required for maximal metabolic effect. However much lower receptor affinities for the rat adipocyte have been reported by others. Gammeltoft and Gliemann (1973) reported a K_d between 1 and 5×10^{-9} M, Kono and Barham (1971) found the K_d to be around 7×10^{-9} M whereas Hammond et al. (1975) found two classes of binding sites with K_d 's of 5×10^{-10} and 3×10^{-9} M. These results suggest that the biological effect is maximum when only a small fraction of the total cell receptors are occupied. This is consistent with the 'spare receptor' theory (see Cuatrecasas and Hollenberg 1976) where large numbers of receptors are present on the cell surface but only a very small number need to be occupied to induce a maximum metabolic effect.

Various workers using rat liver cells found receptor affinities similar to those found by Gammeltoft and Gliemann for the rat adipocyte (Freychet et al. 1974; Terris and Steiner 1975; Olefsky 1975). This correlates well with the almost identical properties of the solubilised receptors from the two tissues. Dissociation constants of the order of 2×10^{-9} M have been reported for rat hepatocytes (Freychet et al. 1974; Freychet 1976) and 3×10^{-9} M for liver plasma membranes (Freychet et al. 1974). Terris and Steiner also report a K_d of 3.5×10^{-9} M for the high affinity component of insulin binding to hepatocytes. Studies using chicken hepatocytes and chicken liver plasma membranes isolated by the method of Neville (1968) showed dissociation constants of 1.3×10^{-9} M and 3.2×10^{-9} M respectively (Simon et al. 1977a).

Insulin receptors have been found in a number of tissues which are not considered to be major target sites for the hormone, such as blood mononuclear cells, thymic lymphocytes and placental tissue. Although numerous studies have been carried out with receptor preparations from these sources the reason for the existence of these specific binding sites is not clear. Due to the ease of isolation and apparent stability, human lymphocytes have been employed to study insulin binding interactions (Gavin et al. 1972; Archer et al. 1973; De Meyts et al. 1973; Gavin et al. 1973; Gavin et al. 1974; van Obberghen et al. 1976). These preparations show high affinity binding sites for insulin and have been used to study the insulin-receptor interaction in a number of pathological states including diabetes (Archer et al. 1973). An uneven distribution of the insulin receptors within the lymphocyte preparation has been reported. Using methods which preferentially isolate T type lymphocytes from peripheral blood, workers have found no evidence for high affinity insulin binding (Olefsky and Reaven 1976; Krug, U. et al. 1972) suggesting that the receptor population resides only in the B type

lymphocytes. Schwartz and co-workers (1975) however, report that the blood monocyte contaminant of the lymphocyte preparation (approx. 10% of total cell number) are the insulin binding cells. Other receptor populations which have been investigated include human peripheral granulocytes (Fussganger et al. 1976), animal placental tissue (Posner 1974; Cuatrecasas and Hollenberg 1975) mouse mammary cells (O'Keefe and Cuatrecasas 1974) and rat (Goldfine et al. 1972) mouse (Soll et al. 1974) and chicken (Simon 1979) thymocytes.

Negative Co-operativity. Original measurements of kinetic and equilibrium constants of the insulin-receptor interaction resulted in conflicting reports. Although some workers found a homogeneous population of receptors (Kono and Barham 1971; Cuatrecasas 1971a,b; Gammeltoft and Gliemann 1973; Gammeltoft 1977; Pollet et al. 1977) other groups have reported a heterogenous population of receptor sites (Hammond et al. 1972; Gavin et al. 1973; Kahn et al. 1974; Posner 1974; Olefsky 1975). The latter workers reported that Scatchard analysis (Scatchard 1949) of the insulin binding data revealed curvilinear plots which indicated the presence of two or more distinct populations of insulin receptors having different affinities for the hormone. These curvilinear plots are often interpreted as resulting from two different receptor populations, one of high affinity but low capacity, and the other of low affinity and high capacity; equilibrium or dissociation constants are frequently cited for both. These plots have also been interpreted as resulting from ligand-ligand interactions with insulin dimerisation at high concentrations inducing rapid dissociation from the receptor (Cuatrecasas and Hollenberg 1975).

De Meyts and coworkers suggested that site-site interactions between receptors may account for the steady state binding data (De Meyts et al. 1973; 1976; 1978). Kinetic studies using monocytes, lymphocytes and

liver membranes showed that the dissociation rate of bound insulin from its receptor was dependent on the fractional hormone occupancy of the sites. It was postulated that high levels of receptor occupancy induced receptor-receptor interactions of a negative co-operative type leading to enhanced dissociation rates of the insulin-receptor complex, thus lowering the average binding affinity. Similar reports of possible negative co-operative interactions have been reported for human granulocytes (Fussganger et al. 1976) and adipocytes (Olefsky and Chang 1978).

The solubilised receptor has been reported to retain negative co-operative interactions and also to dissociate in a reversible manner into subunits when incubated with insulin (Ginsberg et al. 1976, 1977). It was proposed that this insulin induced dissociation of a tetrameric receptor complex was a possible mechanism for negative co-operativity. Recent reports by De Meyts and co-workers indicate that a certain invariable region of the insulin molecule is responsible for the induction of receptor site co-operativity (De Meyts et al. 1978).

As stated previously, nearly all experimental incubations were carried out below physiological temperature (30°C and below) to reduce the amount of hormone and receptor degradation. Gammeltoft and co-workers, when investigating the dissociation of ^{125}I -insulin from isolated rat hepatocytes noted enhanced rates in the presence of native hormone at 12°C but not at the physiological temperature of 37°C (Gammeltoft et al. 1978). Studies at 37°C revealed only a single class of insulin receptors free of negative co-operative interactions with very high affinity (K_d $4.0 \times 10^{-10}\text{M}$) and about 15,000 sites per cell. These results claim a 5 fold higher affinity and approximately a 7 fold decrease in receptor number compared with previous studies of rat hepatocytes, when incubations were carried out at lower temperatures. Dissociation experiments carried out at 37°C by Olefsky and Chang (1978) using rat adipocytes showed

enhanced dissociation rates when incubations also contained native hormone. Therefore, although the solubilized insulin receptors from liver and adipose tissue are reported to be almost identical, there may be more detailed differences between the two when they are incorporated in their native membranes.

Insulin Degradation. Early experimental reports indicated that degradation of insulin by the liver took place via some mechanism independent of hormone binding to the receptor-effector system (Freychet et al. 1972; Gammeltoft and Gliemann 1973; Le Cam et al. 1974). However recent evidence suggests that degradation of insulin by hepatocytes or adipocytes requires prior association with receptor sites (Terris and Steiner 1975, 1976; Gliemann and Sonne 1978; Terris et al. 1979). It was found that the degradation rate of insulin was dependent on the amount of insulin bound to receptor at the steady state and unlike the earlier reports, specificities of binding and degradation were the same. Prebinding of insulin to receptor also increased the degradation rates. Since low temperatures (below 20°C) and plant lectins or antimicrotubular agents inhibit insulin degradation, Steiner and co-workers suggested that certain populations of the insulin-receptor complex first aggregate on the cell surface before being taken into the cell by adsorptive pinocytosis (Silverstein et al. 1977) followed by intralysosomal degradation (Terris et al. 1979).

¹²⁵I-insulin bound to rat adipocytes is rapidly altered or transferred to some compartment less accessible to the external medium (Kahn and Baird 1978). Autoradiographic analyses indicate a time and temperature dependent incorporation of insulin into the intracellular environment of human cultured lymphocytes (Goldfine et al. 1978) or rat hepatocytes (Gorden et al. 1978). Since impermeant sulphydryl blocking agents also inhibit degradation and pinocytosis it is thought that an early degradative

step takes place on the cell surface (Terris et al. 1979). This early degradation may be the splitting of insulin into A and B chains by glutathione-insulin transhydrogenase (Varandi et al. 1972) which is found in a few external microvillous projections of the hepatocyte (Varandi et al. 1978). This may explain the two forms of the receptor previously reported by others (Gliemann 1976; Krupp and Livingston 1978; Olefsky and Chang 1978). Internalisation of the insulin-receptor complex gives support to the finding of specific intracellular binding sites for this hormone (Harvat et al. 1975; Forgue and Freychet 1975; Kahn 1976; Goldfine and Smith 1976; Goldfine et al. 1977; Bergeron et al. 1977); this intracellular bound insulin or some degradative product may be responsible for initiating the intracellular effects associated with this hormone.

Two mechanisms for insulin binding and action have been suggested (as summarised by Steiner 1977); the 'receptor transducer' model in which bound insulin remains in the extracellular environment and acts via the production of some intracellular second messenger which influences protein kinase or protein phosphatase activities, or the 'receptor transducer internaliser' model which allows insulin bound to receptor to enter the cell exposing it to the intracellular degrading system which releases insulin or insulin fragments to regulate various metabolic parameters. Although increasing evidence suggests insulin is internalised and degraded, this process may be independent from the effector system; in this case a second messenger must again be postulated. The second model would also explain the phenomenon of down regulation of receptor number by insulin (Gavin et al. 1974) as is also found for glucagon (Soman and Felig 1978), growth hormone (Lesniack and Roth 1976) and epidermal growth factor (Das and Fox 1978).

The Glucagon Receptor

Rodbell and co-workers (1971a) indicated that glucagon bound specifically to rat liver plasma membranes in a concentration range identical to that observed for activation of adenylate cyclase. Bataille et al. (1974) also noted a close similarity between binding and activation. Birnbaumer and Pohl (1973) however found maximum hormonal effect on adenylate cyclase when only 10 to 20% of the receptors were occupied and likewise Rosselin et al. (1974) and Sonne et al. (1978) found full activation of the cyclase at low receptor occupancy.

The elucidation of the mode of action of the hormone was further complicated by the discovery that physiological concentrations of guanine nucleotides, particularly GTP, can influence both hormone binding to receptor and hormone activation of adenylate cyclase in liver membrane preparations and therefore may possibly have some regulatory function on glucagonic responses (Rodbell 1971b,c; Lad et al. 1977; Rendell et al. 1977; Welton et al. 1977; Yamamura et al. 1977). Using isolated rat liver membrane preparations major discrepancies were reported between the observed binding of ^{125}I -glucagon and glucagon stimulated cyclase activity in the presence of added GTP. The guanine nucleotide decreased the apparent affinity of the receptor for the hormone due to an increased dissociation rate of glucagon from receptor (Rodbell et al. 1971b). However despite this decrease in affinity of the receptor, GTP increases the affinity of glucagon for adenylate cyclase stimulation and the apparent ED_{50} for glucagon action is decreased by approximately 10 fold (values ranging from $2 \times 10^{-9} \text{ M}$ to $7 \times 10^{-9} \text{ M}$ were reduced to values of $2.0 \times 10^{-10} \text{ M}$ to $5 \times 10^{-10} \text{ M}$, Rodbell et al. (1974)). The increased dissociation rate of glucagon from its receptor was not related to cyclase activation. The kinetics of binding of ^{125}I -glucagon to receptor showed that the steady state is reached more slowly than would

be expected from the induction of hormone action (Rodbell et al. 1974) indicating that some complex interaction between hormone receptor, adenylate cyclase and GTP was necessary to explain the observed effects.

The binding site for GTP is distinct from the glucagon binding site and is thought to have an allosteric action on the system (Rodbell et al. 1971b,c). Some evidence has been put forward for the possibility of two distinct guanine nucleotide sites; one for regulation of receptor binding and the other for cyclase activation (Lad et al. 1977). Welton and co-workers (1977) have shown that solubilisation of liver membrane preparations yields distinct receptor and cyclase populations each having GTP regulatory sites. Another suggestion is that the guanine nucleotide is required for coupling the receptor and cyclase (Pecker and Hanoune 1977). The cyclase solubilised from pigeon erythrocytes can be inactivated by dissociation into two protein fractions by GTP-Sepharose affinity columns; only one protein retains the nucleotide binding site (Pfeuffer 1977). Increasing evidence suggests that this nucleotide binding protein regulates cyclase activation by both hormones and fluoride (Rodbell 1978). Work carried out on the solubilised myocardial cyclase suggests that acid phospholipids play an important role in coupling the receptor with catalytic units (Levey 1975; Levey et al. 1975). It is not known if GTP has a role in this lipid involvement although it has been shown that digestion of membranes with phospholipase C (which cleaves acid phospholipids) abolishes the effect of GTP on dissociation of glucagon and enhances cyclase activity (Rubaclava and Rodbell 1973). The system is further complicated by the discovery of another GTP dependent site which in the presence of chelating or thiol reducing agents inhibits cyclase activity without altering the stimulating effects of GTP or hormones (Yamamura et al. 1977).

Glucagon Binding Sites

The majority of investigations into glucagon binding to its receptors have been carried out using iodinated hormone as the tracer. Iodination of the tyrosyl residues of glucagon produces two opposing biological effects in the molecule. It has been reported that the covalent insertion of an iodine atom increases the hydrophobic interaction of the hormone with its receptor thus increasing the affinity, whereas the lowered isoelectric point due to the inductive effect of the iodine atom increases the proportion of ionised phenoxy groups, resulting in a loss of binding capacity. Due to the heterogenous tracer population, variations in pH can alter the overall biological affinity observed (Rodbell et al. 1976; Lin et al. 1976), which will depend on the extent of iodine incorporation into the hormone. A heterogenous tracer population may also explain the anomalies between the kinetics of binding and activation (Lin, MC et al. 1977). When [³H]-glucagon was used in membrane preparations as the tracer it was possible to show that only 90% of the receptor population had a reduced affinity in the presence of GTP, with 10% remaining as high affinity receptors (Lin, MC et al. 1977). A sigmoidal affinity profile with K_d of 2×10^{-9} M was changed into a complex binding curve composed of low and high affinity sites in the presence of GTP. These high affinity receptors may be responsible for the transmission of glucagon action with the low affinity sites acting as spare receptors. Due to the low specific activity of the tritiated hormone it was not possible to accurately investigate the high affinity receptor.

Other workers have found variations in iodoglucagon affinity. Bromer and coworkers (1973) found monoiodoglucagon to be five times more potent than native hormone and likewise, Desbuquois also noted an increased ability of iodinated hormone to activate adenylate cyclase,

and to a lesser extent, bind to receptor (Desbuquois 1975, 1977). A maximum adenylate cyclase stimulation of 5 fold was found with the tetrasubstituted hormone and maximal increase in binding of 2 fold with the trisubstituted. However Sonne et al. (1978), when using iodinated tracer preparations with 0.2 to 0.5 atoms of ^{125}I per molecule of glucagon, found no difference in the binding properties in isolated hepatocytes at pH 6.5 or pH 7.4, indicating the tracer population behaved identically irrespective of the percentage of ionisable tyrosines. Although similar results were indicated at different pH's the tracer itself however may not exhibit identical binding properties to the native hormone.

Sonne et al. (1978) found the receptor population to be a heterogenous nature with both high affinity low capacity (K_d $7.0 \times 10^{-10}\text{M}$; 20,000 sites per cell) and low affinity high capacity (K_d $13 \times 10^{-9}\text{M}$; 200,000 sites per cell) components on Scatchard analysis. No evidence for co-operativity between receptors was found although it was previously reported that co-operative interactions may exist (Birnbaumer and Pohl 1973). Reports of glucagon-receptor affinity for rat liver membranes on isolated hepatocytes are not as numerous as for insulin. Half-maximal binding constants of $4 \times 10^{-9}\text{M}$ (Rodbell et al. 1971a) and $1.5 \times 10^{-9}\text{M}$ (Bataille et al. 1974) have been reported for rat liver membrane preparations and $3 \times 10^{-9}\text{M}$ (Freychet et al. 1974) for rat hepatocytes. Direct comparison with the values given by Sonne et al. are difficult since the latter workers did not analyse the data for more than one class of binding site. Freychet et al. (1974) reported a total of 110,000 binding sites per cell for glucagon but only around 11,000 need be filled for half maximum activation of adenylate cyclase. Shaltz and Marinetti (1972) using rat liver plasma membranes found two relatively high dissociation constants of $1 \times 10^{-7}\text{M}$ and $3 \times 10^{-5}\text{M}$ whereas Giorgio et al. (1974) reported that a purified Lubrol PX

extract of liver membrane receptor had a high affinity for glucagon with K_d of 1×10^{-10} M.

Although most workers report similar kinetic parameters the conditions under which the various experiments were carried out must be considered before valid comparisons can be made of the quantitative results. In particular, variations in the specific radioactivities of the tracer hormones makes detailed comparisons difficult.

Reports on degradation of 125 I-glucagon by the liver are relatively scarce. Although liver membranes rapidly degrade glucagon (Rodbell et al. 1971a) the inactivation of the hormone is thought to take place at a site independent of adenylate cyclase activation (Pohl et al. 1972). Desbuquois et al. (1974) have shown that inhibitors of glucagon degradation such as bacitracin can increase hormone binding by up to 80% and also increase the apparent affinity for adenylate cyclase activation, again suggesting independent sites for action and degradation. Since the liver is considered to be the major site of glucagon clearance from the blood (Buchanan et al. 1968) it has been suggested that the plasma membrane fraction accounts for the most of this due to high rates of degradation (Pohl et al. 1972). However, recent work using kidney membranes showed that glucagon degrading activity per mg protein was 20-fold higher than found in liver membranes, with an affinity of the degrading system (K_m 2.4×10^{-6} M) similar to that found for the liver (Duckworth 1978). Therefore the extent the liver plays in glucagon degradation, and the mechanism by which this is carried out, require more investigation.

Allowing for the observations reported on glucagon binding and stimulation of adenylate cyclase with the requirements for GTP, and also the poor evidence for the method of degradation, it is very difficult to propose a model system which will allow for all possible modes of action of the hormone. However various models have been proposed by some

investigators to explain hormone binding and also activation of adenylate cyclase with some models incorporating a role for GTP or analogues (Rodbell 1973; Bennet et al. 1975; Helmreich et al. 1976; Cuatrecasas and Hollenberg 1976; Rodbell 1978).

The APP Receptor

Investigations into the receptor binding properties of APP have been confined to isolated chicken adipocytes (McCumbee and Hazelwood 1977).

The iodinated hormone was found to bind specifically to two classes of receptor site; a high affinity low capacity site ($K_d = 5.6 \times 10^{-9}$ M; 40,000 sites per cell) and a low affinity high capacity site ($K_d = 8.3 \times 10^{-7}$ M; 190,000 sites per cell). Neither insulin nor glucagon competed for the APP binding site. There have been no reports on APP binding to chicken hepatocytes or chicken liver plasma membranes.

It is known that high intravenous concentrations of APP cause depletion of liver glycogen and a decrease in plasma glycerol (Hazelwood et al. 1973). It is not yet known if these metabolic disturbances are due to a direct effect of APP at the hepatic level or if indeed they are secondary to another major effect of the hormone.

Experimental Approach

The binding of iodinated insulin, glucagon and APP to the chicken hepatocyte preparation described in Chapter 3 was investigated. Concomitantly, studies were performed of the metabolic effects of these hormones; these are described in Chapters 5-6.

Method

Experimental Incubations. Cell suspensions were prepared using either batch 17 or 25 collagenase. No significant variations in hormone sensitivity were found in cells isolated using either collagenase batch, therefore all results were used to calculate average affinity values. Unless otherwise indicated all binding experiments using labelled insulin,

glucagon or APP, were carried out using cell concentrations less than 10×10^6 cells/ml. Concentrated cell suspension was added to 1 ml gassed KRB buffer containing tracer in 25 x 75 mm plastic incubation pots. The pots were then gassed with 95% O₂/5% CO₂ for approximately 20 seconds, lids sealed, and incubated for the desired time in a reciprocal shaking water bath (120-140 strokes/min) at room temperature (22°C). A sample of cell suspension (1 ml) was then removed and cells pelleted by centrifugation in a Beckman microfuge B (10,000 rpm 7000 g) for 1 minute. The supernatant was aspirated and 1 ml of ice cold KRB buffer added to the tube without resuspension of the pellet. The tube was then recentrifuged as above for 30 seconds, supernatant aspirated and the tube allowed to drain. The tip of the tube containing the cell pellet was excised and radioactivity determined on a Tracerlab gamma counter.

Porcine insulin and glucagon and chicken pancreatic polypeptide were used throughout the binding studies.

Results

1. ¹²⁵I-Insulin-Receptor Interactions

a) Tracer viability. Monocomponent porcine insulin was iodinated with ¹²⁵I by the chloramine-T method as described in Chapter 3. The quality of the tracer was assessed by radioimmunoassay, paper chromatoelectrophoresis, TCA and PCA precipitability, binding to talc and cellulose (for details of procedures and results see Chapter 3). The cellulose column purification procedure removed all unreacted iodide and nearly all damaged protein. However a large fraction of ¹²⁵I-insulin which was considered viable under conditions of chromatoelectrophoresis and by binding to talc, did not adsorb to the column and was washed through with the unwanted reaction products. Fig. 4.1 shows a typical paper chromatographic separation of the reaction products before and after purification on the cellulose column. No method was available to test

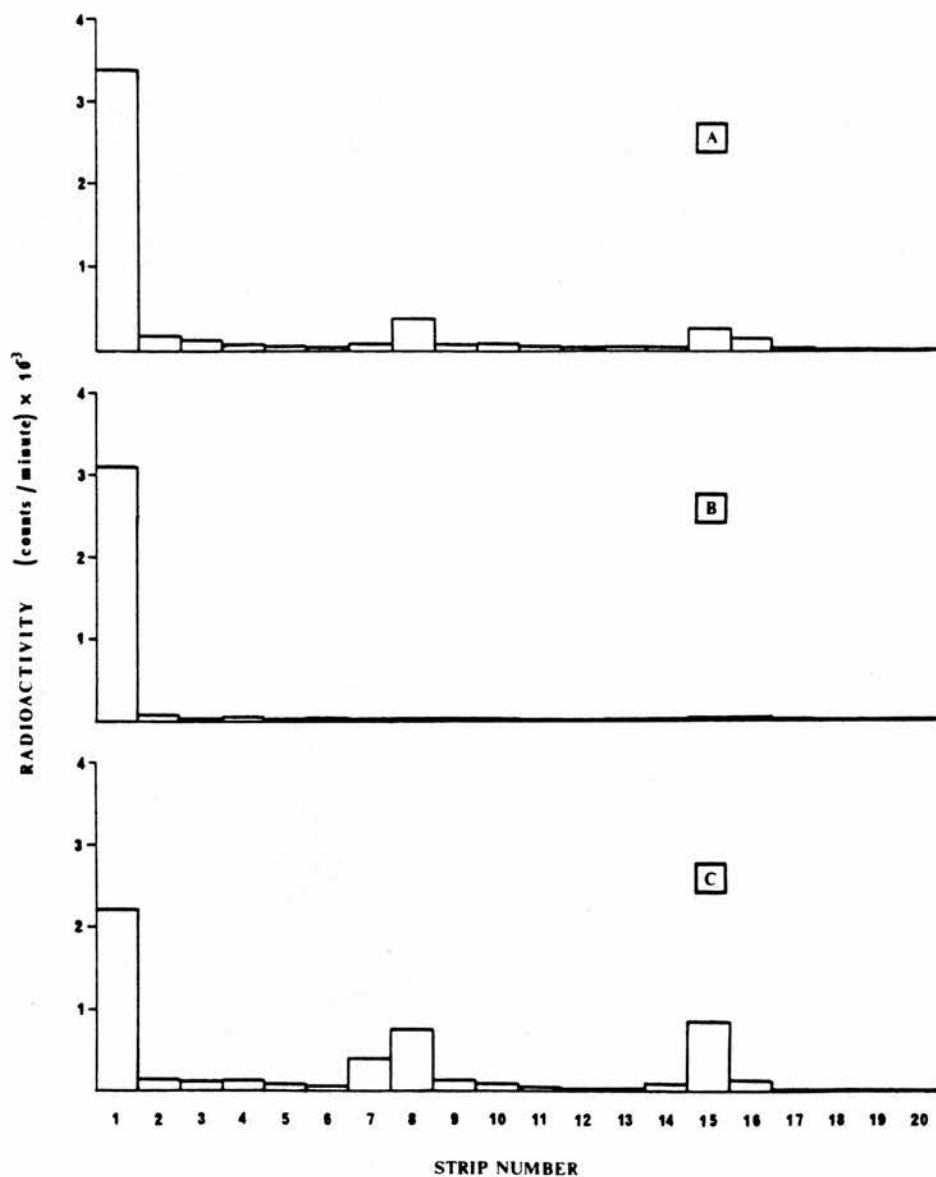


Fig. 4.1. Paper chromatoelectrophoretic pattern of iodinated insulin from A. the iodination mixture, B. the 24% BSA eluate from cellulose column and C. initial wash from cellulose column.

the biological activity of the tracer since insulin was found to have no metabolic effect on the chicken hepatocytes (see Chapters 5 and 6).

- b) Time course of ^{125}I -insulin binding. ^{125}I -insulin (0.35 ng/ml) was incubated with cell suspension (9.7×10^6 cells/ml) for various times at either 10°C , 22°C or 40°C . Another series of pots containing tracer plus 100 $\mu\text{g/ml}$ native insulin were also incubated under similar conditions to estimate counts nonspecifically bound. Specific binding of tracer was determined by subtraction of the counts bound in the presence of the high native hormone concentration. The time course for specific ^{125}I -insulin binding is shown in Fig. 4.2. As expected binding was much faster at 40°C , reaching a maximum at around 20 minutes. Total binding at this temperature, however, only reached approximately 60% of that at lower temperatures. The steady state binding level at 40°C was also unstable and with prolonged incubations the binding of ^{125}I -insulin rapidly decreased. Although the binding rate was quicker at 22°C than at 10°C , maximal binding was approximately the same and remained relatively constant for 2 hours incubation. Cell suspensions were regassed with O_2/CO_2 after 60 minutes of incubation at these temperatures. ^{125}I -insulin binding therefore appears to reach a steady state after 45 minutes incubation at 22°C . Addition of small amounts of cold hormone (2 ng/ml) or decreasing the cell suspension concentration to 5×10^6 cells/ml did not markedly effect the rate of binding at 22°C . Binding capacity was found to be linearly proportional to cell concentration at least up to 14.0×10^6 cells/ml. Addition of glucagon or APP at 500 ng/ml did not effect the steady state binding level of ^{125}I -insulin.
- c) Effect of preincubation of cell suspension. Cell suspensions (10 to 12×10^6 cells/ml) after gassing with O_2/CO_2 were preincubated at 22°C , 32°C or 38°C for various periods of time before adding ^{125}I -insulin (0.35 ng/ml) and then reincubating for a further 45 minutes at 22°C .

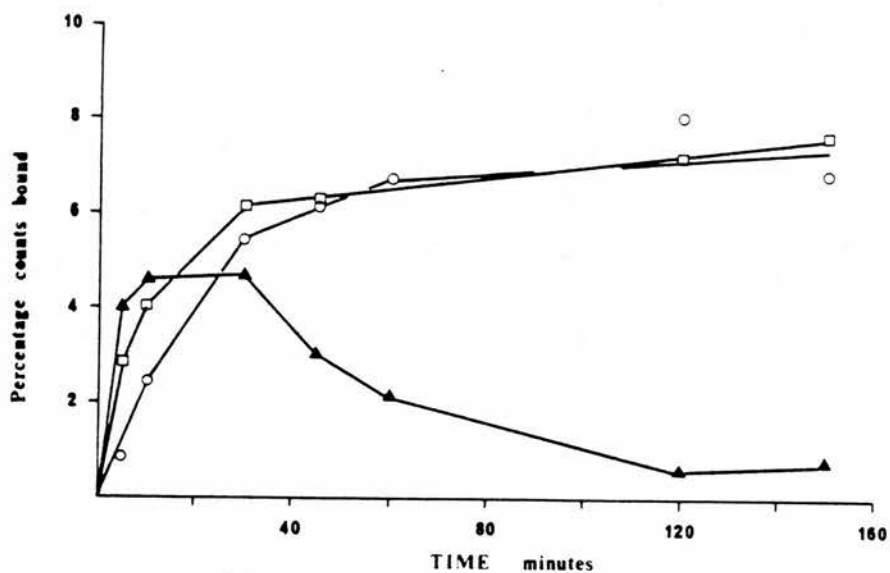


Fig. 4.2 Binding of ¹²⁵I-insulin (0.35 ng/ml) to cells incubated at 10°C (O-O), 22°C (□-□) and 40°C (▲-▲).

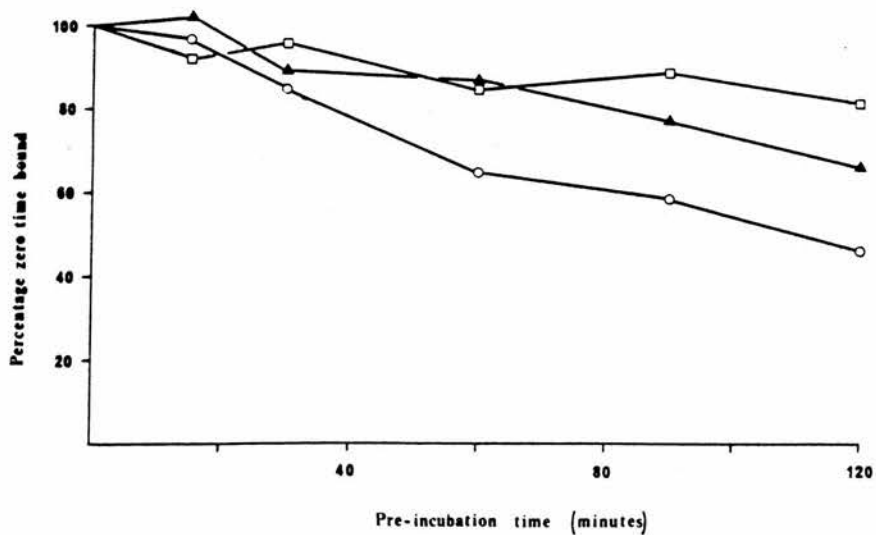


Fig. 4.3 Effect of preincubation of cell suspension at 25°C (□-□), 32°C (▲-▲) and 38°C (O-O) on subsequent binding of ¹²⁵I-insulin (0.35 ng/ml) at 22°C.

Determination of specifically bound tracer was achieved by running parallel incubations containing 10 µg/ml native hormone. Tracer binding is shown in Fig. 4.3. Preincubation at higher temperatures results in a progressive decrease in the amount of ^{125}I -insulin bound. This does not correlate with a decrease in cell number nor an increase in trypan blue staining with time (see Chapter 3). After 2 hours incubation at 38°C up to half the insulin binding capacity is lost whereas at 22°C less than 20% of the insulin binding is affected after this time. After incubation for 45 minutes at 22°C (the time used to attain steady state binding) only approximately 10% of the binding capacity is lost.

d) Binding affinity. ^{125}I -insulin (0.4 ng/ml) plus varying amounts of native insulin (0.78 to 10,000 ng/ml) were incubated with cell suspension (2.0 to 3.0×10^6 cells/ml) at 22°C for 45 minutes. Binding was assessed by centrifugation as before, with radioactivity still bound in the presence of 1 to 5 µg/ml native hormone taken to be non-specific binding. Specifically bound hormone and free hormone was calculated and data processed for Scatchard plot analysis (Scatchard 1949). Fig. 4.4 shows a typical curvilinear plot indicating the existence of more than one class of binding site or negative co-operative interactions between sites. The data were analysed as if they resulted from two distinct classes of receptor. The linear portions of the extremities of the plot were extrapolated to the axes and the binding contamination of the high affinity sites by the low affinity sites was determined and subtracted (see Fig. 4.4). The corrected data for the high affinity component were replotted (Fig. 4.5) and the best straight line drawn with weighting to the points of low receptor occupancy. Four individual binding experiments were carried out using different cell preparations. The results from these experiments are given in Table 4.1 with both binding affinities and number of sites per cell reported. The high

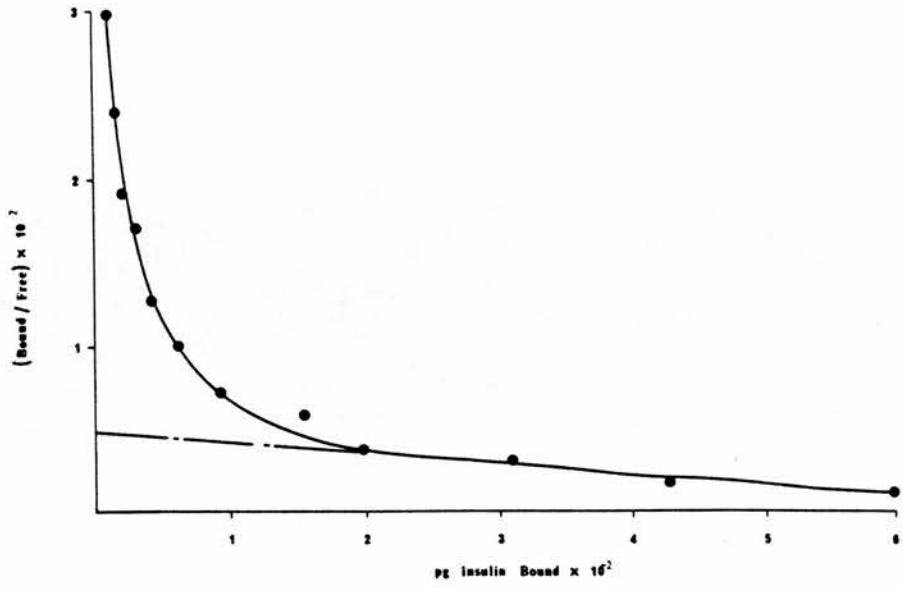


Fig. 4.4 Scatchard plot of specific insulin binding to cells.

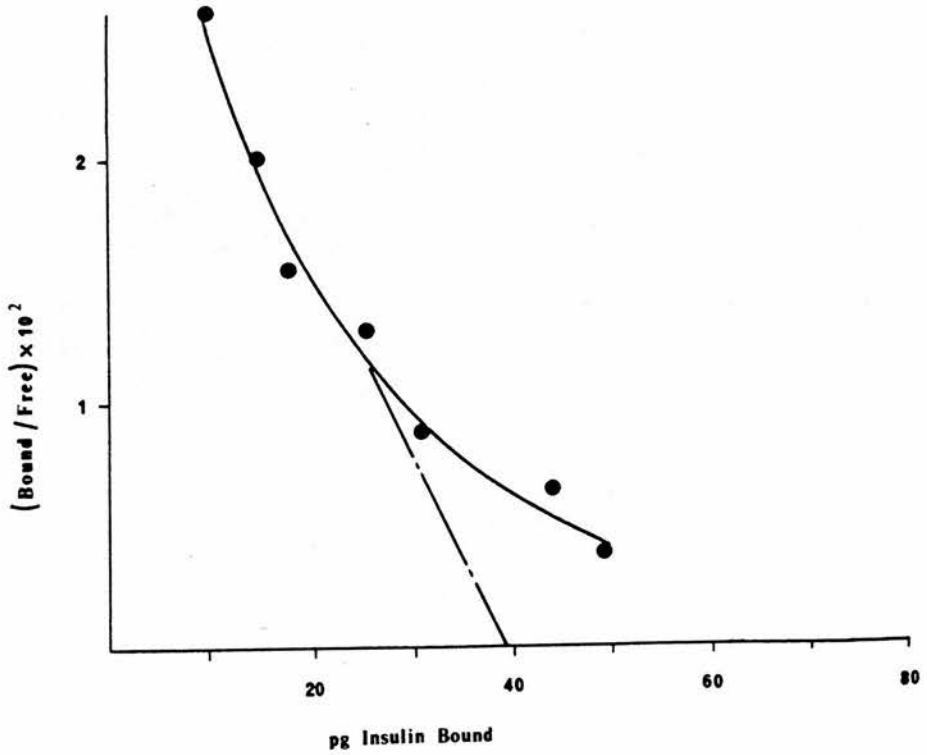


Fig. 4.5 Scatchard replot of high affinity insulin binding sites after subtraction of low affinity values.

Table 4.1: Insulin Binding - calculated affinity constants and binding capacities from Scatchard analyses.

Experiment Number	High affinity site		Low affinity site	
	$K_d \times 10^{-10} \text{ M}$	Binding capacity sites per cell	$K_d \times 10^{-7} \text{ M}$	Binding capacity sites per cell
1	4.32	2415	3.6	~750,000
2	3.07	3178	2.4	~600,000
3	2.19	1854	1.5	~550,000
4	2.42	1264	0.3	~950,000

High affinity site

Average $K_d = 3.00 \times 10^{-10} \pm 0.96 \times 10^{-10} \text{ M}$ (SD; n=4).

Average binding capacity = 2178 ± 816 (SD; n=4).

Low affinity site

Average $K_d = 1.95 \times 10^{-7} \pm 1.40 \times 10^{-7} \text{ M}$ (SD; n=4).

Average binding capacity = $713,000 \pm 180,000$ (SD; n=4).

affinity binding sites showed an averaged dissociation constant ($K_d^{av.}$) of $3.00 \times 10^{-10} M \pm 0.96 \times 10^{-10} M$ (S.D.) with approximately 2,000 receptor sites per cell. The low affinity sites were much more variable with K_d ranging from $0.3 \times 10^{-7} M$ to $3.6 \times 10^{-7} M$ and approximately 700,000 receptor sites per cell.

- e) Dissociation of ^{125}I -insulin from hepatocytes. Cell suspension (5.0 to 8.0×10^6 cells/ml) was incubated with ^{125}I -insulin (0.35 ng/ml) for 45 minutes at $22^\circ C$. Cells were then pelleted at 60 g for 2 minutes and resuspended to the same volume in fresh KRB buffer. Aliquots of resuspended cell suspension were diluted ten fold with KRB buffer or KRB buffer containing 1.0 $\mu g/ml$ native insulin and further incubated for various times at one of four temperatures - $10^\circ C$, $22^\circ C$, $30^\circ C$ or $40^\circ C$. After preset times, aliquots (1 ml) of suspension were removed and cell bound radioactivity determined as before. Parallel initial incubations were carried out in the presence of native insulin (5 $\mu g/ml$) to estimate the extent of nonspecific binding. The results were all corrected and reported as specific insulin binding. The rate of dissociation of labelled insulin from hepatocytes was dependent on temperature (Fig. 4.6). The presence of added native insulin to incubations after dilution increased the dissociation rate at all temperatures used. The dissociation rates in the presence or absence of native insulin were not linear with the time and semi-logarithmic plots were also non-linear (Fig. 4.7).
- f) ^{125}I -insulin degradation by hepatocytes. ^{125}I -insulin (0.5 ng/ml) was added to cell suspension (9.0×10^6 cells/ml) and incubated at $37^\circ C$ for various times before the addition of an equal volume of ice cold 10% TCA. Samples were shaken, left at room temperature for 30 minutes to allow maximum precipitation of protein and then centrifuged (2750 g; 1 minute). A sample of the supernatant was removed to determine the amount of non-protein radioactivity remaining in the supernatant.

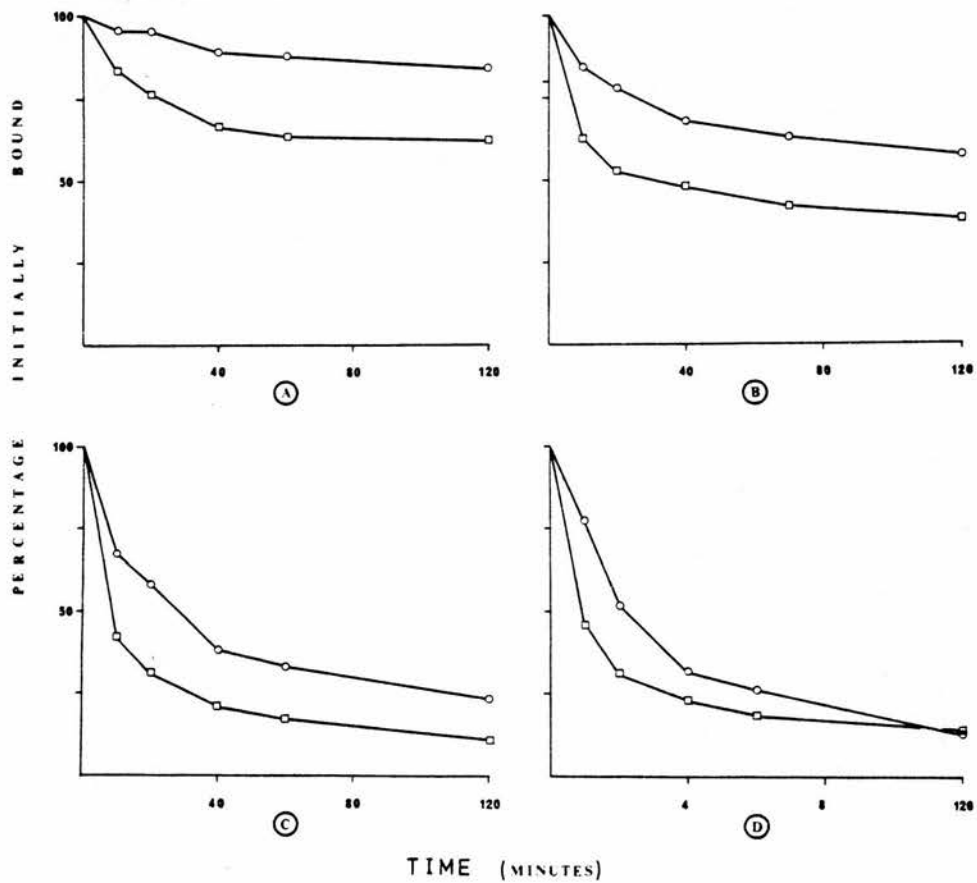


Fig. 4.6 Dissociation of bound ^{125}I -insulin from cells at A. 10°C , B. 22°C , C. 30°C or D. 40°C in the absence (O-O) and presence (□-□) of unlabelled insulin ($1\ \mu\text{g}/\text{ml}$).

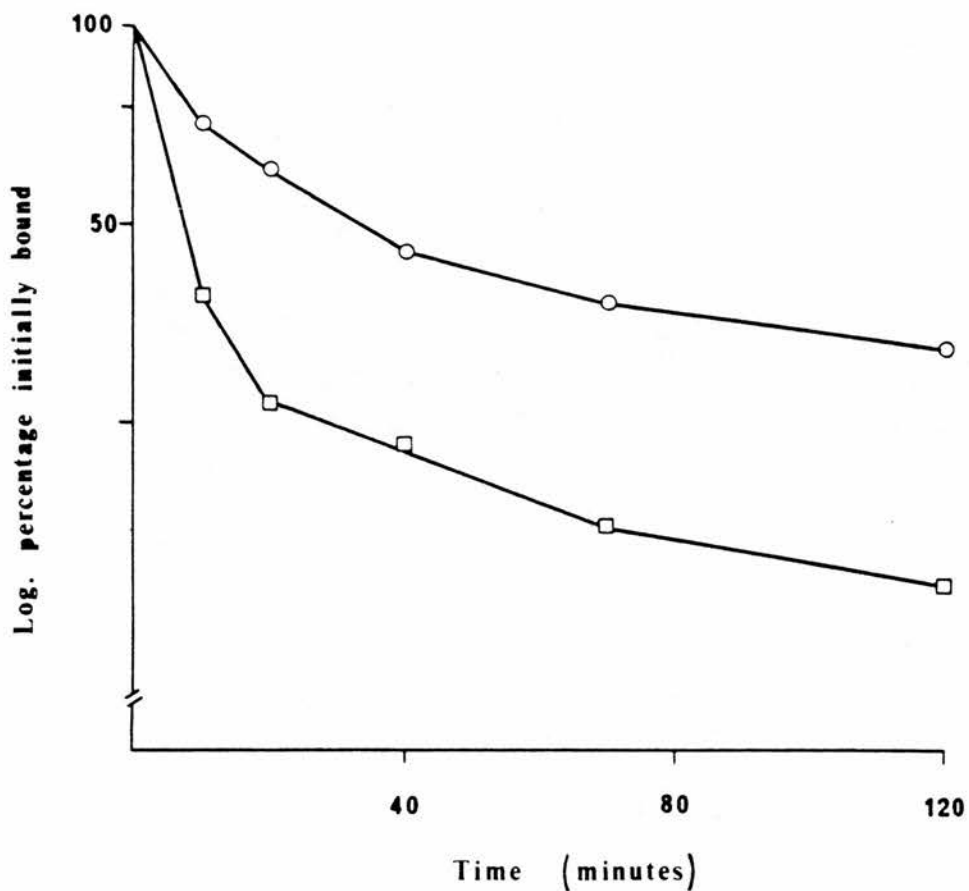


Fig. 4.7 Semi-logarithmic plot of ^{125}I -insulin dissociation from cells incubated at 22°C in the absence (O-O) and presence (□-□) of unlabelled insulin ($1\ \mu\text{g}/\text{ml}$).

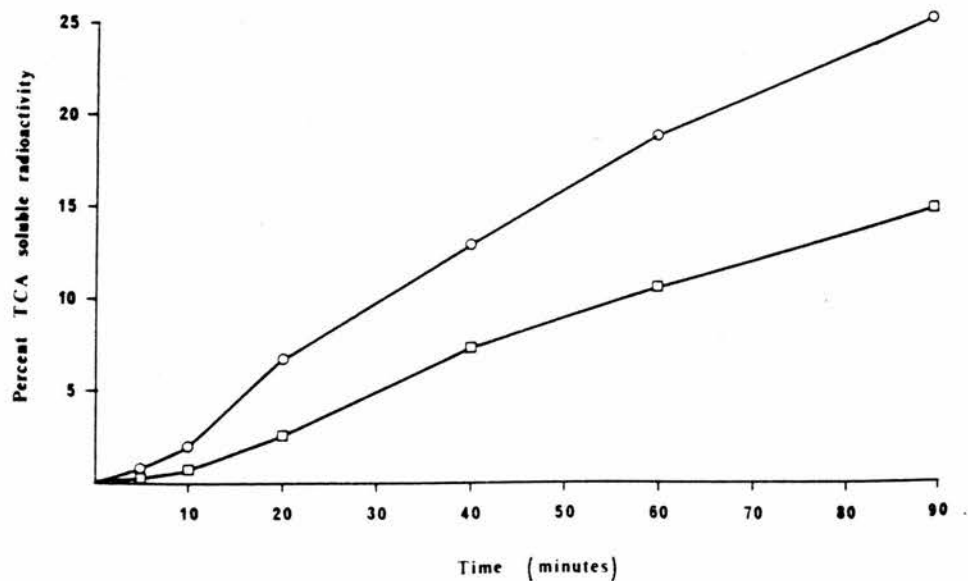


Fig. 4.8 Degradation of ^{125}I -insulin in cell suspensions incubated at 37°C in the absence (O-O) and presence (□-□) of bacitracin ($0.75\ \text{mg}/\text{ml}$).

Fig. 4.8 shows the increase in radioactivity in the supernatant with time. Zero time soluble radioactivity (approximately 2 to 3% of total) was subtracted from all other results obtained. Incubations containing 0.75 mg/ml bacitracin had an inhibitory effect on ^{125}I -insulin degradation but did not prevent degradation completely. The effect of cell concentration on insulin degradation at 22°C is shown in Table 4.2. Only very low levels of TCA soluble radioactivity are present after 45 minutes even with cell concentrations above 20×10^6 cells/ml.

^{125}I -Glucagon-Receptor Interactions

a) Viability of tracer. For details of iodination and other aspects of tracer preparation and viability see Chapter 3. Cellulose column purification increased the proportion of viable iodinated hormone in the sample. Results of typical chromatoelectrophoretic patterns of iodinated tracer solution before and after cellulose or urea-DEAE column purification are shown in Figs. 4.9 and 4.10. In all ^{125}I -glucagon preparations purified by cellulose chromatography, 5 to 15% of the total radioactivity of the tracer was not present as viable glucagon; this was taken into consideration when calculating results. ^{125}I -glucagon purified by urea-DEAE cellulose column was not used in binding experiments.

The biological activity of ^{125}I -glucagon was also examined. Isolated chicken hepatocytes are very sensitive to glucagon with very low concentrations inducing marked glycogenolysis (see Chapter 6). The extent of induced glycogenolysis by various dilutions of ^{125}I -glucagon was compared to a standard dose response curve for native glucagon (Fig. 4.11). Doubling the amount of ^{125}I -glucagon added resulted in a larger stimulation of glycogenolysis than was expected. ^{125}I -glucagon always showed an increased sensitivity over native glucagon. From the results obtained in the glycogenolysis experiments the relative potencies of ^{125}I -glucagon and native glucagon were determined with the former being approximately 150% as active as the latter.

Table 4.2: Effect of cell concentration on insulin degradation over 45 minutes at 22°C.

Cell concentration x 10 ⁶ cells/ml	Percentage TCA soluble radioactivity
2.9	0.1
5.8	1.8
11.7	3.0
23.4	4.3

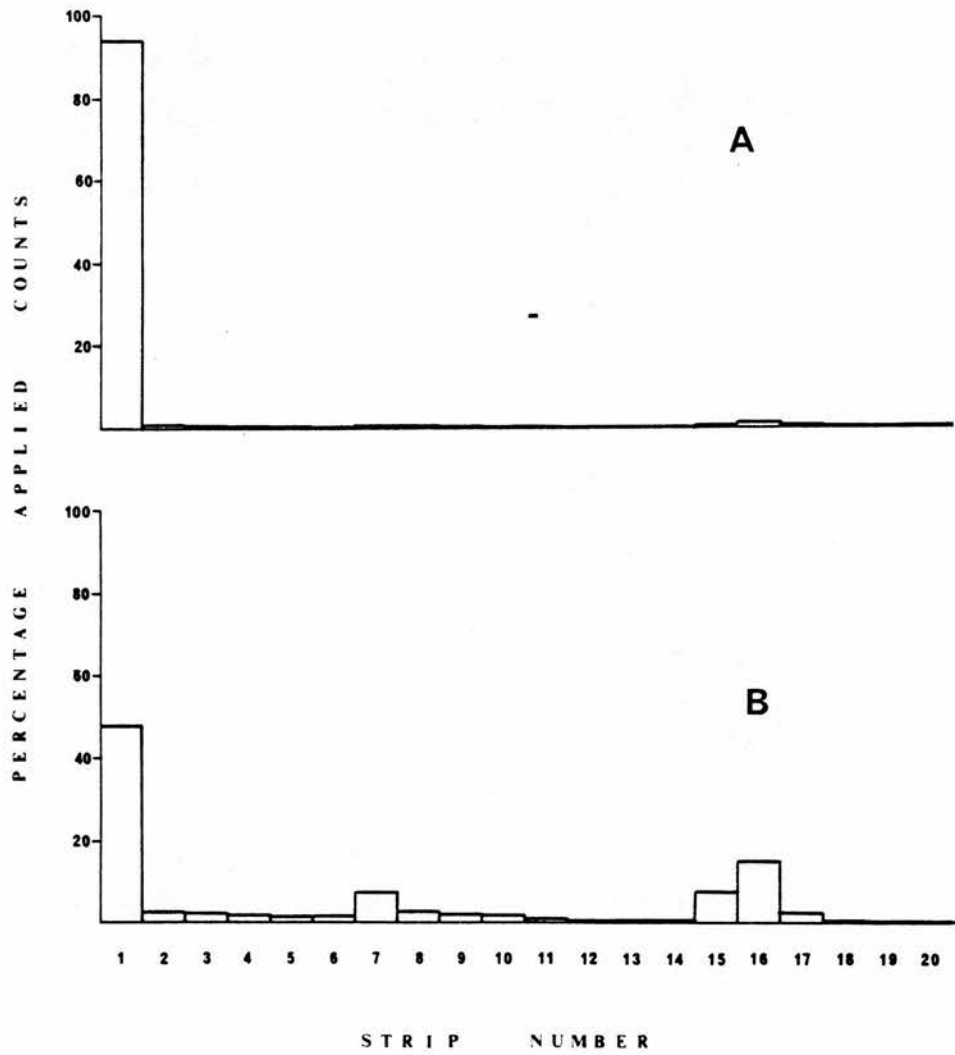


Fig. 4.9 Paper chromatoelectrophoretic pattern of ^{125}I -glucagon from, A. cellulose column purification; B. original iodination mixture.

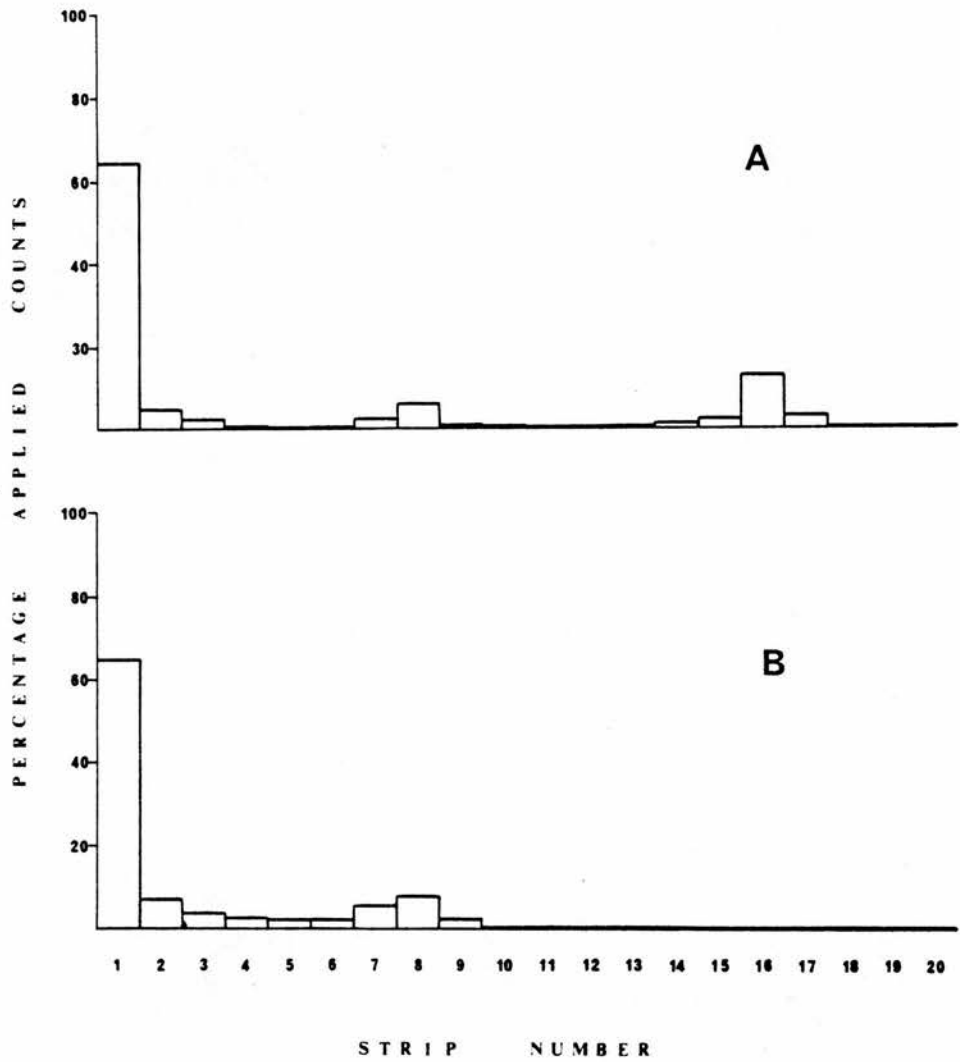


Fig. 4.10 Paper chromatoelectrophoretic pattern of ^{125}I -glucagon from, A. original iodination mixture; B. urea-DEAE column purification procedure.

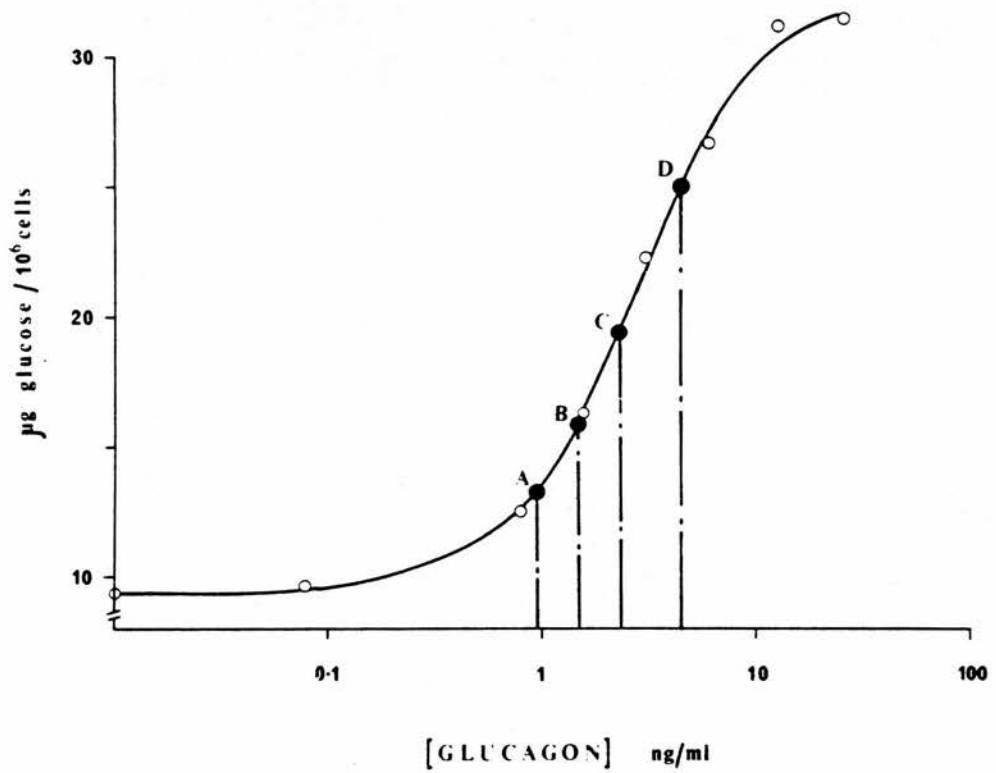


Fig. 4.11 Glucagon dose response curve to cellular glucose release. Unlabelled glucagon (O-O) and ^{125}I -glucagon (●) stimulated. Volumes of ^{125}I -glucagon stock added - A. 5 μl ; B. 7.5 μl ; C. 10 μl ; D. 15 μl .

b) Time course of ^{125}I -glucagon binding. Cell suspension (8.0×10^6 cells/ml) containing ^{125}I -glucagon (0.22 ng/ml) was incubated at 22°C for various times. Aliquots (1 ml) were then removed and radioactivity bound to cells determined by centrifugation as in the insulin studies (Fig. 4.12). Incubations were also carried out with native hormone at $10 \mu\text{g/ml}$. Counts still bound in the presence of $10 \mu\text{g/ml}$ native glucagon were taken to represent non-specific binding. At 22°C glucagon binding reached a steady state equilibrium after 20 minutes. All future incubations were carried out for 30 minutes to ensure steady state binding. Addition of small amounts of native hormone or decreasing cell concentration by half did not have a marked effect on the binding rate; binding was proportional to the cell number. High concentrations of insulin ($10 \mu\text{g/ml}$) or APP ($5 \mu\text{g/ml}$) did not decrease the amount of ^{125}I -glucagon bound.

c) Binding affinity. Cell suspensions (3.0×10^6 cells/ml and below) were incubated for 30 minutes in the presence of tracer (0.2 ng/ml) with native hormone at a range of concentrations (0.4 to $10,000 \text{ ng/ml}$). Dose response binding curves showed a high proportion of low affinity specific sites. High concentrations of native hormone (10 to $20 \mu\text{g/ml}$) were needed to successfully reduce tracer binding by 80 to 90% (Fig. 4.13). Radioactivity bound to cells in the presence of $10 \mu\text{g/ml}$ native hormone was considered non-specific although increasing the native hormone concentration to $20 \mu\text{g/ml}$ consistently removed another 3 to 5% of the bound radioactivity. Scatchard plots again revealed more than one class of binding site, but the analysis of the data was very difficult due to the high levels of low affinity sites (Fig. 4.14). Like that of insulin, the results were processed as if resulting from two classes of sites. Extrapolation and subtraction of the high values for the low affinity component from the values for the high affinity component unavoidably

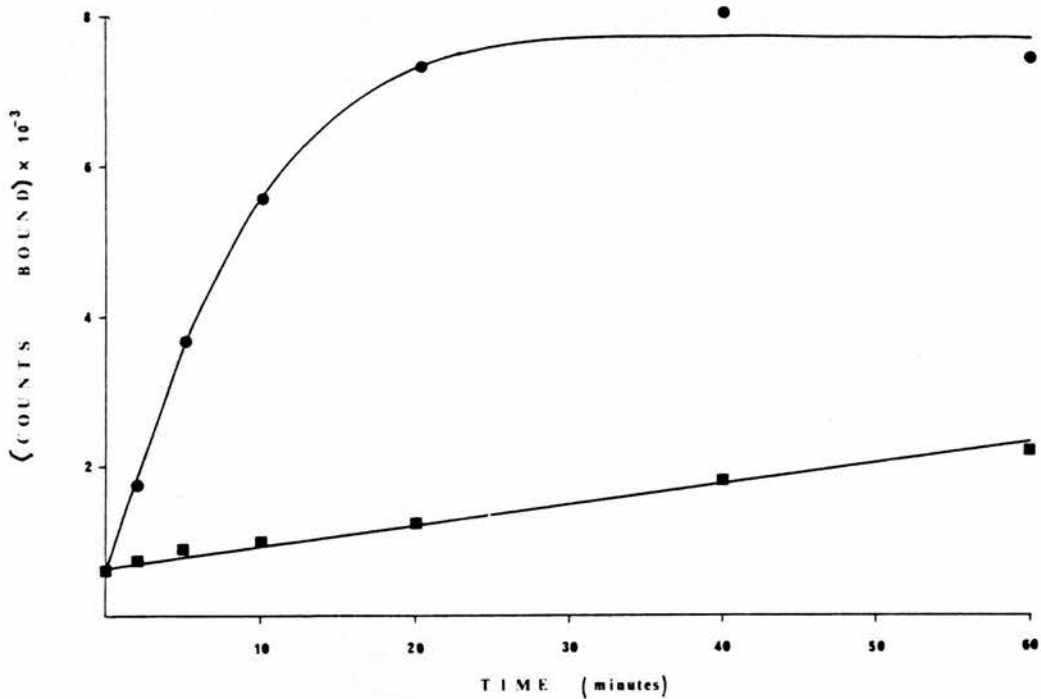


Fig. 4.12 Time course of ^{125}I -glucagon (0.22 ng/ml) binding in the absence (●-●) and the presence (■-■) of unlabelled glucagon (10 $\mu\text{g/ml}$).

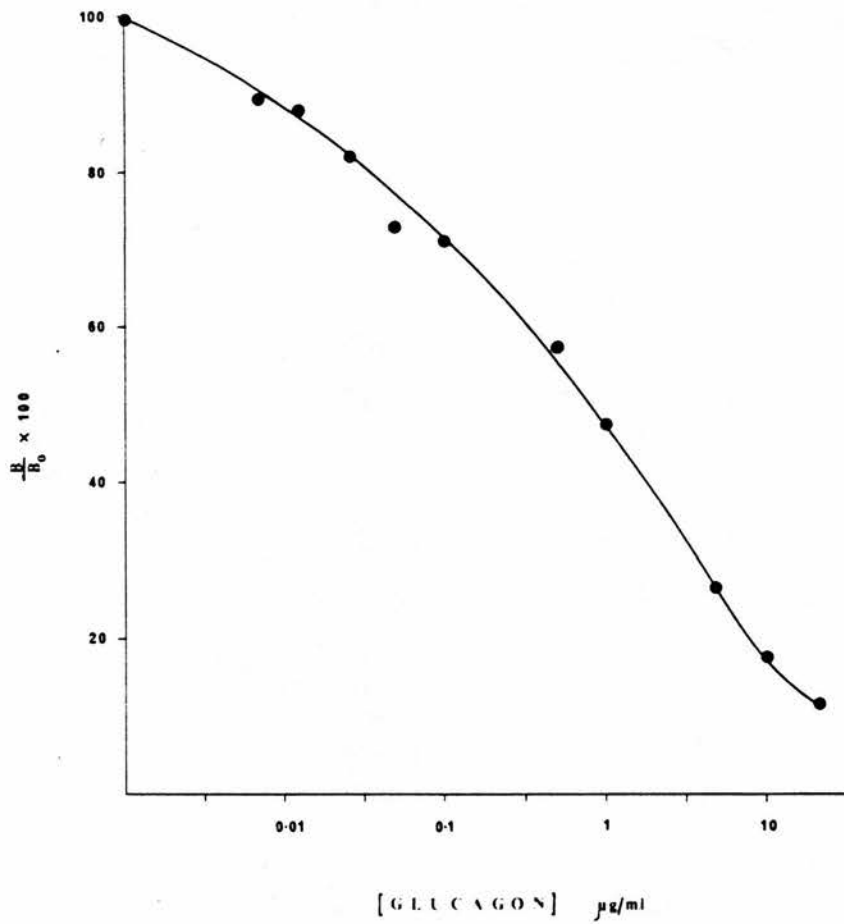


Fig. 4.13 Reduction in ^{125}I -glucagon (0.2 ng/ml) bound to cells by increasing unlabelled glucagon. B-radioactivity bound
 B_0 - radioactivity bound on the absence of unlabelled glucagon.

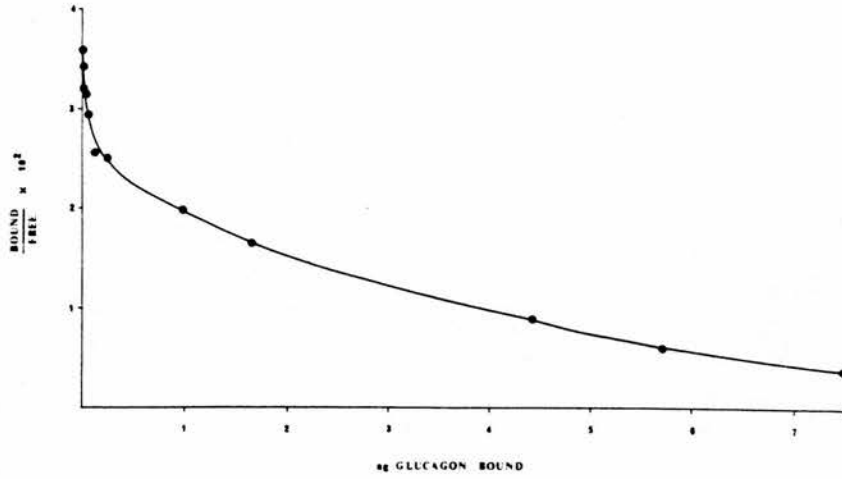


Fig. 4.14 Scatchard plot of glucagon binding to cells.

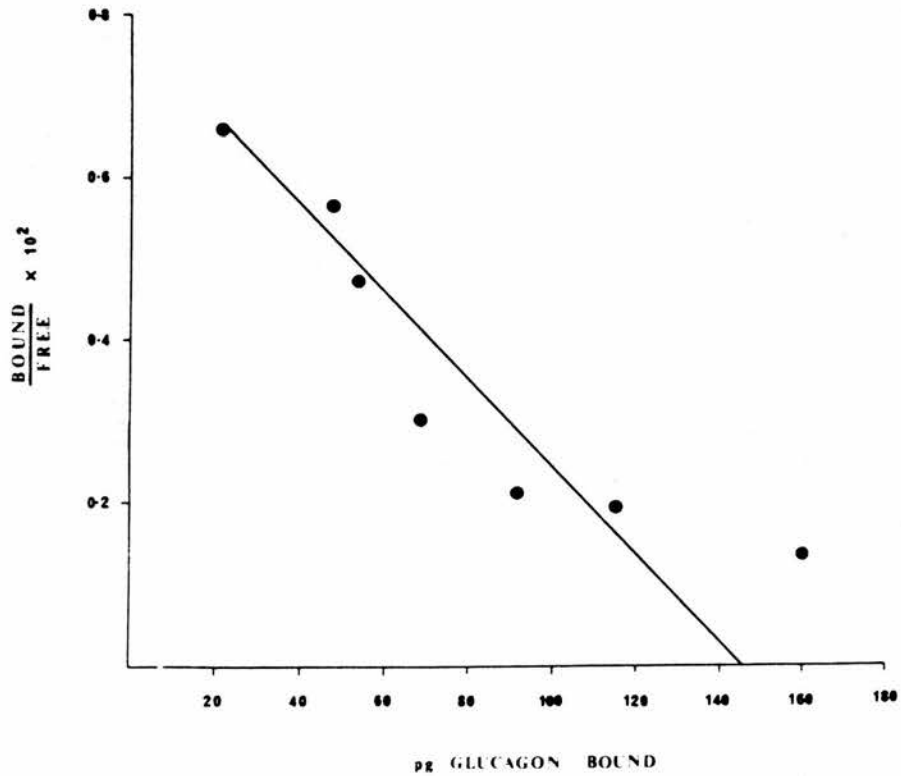


Fig. 4.15 Scatchard replot of high affinity binding to cells. Low affinity component subtracted from all high affinity values.

resulted in large errors. Fig. 4.15 shows a Scatchard plot of the recalculated high affinity values for one experiment. Affinity constants and number of binding sites were determined for the high affinity sites only, by using the values found for the slope and intercept of the replotted data and are given in Table 4.3. Values for the low affinity site proved difficult to estimate but generally resulted in a K_d of 4×10^{-5} M or higher and around 1,500,000 sites/cell. The average affinity of the glucagon receptor was calculated $K_d^{av} 9.4 \times 10^{-9} \pm 3.1 \times 10^{-9}$ M (SD - five observations) and the number of binding sites found to be in the order of 13,000 sites per cell.

d) 125 I-glucagon dissociation. 125 I-glucagon (0.2 ng/ml) was preincubated with cell suspension (3.5×10^6 cells/ml) for 30 minutes at 22°C . Cells were pelleted by centrifugation (60 g: 2 min) and resuspended to the original volume with fresh KRB buffer. The suspension was diluted ten fold with KRB buffer or KRB buffer containing 2 $\mu\text{g/ml}$ native glucagon and incubated for the times indicated at 37°C (Fig. 4.16). Only 50% of the total 125 I-glucagon bound was released after incubations for up to 90 minutes in duration with the majority of the tracer (approx. 40%) having dissociated within the first 20 minutes. The presence of native glucagon decreased the amount bound by approximately another 10% after 90 minutes but did not increase the initial rate of dissociation. Semi-logarithmic plots of the dissociation figures still were non-linear (Fig. 4.17).

e) 125 I-glucagon degradation. Degradation was again judged by the appearance of radioactivity in a TCA precipitated cell supernatant fraction. Soluble counts at zero time (10% of total or less) have been subtracted from all results. The effect of cell concentration on degradation of tracer at 22°C and 37°C is shown in Fig. 4.18. At 22°C , concentrations of 3.0×10^6 cells per ml or below show the same proportion of tracer degradation rate after 30 minutes incubation. Higher cell concentrations show

Table 4.3: Glucagon Binding - calculated affinity constants and binding capacities from Scatchard analyses. High affinity binding only.

Experiment Number	K_d $\times 10^{-9}$ M	Binding capacity sites per cell
1	5.6	15,000
2	7.2	6,900
3	9.34	10,177
4	12.5	12,933
5	12.5	19,700

Average $K_d = 9.4 \times 10^{-9} \pm 3.1 \times 10^{-9}$ M (SD; n=5).

Average binding capacity = $12,942 \pm 4849$ (SD; n=5).

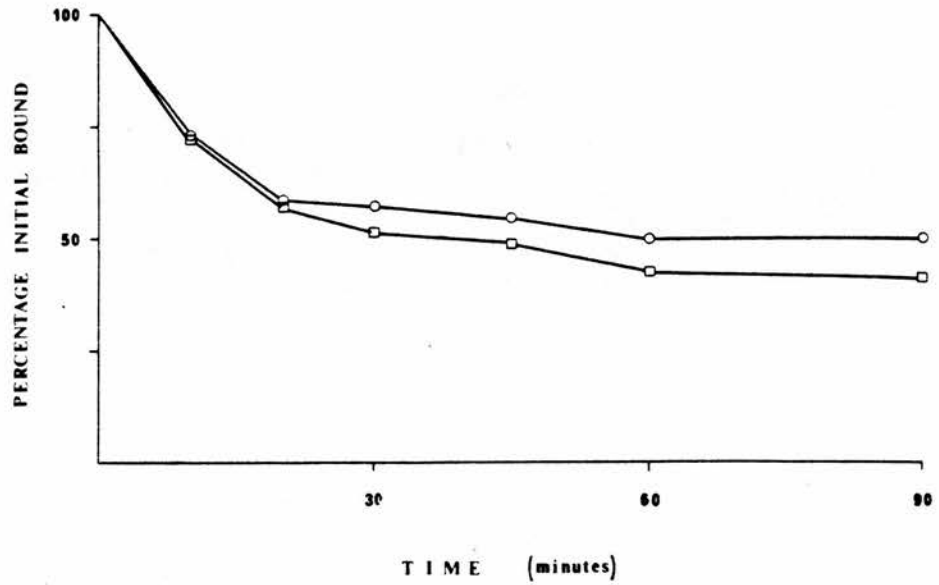


Fig. 4.16 Dissociation of bound ^{125}I -glucagon from cells incubated in the absence (O-O) or presence (□-□) of unlabelled glucagon (2 $\mu\text{g}/\text{ml}$).

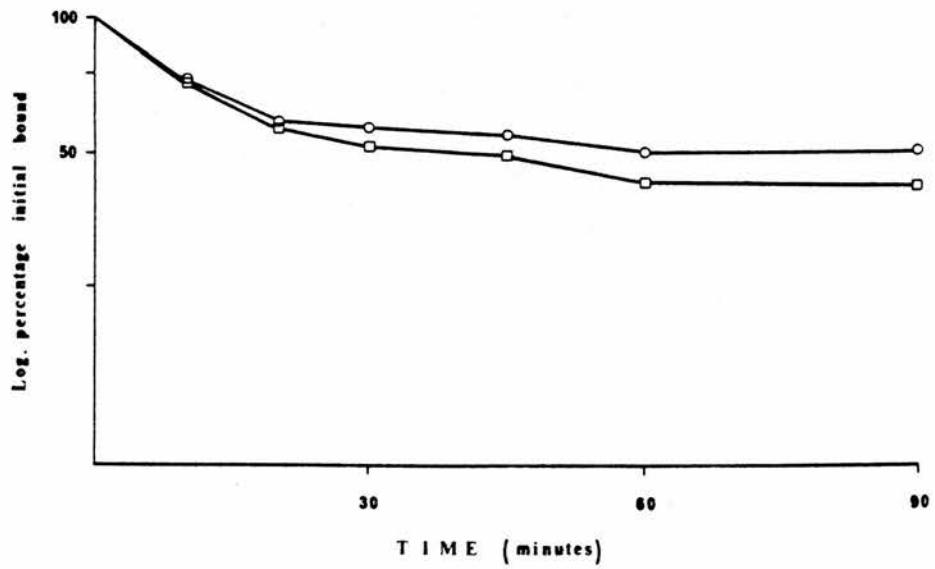


Fig. 4.17 Semi-logarithmic plot of dissociation of bound ^{125}I -glucagon in the absence (O-O) or presence (□-□) of unlabelled glucagon (2 $\mu\text{g}/\text{ml}$).

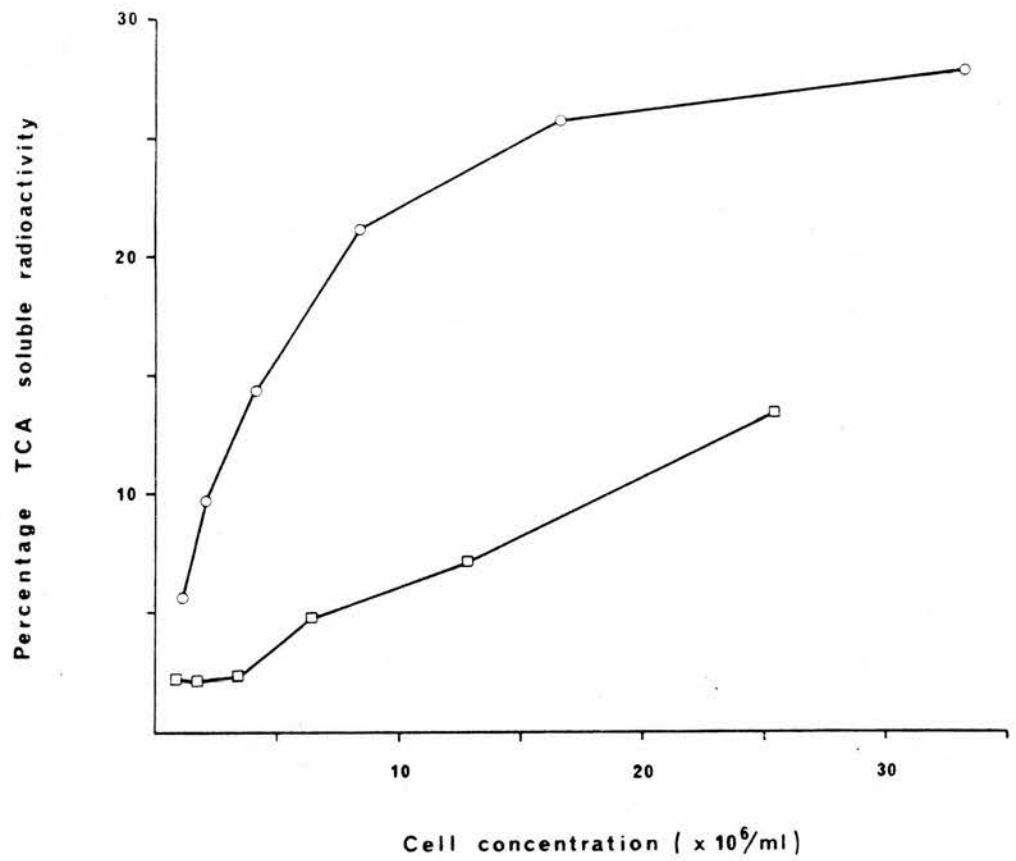


Fig. 4.18 Effect of cell suspension concentration on the degradation of ¹²⁵I-glucagon (0.2 ng/ml) after 30 minutes incubation at 37°C (O-O) or 22°C (□-□).

substantial increases in degradation especially at 37°C where a hyperbolic relationship exists between cell concentration and fraction of hormone degraded. The degradation rate of ^{125}I -glucagon at 37°C was found to vary considerably between individual cell preparations with degradation rates of up to half that plotted in Fig. 4.18 being found. Bacitracin (0.75 mg/ml) only slightly inhibited the degradation of ^{125}I -glucagon at 37°C (Fig. 4.19). At the low cell concentration and temperature used for steady state binding studies the degradation of tracer is approximately linear up to 1 hour with loss of only 2-3% of the tracer in 30 minutes (Fig. 4.20).

^{125}I -APP-Receptor Interactions

- a) Tracer viability. As with insulin there was no biological assay available to determine the viability of the iodinated hormone. Unlike insulin however there was also no antibody to APP available to test the immunological properties of the labelled hormone. The methods used to estimate viability and the values obtained for acid precipitability and binding to talc are given in Chapter 3. The chromatoelectrophoretic pattern of iodinated products before and after gel column purification are shown in Fig. 4.21.
- b) Time course of ^{125}I -APP binding. ^{125}I -APP (0.5 ng/ml) was incubated with cell suspension (6.0×10^6 cells/ml) at 22°C for various times. Radioactive bound tracer was determined as before. A parallel incubation containing 2 µg/ml native APP was also carried out to estimate nonspecific binding (Fig. 4.22). Binding of tracer reached a steady state after 40 minutes incubation. The high native APP concentration; although successfully depressing the steady state binding by more than 50% failed to completely inhibit specific binding.
- c) Binding affinity. ^{125}I -APP (0.5 ng/ml) and native hormone at various concentrations (1.28 to 10,000 ng/ml) were incubated with cell suspension

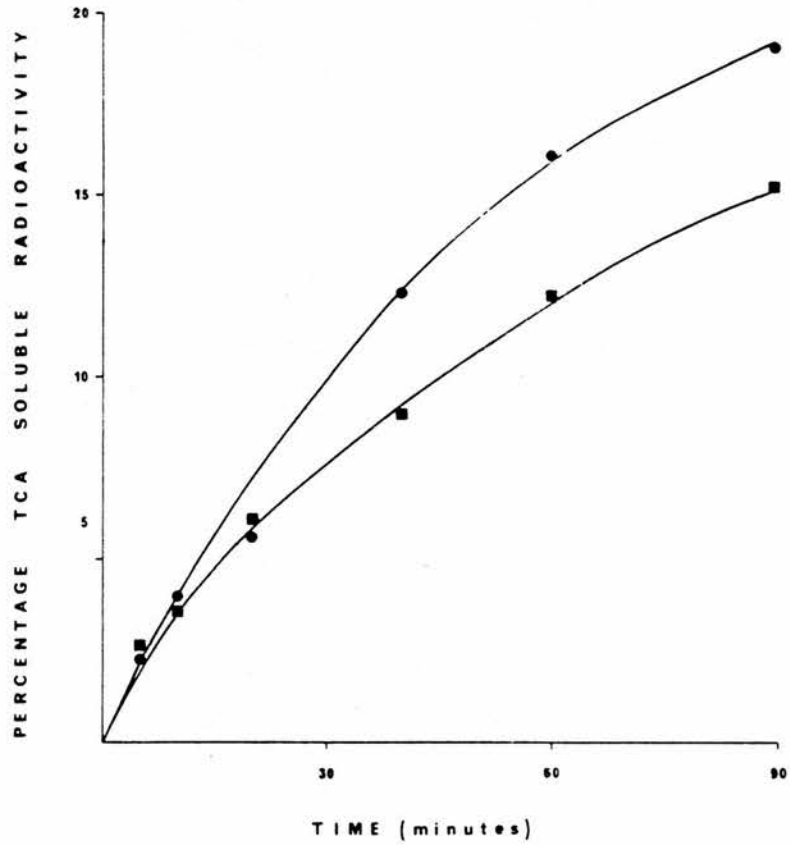


Fig. 4.19 Degradation of ^{125}I -glucagon (0.2 ng/ml) in cell suspension (2.6×10^6 cells/ml) incubated at 37°C in the absence (●-●) or presence (■-■) of bacitracin (0.75 mg/ml).

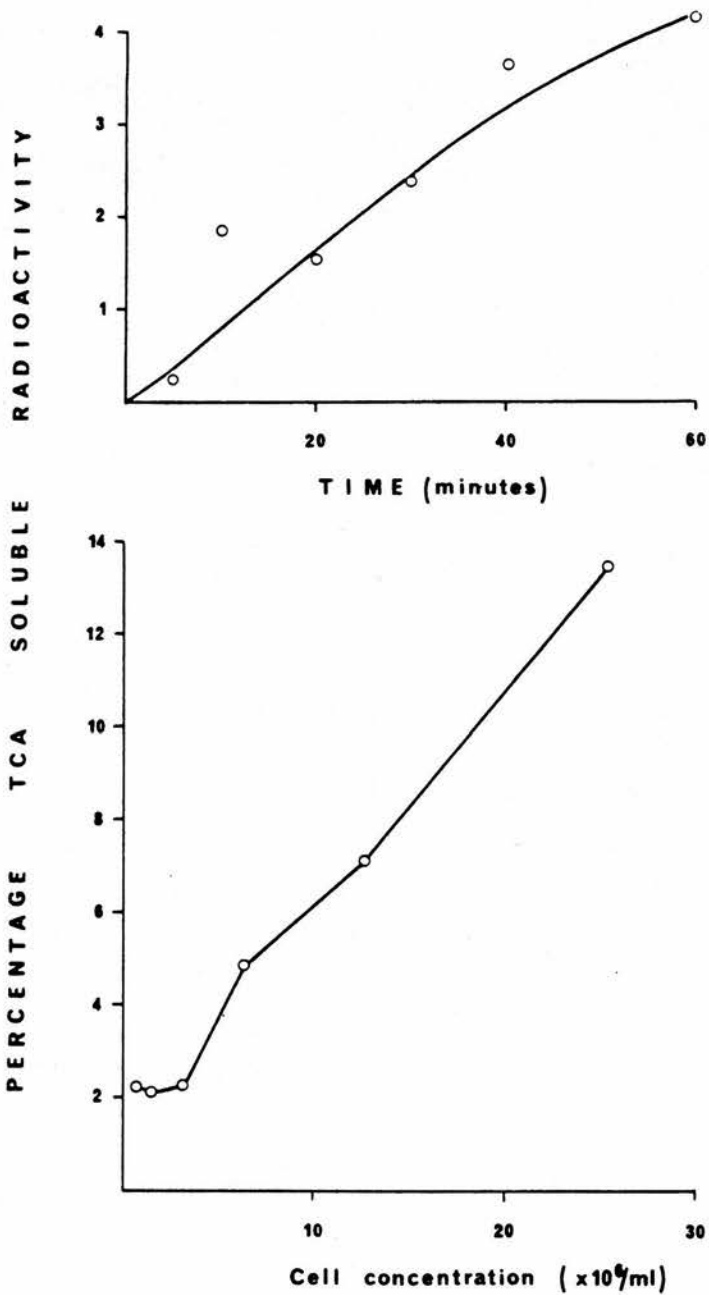


Fig. 4.20 Top - ¹²⁵I-glucagon degradation rate at low concentration of cell suspension (less than 5×10^6 cells/ml) incubated at 22°C. Bottom - Effect of cell concentration on degradation after 30 minutes at 22°C.

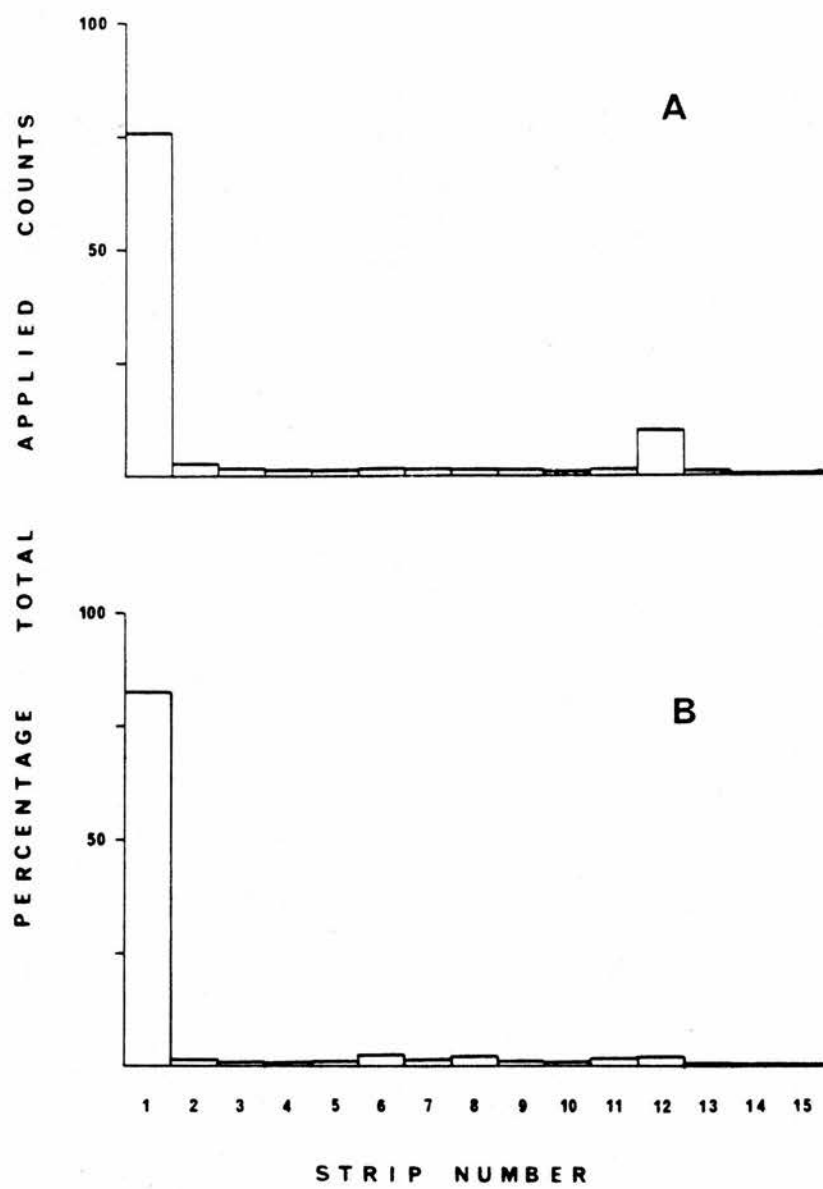


Fig. 4.21 Chromatoelectrophoretic pattern of ^{125}I -APP. A. sample of the iodination mixture. B. sample after sephadex G-25 column purification.

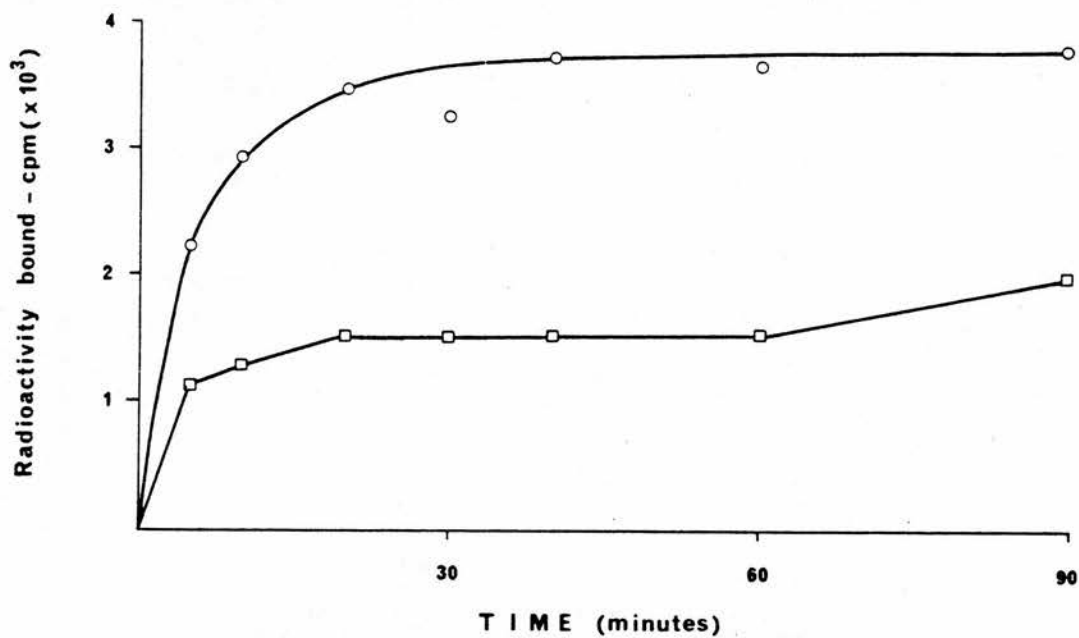


Fig. 4.22 Time course of ^{125}I -APP (0.5 ng/ml) binding to cell suspension (6.0×10^6 cells/ml) incubated at 22°C in the absence (O-O) or presence (□-□) of unlabelled APP (2 $\mu\text{g/ml}$).

(6.0 to 8.0 x 10⁶ cells/ml) for 40 minutes at 22°C. The amount of radioactivity bound after incubation was determined by centrifugation. Counts remaining bound in the presence of 10 µg/ml native hormone was taken as nonspecific binding and data interpreted by Scatchard plot analysis. Scatchard plots of the data resulted in only one class of binding site (Fig. 4.23). Processing of the data revealed a very low affinity high capacity binding site with K_d of approximately 2 x 10⁻⁶ M with nearly 300,000 sites per cell.

d) Degradation of ¹²⁵I-APP. Degradation of ¹²⁵I-APP was estimated by acid precipitation, using the same method as with ¹²⁵I-insulin and ¹²⁵I-glucagon. A low rate of appearance of TCA soluble radioactivity indicated a slow rate of APP degradation. Even at 37°C and at relatively high cell concentrations (up to 16.0 x 10⁶ cells/ml) less than 5% of the total radioactivity was found in an acid soluble extract of suspension after incubation for 40 minutes (Fig. 4.24).

Discussion

Before attempting to correlate the binding results obtained using the three hormones, insulin, glucagon and APP, and comparing them with the findings of other workers using chicken and rat hepatocytes it must first be noted that all results obtained depend on the individual characteristics of the iodinated hormones. Several workers have succeeded in isolating the monoiodo-derivatives of insulin and glucagon from iodination mixtures by DEAE cellulose or DEAE sephadex chromatography (Freychet et al. 1971; Nottey and Rosselin 1971; Sodoyez et al. 1975) thus at least ensuring a homogeneous tracer population as well as a high specific activity. Mono-iodoinsulin has been reported to have almost identical biological activity to that of the native hormone (Bihler and Morris 1972; Hamlin and Arquilla 1974; Sodoyez et al. 1975) whereas mono-iodoglucagon has an enhanced binding affinity as well as enhanced

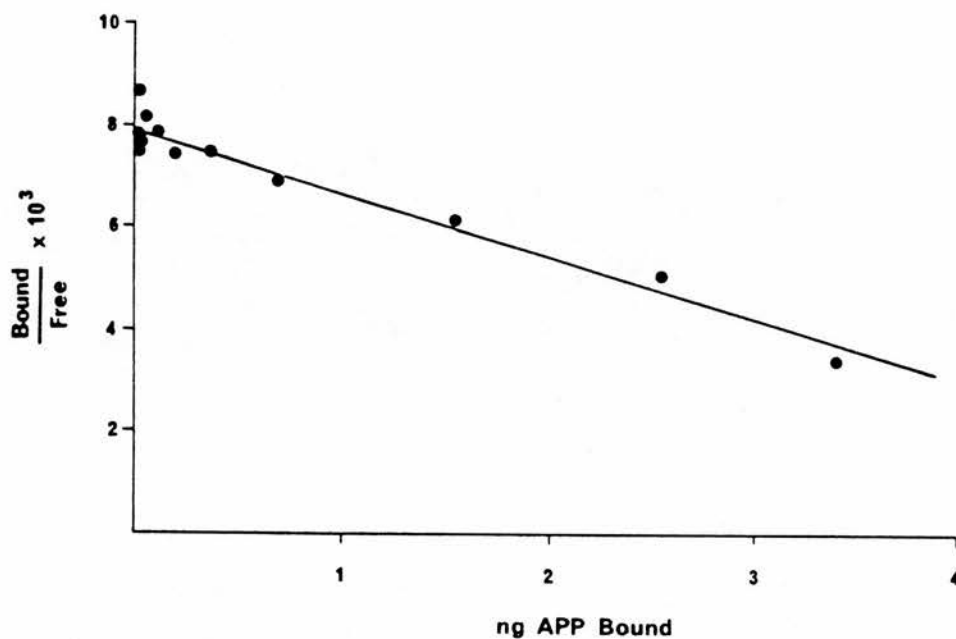


Fig. 4.23 Scatchard plot of APP binding to cells.

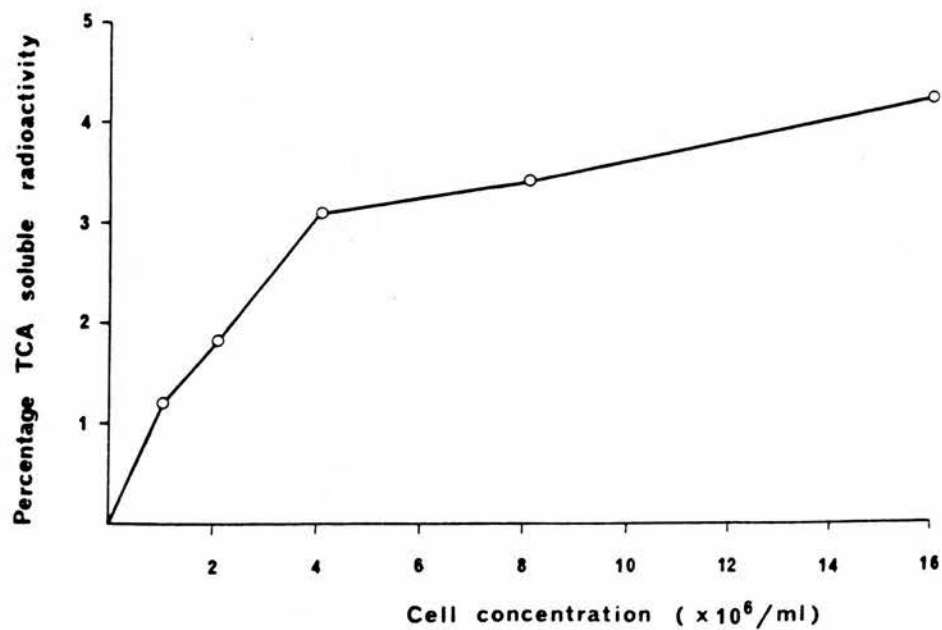


Fig. 4.24 Effect of cell concentration on the degradation of ^{125}I -APP in suspensions incubated for 40 minutes at 37°C .

biological potency (Desbuquois 1975). Assessing the biological potency of the hormone as a guide to tracer viability may not give an accurate assessment of binding affinity. Desbuquois (1975) has shown that when using multi-iodinated derivatives of glucagon, binding characteristics and potency of activating adenylate cyclase are not directly proportional.

Although very few studies of APP have been made it has been found that a ^{125}I -APP preparation with iodide to protein ratios of 1.6 to 1 still retains biological activity in chicken adipocytes, although potency is lower than that of the native hormone (McCumbee and Hazelwood 1977).

Therefore when calculating binding affinities using ^{125}I -labelled hormones it must be accepted that the values obtained for the dissociation constants and rate constants etc., will only be as accurate as the tracer allows. Because of this and due to the relatively large experimental errors that accompany these type of radioactive experiments the binding affinities obtained by mathematical treatment of the experimental results given in this Chapter may only be accurate to within one order of magnitude. However this may be sufficient for interesting comparisons to be made with results obtained by other workers using rat and chicken hepatocytes.

Insulin Binding

Freychet and coworkers (Simon et al. 1977a) recently reported that there was a lower number of insulin receptor sites per cell, and indeed per unit surface area of plasma membrane, in chicken hepatocytes than in rat hepatocytes. Chicken hepatocytes isolated by the method of Capuzzi et al. (1974) and chicken liver plasma membranes isolated by the method of Neville (1968) showed half maximum binding constants of $1.3 \times 10^{-9}\text{M}$ and $3.2 \times 10^{-9}\text{M}$ respectively. This compares well with values obtained for rat hepatocytes and liver membranes of $2.0 \times 10^{-9}\text{M}$ and $3.0 \times 10^{-9}\text{M}$ respectively, derived from experiments carried out under the same conditions by the same group of workers (Freychet et al. 1974).

Values found in the present work were of the order of 3×10^{-10} M.

Gammeltoft et al. (1978) however, also found that the receptor on rat hepatocytes had a higher affinity than that reported by Freychet's group, steady state binding experiments giving dissociation constants of $4-5 \times 10^{-10}$ M.

There appears to be a great deal of variation between individual workers on reported affinity constants for insulin binding to receptor. Affinity values have been reported which vary over three orders of magnitude with ' K_d ' ranging from 5×10^{-11} M to 8×10^{-8} M. The main discrepancy seems to lie not with the individual tracer preparation or receptor preparation or even variations in the experimental details such as incubation temperatures, but in how individual workers interpret the data obtained. Many investigators report figures that give half maximum inhibition of tracer binding even although it is sometimes apparent that a heterogenous population of receptor sites exists. The figures derived by this method will obviously be dependent on the proportion of the low affinity component as well as that of the high affinity component and therefore intermediate binding constants are found. Examples of this type of interpretation for rat hepatocytes include work by Freychet et al. (1974) and Freychet (1976) who report a K_d of 2×10^{-9} M, Terris and Steiner (1975) K_d 3.5×10^{-9} M, Olefsky et al. (1975) 4×10^{-9} M and also, for chicken hepatocytes, by Simon et al. (1977a) K_d 1.3×10^{-9} M. Similar results are obtained when purified plasma membrane fractions are used as the receptor preparation. Reports of half maximal inhibition constants for rat liver membranes of 3.0×10^{-9} M (Freychet 1974) and chicken liver membranes of 3.2×10^{-9} M (Simon et al. 1977a) have been reported. However when binding data are analysed for the presence of two or even three individual binding components much lower dissociation constants are recorded for the higher affinity site. Reports of steady

state binding data analysed into separate components show high affinity dissociation constants, with K_d of 4×10^{-10} M for rat hepatocytes (Gammeltoft et al. 1978) and 5.0×10^{-10} M for rat liver membranes (Kahn et al. 1974). Values obtained for insulin binding to chicken hepatocytes as given in this work compare well with those of Gammeltoft et al. and Kahn et al. with K_d^{av} of 3.0×10^{-10} M.

Although there may be some discrepancy in the interpretation of affinity values for insulin binding it appears that the receptors affinity for the chicken liver insulin receptor is approximately the same as that found in rat liver whether the data are treated as arising from single or multicomponent sites. Half maximal binding constants although reporting a much lower overall affinity also indicate that a similar receptor population exists in rat and chicken liver (Simon et al. 1977_{a,b})

Reported binding capacities for the hormone are also dependent on the treatment of the data, as well as temperature of incubation. In general, low incubation temperatures result in higher binding capacities. Results which have been analysed with respect to two classes of sites yield binding capacities for rat hepatocytes of about 15,000 sites per cell (Gammeltoft et al. 1978) and for rat liver membranes of 4×10^{10} sites per mg protein (Kahn et al. 1974). Analyses in terms of a single class of sites result in higher capacities being reported, with rat liver having 80 to 90×10^3 sites per cell or around 160×10^{10} sites per mg protein (Freychet et al. 1974; Freychet 1976) and chicken liver 4.6×10^3 sites per cell or 30×10^{10} sites per mg protein (Simon et al. 1977_a). Values found in the present study for chicken hepatocytes by analysis of two individual sites indicate a binding capacity of the higher affinity component of about 2.0×10^3 sites per cell. This figure is ten times lower than that reported by Gammeltoft and coworkers for rat hepatocytes. If it is assumed that the rat and chicken hepatocytes

are spherical with diameters of 24 μm and 12 μm respectively it can be calculated that there is approximately a 2 to 3 fold decrease in receptor density on the surface of the chicken hepatocytes compared to that of the rat. Simon et al. (1977a) report a twenty fold difference in binding capacity per cell between chicken and rat hepatocytes indicating an approximate 5 fold decrease in receptor sites per unit surface area of plasma membrane. Although the quantitative results of affinity and receptor number reported vary considerably depending on the type of mathematical treatment, the qualitative results between chicken and rat hepatocytes are nearly identical. Ratios of receptor number between chicken and rat liver membranes also appear reasonably similar no matter which analysis is used. It must be added that direct comparison of work in this respect to that of Gammeltoft must allow for the differences in incubation temperature used (22°C compared to 37°C respectively) thus slightly higher binding capacities will be reported for this work.

It therefore appears that the chicken liver insulin receptor has approximately the same binding affinity as that found in rat liver but the receptor number per unit area of the plasma membrane may be decreased by 2 to 5 fold. Since chicken insulin has been reported to be about twice as potent as mammalian insulins (Simon et al. 1977b) this apparent decrease in receptor site number could well be counteracted by the increased binding affinity of the chicken insulin.

2) Insulin-receptor dissociation

Dissociation rates of ^{125}I -insulin from receptor were shown to be non-linear (Figs. 4.6-4.7) indicating that the rate of dissociation from receptors is not first order with respect to receptor occupancy. The slow dissociation rate of a fraction of receptor bound ^{125}I -insulin may be due to site heterogeneity with the higher affinity sites releasing insulin at a far slower rate (Gavin et al. 1973; Kahn et al. 1974); alternatively, certain insulin-receptor complexes may eventually lead to insulin

degradation (Terris and Steiner 1975; Gammeltoft et al. 1978; Gliemann and Sonne 1978) having different dissociation characteristics.

3) Glucagon Binding

The dissociation constant determined for the high affinity binding component of ^{125}I -glucagon (K_d^{av} of $9.4 \pm 3.1 \times 10^{-9}\text{M}$) was 30 fold that found for insulin, indicating a relatively lower affinity receptor for this hormone. This value correlates well with reports of half maximal binding constants of $4 \times 10^{-9}\text{M}$ (Rodbell et al. 1971a) and about $1.5 \times 10^{-9}\text{M}$ (Bataille et al. 1973; 1974) for rat liver membranes and $3 \times 10^{-9}\text{M}$ (Freychet et al. 1974) for rat hepatocytes. Simon et al. (1977a) reported binding curves for ^{125}I -glucagon binding to chicken liver membranes and although no figures were given it was concluded that specific glucagon binding sites were less numerous in chicken membranes than in rat at low glucagon concentrations (5 ng/ml) but more numerous at high concentrations (100 ng/ml). Sonne et al. (1978), treating the binding data in terms of two classes of receptor site, found the high affinity component of rat liver membranes to have a K_d of $7.0 \times 10^{-10}\text{M}$ and a capacity of approximately 20,000 sites per cell. The method of calculation of data is directly comparable with that used in the present work using the chicken hepatocyte, suggesting that there is approximately a 10 fold difference between the binding affinities of the chicken and rat liver glucagon receptors. This is similar to the conclusions of Simon et al. referred to above.

The binding capacity of the chicken hepatocyte for the high affinity site is approximately 13,000 sites per cell. Again assuming that both rat and chicken hepatocytes are spherical with respective diameters of $24\ \mu\text{m}$ and $12\ \mu\text{m}$ there appears to be 2 to 3 times more glucagon receptors per unit surface area of the chicken hepatocytes. However it must again be stressed that due to the enhanced potency of various iodinated glucagon preparations (Desbuquois 1975; Fig.4.11) large discrepancies can occur in

the calculated affinity depending on the iodine to hormone ratio of the tracer. Since iodination always increases the binding affinities of the hormone at physiological pH, the calculated dissociation or half maximum binding constants will always be too high since higher native glucagon concentrations will be required to displace the more potent iodinated species.

Chicken glucagon, although only differing from mammalian glucagons at one position in the amino acid sequence, has been reported to have an enhanced biological potency (Hazelwood 1976). It is not known if this increased potency of the hormone or the reported increase in receptor concentration on the cell surface will in some way compensate the apparent low affinity of the receptor population.

Dissociation of ^{125}I -glucagon from the hepatocytes was not accelerated by the presence of native glucagon (Fig. 4.16) thus indicating an absence of the apparent negative co-operative effect that is seen with the insulin receptor. Sonne et al. (1978) also failed to note any evidence for negative co-operativity of the glucagon receptor in rat hepatocytes. The dissociation however did not show first order kinetics with respect to receptor occupancy, indicating that more than one type of receptor population was present. After prolonged incubation approximately 50% of the bound tracer remained bound to the hepatocytes. This suggests that a large fraction of glucagon bound to the hepatocytes at 37°C may be altered in some way or transferred to some compartment which is less accessible to the external medium. This process may be linked to the high degradation rates observed with glucagon at 37°C (Fig. 4.19). Hormone degradation was found not to be linearly dependent on cell concentration at this temperature and a hyperbolic response was found. This may be due to the heterogeneity of the tracer population where highly iodinated, highly potent glucagon molecules may be preferentially degraded by the

hepatocytes. If heterogeneity of substrate is not responsible for this response the increasing cell concentration must in some way regulate the maximum degradation rate of the suspension as a whole. Even although a large fraction of the iodinated tracer is degraded (up to 20%) this decrease in substrate would not solely account for the lower degradative capacity at higher cell concentrations.

4) APP Binding

In contrast to the findings of McCumbee and Hazelwood (1977) who demonstrated both high affinity ($K_d = 5.6 \times 10^{-9}$ M) low capacity (approx. 40×10^3 sites per cell) and low affinity ($K_d = 8.3 \times 10^{-7}$ M) high capacity (approx. 19×10^5 sites per cell) binding components for APP in chicken adipocytes, only a low affinity binding site was found using chicken hepatocytes. Values obtained for the affinity of the hepatic site were two fold lower than the low affinity value reported for adipocytes with K_d of about 2.0×10^{-6} M however the capacities were similar with approximately 300,000 sites per hepatocyte. Whether this low affinity site has any biological significance is questionable.

Hepatocytes were also found to degrade 125 I-APP at a very slow rate compared to equal concentrations of 125 I-insulin or 125 I-glucagon, indicating the absence of a specific site for degradation of this hormone in the liver (Fig. 4.24). Although it has been reported that APP lowers liver glycogen content in vivo (Hazelwood et al. 1973) it is very unlikely that this effect is mediated through the low affinity binding site found and is probably secondary to some major effect of the hormone acting elsewhere in the body.

The physiological significance of the low affinity binding sites for insulin and glucagon are also questionable since binding affinities are much lower than the physiological concentrations of the hormones found in the blood. Although individual cell preparations showed variations

in the extent of low affinity binding for both insulin and glucagon, the high affinity components showed remarkable consistency between experiments. It is therefore suggested that the high affinity binding sites are solely responsible for any hormone regulation of biological effects in the chicken liver. Since no high affinity receptor was found for APP it is concluded that this hormone has no direct biological action in the chicken liver.

CHAPTER 5

'SECOND MESSENGERS' OF HORMONE ACTION

Introduction

The regulation of many intracellular processes is dependent on covalent modification of certain key enzymes. This modification leads to altered activities of the rate controlling enzymes with a resulting alteration in metabolic flux. The most common type of covalent modification found in biological systems involves specific phosphorylation and dephosphorylation processes. The earliest report of a reversible phosphorylation of an enzyme leading to altered activity was in 1955 for glycogen phosphorylase (Fischer and Krebs 1955). Since then over twenty different enzymes have been found to be regulated by phosphorylation - dephosphorylation mechanisms carried out by protein kinases and phosphoprotein phosphatases. The regulation and action of these kinases and phosphatases has been the subject of recent review articles (Ruben and Rosen 1975; Nimmo and Cohen 1977; Krebs and Beavo 1979).

Most of the evidence for the structure and function of protein kinases and phosphoprotein phosphatases has been obtained from tissues other than liver, particularly rabbit skeletal or cardiac muscle. Five different classes of protein kinase have been reported which are regulated by different factors (Krebs and Beavo 1979). Two cAMP dependent protein kinases (type I and type II) and a single cGMP dependent protein kinase have been found. Both cAMP dependent protein kinases comprise two different subunits - a regulatory subunit which binds cAMP and a catalytic subunit which dissociates on cAMP binding. The cGMP dependent protein kinase contains two identical subunits which have both cGMP binding and catalytic activity at two distinct functional sites on the same molecule.

In addition two calcium dependent protein kinases have been found - phosphorylase kinase and myosin light chain kinase. With myosin light chain kinase the calcium sensitivity is induced by a calcium dependent

molecular protein that is usually associated with activating cyclic nucleotide phosphodiesterase (Dabrowski et al. 1978; Yagi et al. 1978; Warsman et al. 1978). The same modulator protein may be responsible for inducing Ca^{2+} sensitivity on phosphorylase kinase (Cohen et al. 1978).

It is therefore obvious that any fluctuations in basal intracellular levels of cAMP or cGMP as well as those of free Ca^{2+} may activate a particular protein kinase with resulting enzyme phosphorylation. Depending on the specificities of individual protein kinases in the liver cell, alterations in basal hepatic metabolism are possible. It is now well established that activation of phosphorylase kinase by cAMP dependent protein kinase leads to phosphorylase b to a conversion with a resulting increase in the rate of glycogenolysis. The mechanism of the glycogenolytic cascade effect has been reported in more detail by Nimmo and Cohen (1977).

As mentioned above, phosphorylase kinase can also be directly activated or deactivated by alterations in intracellular free calcium levels (Heilmeyer et al. 1970). Therefore both cAMP and intracellular calcium can regulate the glycogenolytic rate. Various models for the interdependent actions of calcium and cAMP in different tissues have been put forward by Berridge (1975).

Cyclic GMP protein kinase has been reported to have similar specificity to the cAMP dependent enzyme. Both enzymes can phosphorylate phosphorylase kinase, glycogen synthetase (Lincoln and Corbin 1977) and pyruvate kinase (Lincoln and Corbin 1977; Ljungstrom and Ekman 1977; Riou et al. 1978), as well as hormone sensitive lipase, cholesterol esterase (Khoo et al. 1977) and fructose 1,6-diphosphatase (Riou et al. 1977). In view of their physical and biological similarities, it has been suggested that cAMP and cGMP kinases may have evolved from a common ancestral protein (Lincoln and Corbin 1977, 1978).

Alteration of the basal intracellular levels of the two nucleotides or calcium by extracellular hormone binding would be a possible mechanism for hormonal regulation of metabolism. Both glucagon and adrenaline stimulate liver adenylate cyclase with concomitant increases in intracellular cAMP resulting in activation of cAMP dependent protein kinase. The hormone stimulation of cAMP in rat hepatocytes has been investigated by several workers (Johnson et al. 1972; Garrison and Haynes 1973; Pilkis et al. 1975; Sonne et al. 1978; Westwood and Siddle 1979). Insulin has been reported to inhibit hormone stimulated cAMP levels (Exton and Park 1972; Pilkis et al. 1975; Westwood and Siddle 1979). Although some laboratories have reported a direct inactivation of adenylate cyclase by insulin (Illiano and Cuatrecasas 1972; Hepp and Renner 1972), other workers have found no effect (Pohl et al. 1971; Thompson et al. 1973; Pilkis et al. 1974).

Although adrenaline stimulates cAMP production in the rat hepatocyte, recent evidence suggests that its effects on gluconeogenesis and glycogenolysis are mediated through the cAMP independent α -receptor (see Chapter 1). Binding studies indicate the presence of two α -adrenergic binding sites in the liver plasma membrane with only one of these believed to be physiologically important (El-Refai et al. 1979). Low levels of glucagon, insufficient to stimulate cAMP accumulation or protein kinase activation, have been reported to activate liver phosphorylase (Birnbaum and Fain 1977), and Keppens et al. (1977) reported that calcium may be an additional messenger for glucagon, besides cAMP. The same workers suggested that calcium may act by activation of liver phosphorylase b kinase, in view of the well-documented calcium dependence of the muscle enzyme (see review by Krebs and Beavo 1979). Hughes and Coore (1978) reported diminished calcium binding to rat liver plasma membranes after binding of glucagon.

They suggested that this may be part of the mechanism stimulating adenylate cyclase. However this could also lead to increased intracellular calcium and activation of phosphorylase kinase at low glucagon concentrations.

The relationship between guanylate cyclase, cGMP and the regulation of metabolism has recently been reviewed (Goldberg and Haddox 1977; Mittal and Murad 1977). The role of cGMP as a possible second messenger for hormone action is rather obscure. Unlike adenylate cyclase and cAMP there has been little conclusive evidence for hormone stimulation of guanylate cyclase. Illiano et al. (1973) reported that insulin and cholinergic agents such as acetylcholine increased the intracellular level of cGMP in isolated fat cells and in liver slices. Similarly Pointer et al. (1976) reported increased levels of cGMP in isolated rat hepatocytes exposed to the catecholamines, adrenaline, isoproterenol and phenylephrine as well as carbachol, insulin, ionophore A23187 and to a lesser extent glucagon. However cGMP levels did not correlate with the observed biological effects of the various hormones. Both catecholamines and insulin increased cGMP levels in the hepatocytes but had opposing effects on glycogenolysis. Numerous other workers have failed to note any effect of several hormones, including adrenaline, noradrenaline, insulin and glucagon, on cGMP accumulation (see Goldberg and Haddox 1977). Therefore the role of cGMP, if any, in hormone regulation remains to be established.

The only means of removal of cAMP or cGMP from the cell cytoplasm is by degradation by their specific cyclic nucleotide phosphodiesterases to 5'AMP or 5'GMP. Alterations in cyclic nucleotide phosphodiesterase activity would also serve as a control for nucleotide regulation. The phosphodiesterases can be activated by calcium ions in the presence of a heat stable protein modulator (Smoake et al. 1974; Kakiuchi et al.

1975). Two kinetic forms of both cAMP and cGMP phosphodiesterases have been found in muscle, liver and nervous tissues from many different animal species (Arch and Newsholme 1976). Numerous workers have reported activation of the particulate bound low K_m enzyme from adipose tissue and intact adipocytes by insulin and adrenaline (Loten and Sneyd 1970; Manganiello and Vaughan 1973; Paulson et al. 1974; Kono et al. 1975; Zinman and Hollenberg 1974). This low K_m hormone-sensitive enzyme has been successfully solubilised (Lovell-Smith et al. 1977). Work by Phelps and Varandi (1977) suggests that glutathione-insulin transhydrogenase-catalysed sulphhydryl-disulphide exchange with insulin may be a pre-requisite for insulin stimulation of phosphodiesterase activity. This again indicates that insulin degradation and internalisation, as postulated with the liver (Terris et al. 1979), may be necessary for intracellular effects on metabolism.

Thompson et al. (1973) reported that following injection of insulin into rats, the liver high affinity cAMP phosphodiesterase exhibited enhanced activity. Others have also reported an insulin sensitive phosphodiesterase in rat liver (House et al. 1972; Tria et al. 1976). Glucagon has also been reported to stimulate the enzyme in the rat hepatocytes (Allen and Sneyd 1975). These reports have recently been confirmed by Loten et al. (1978) who found stimulation of the low K_m enzyme from liver by preincubating intact hepatocytes with insulin or glucagon. The mechanisms of activation by the two hormones may be different and the effect of glucagon could be responsible for the failure to produce sustained increases in cAMP levels in rat hepatocytes. Similar conclusions were reported when studying the effect of adrenaline on cAMP production in rat adipocytes (Zinman and Hollenberg 1974). Loten et al. (1978) also suggested that the altered phosphodiesterase activity may be a result of independent phosphorylations of the protein

stimulated by glucagon- and insulin-induced specific protein kinases. However, variations in free calcium could also effect the phosphodiesterase activity by binding or dissociating from the modulator protein. Nemecek (1978) recently reported that ionophore A23187 and calcium stimulated cyclic nucleotide phosphodiesterase activity in isolated hamster fat cells with no associated increase in cAMP.

To date, all investigations into possible intracellular mediators of hormone action have been carried out using tissues isolated from mammalian sources and no direct studies have been undertaken using isolated chicken hepatocytes. It is not known if glucagon and adrenaline have similar effects on intracellular cAMP levels in the chicken hepatocyte or indeed if cGMP, Ca^{2+} and cAMP phosphodiesterase are also involved in hormone control. Elucidation of these problems was the objective of the work to be reported in this Chapter.

Methods

Concentrated cell suspension (0.5 ml of a 20 to 30 x 10⁶ cell/ml solution) was added to 25 mm x 75 mm plastic incubation pots containing fresh KRB buffer (1 ml) equilibrated with 95% O₂/5% CO₂ at 37°C and containing any required effector. Pots were then gassed with 95% O₂/5% CO₂ for 10 to 15 seconds and lids sealed. All incubations were carried out at 37°C in reciprocal shaking water baths (140 to 160 strokes/min). Termination of cell incubations was carried out by

- a) addition of a cell suspension sample (1 ml) to an equal volume of ice cold 6% PCA (cAMP determinations) or ice cold 10% TCA (cGMP determinations) for estimation of cyclic nucleotide content.
- b) centrifugation (2750 g; 1 min) and resuspension of cell pellet in 10 mM tris-HCl buffer pH 7.4 by homogenisation (approximately 30 strokes of the tight fitting pestle) for determination of adenylate cyclase activity.

c) centrifugation (2750 g; 1 min) and resuspension of cell pellet in a 50 mM tris-HCl extraction buffer pH 7.4 by homogenisation (as above) for determination of phosphodiesterase activity.

Complete details of these assay procedures are given in Chapter 2.

Results

1. Cyclic AMP

In comparing the experimental results in this section it will become obvious that discrepancies exist between glucagon or adrenaline concentrations and cAMP levels for different experiments. This problem was introduced by the unavoidable changes in collagenase preparations obtained from the Boehringer Corporation Ltd. Consistent results were obtained when the same batch of collagenase was used to prepare the cells. Discrepancies are presumably due to associated non-specific protease activity. Time course experiments were carried out using batch number 17 collagenase whereas dose response curves were carried out using batch 25 collagenase.

a) Basal Cellular cAMP Content. Immediately after isolation concentrated cell suspensions ($20-30 \times 10^6$ cells/ml) were found to have intracellular cAMP levels ranging from 1 to 4 pmols/ 10^6 cells. Preincubation of the suspension for 15 minutes at 37°C with constant gassing with O_2/CO_2 generally reduced the basal levels to a steady state around 1 pmol/ 10^6 cells. Fig. 5.1 shows a typical time course of basal cAMP content of hepatocytes with incubation at 37°C . In all other experiments reported the cell suspension was preincubated for 15 minutes at 37°C with constant gassing with O_2/CO_2 to reduce basal levels to stable values. In some experiments however the basal levels of cAMP did not fall to the values mentioned. Cells prepared by particular batches of collagenase (25 or 27) consistently showed slightly elevated cAMP levels which remained stable during incubations up to 40 minutes at 37°C . Centrifugation (50, 350 or 2750 g; 1 to 5 minutes)

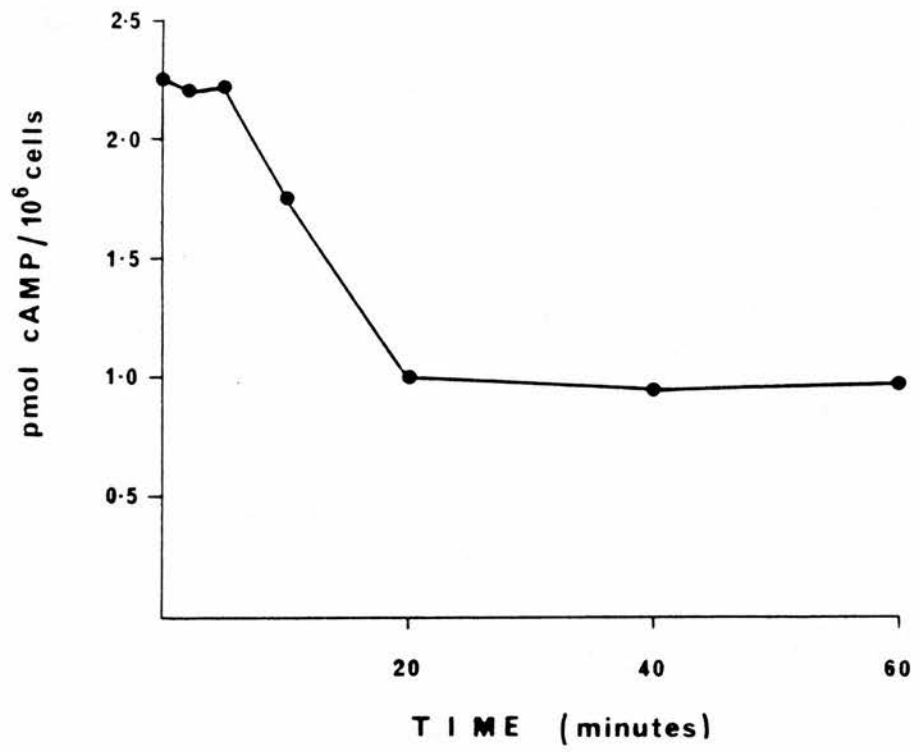


Fig. 5.1 Effect of incubation on basal cAMP content of the cells.

or rapid changes in medium temperature (addition of equal volume of ice cold KRB) did not alter basal or hormone stimulated cAMP levels. Therefore the physical procedures carried out during cell isolation were not considered to be responsible for the elevated nucleotide levels.

b) Time course of Hormonal Effects on total cAMP. The time course of cAMP accumulation in the suspensions in the presence of glucagon (25, 10 and 5 ng/ml) and adrenaline (1 $\mu\text{g/ml}$) are shown in Figs. 5.2 and 5.3 respectively. High concentrations of glucagon (100 or 25 ng/ml) produce maximum levels of cAMP after approximately 5 to 10 minutes. The lower glucagon concentrations (5 ng/ml) show maximum cAMP after only 1 or 2 minutes. After this peak, cAMP levels fall in a biphasic manner. Even after 40 minutes incubation there is still a substantial elevation of cAMP above basal levels. Cyclic AMP production in response to adrenaline is similar to that of glucagon except that absolute levels of cAMP are lower. Basal levels of cAMP remain relatively constant for the period of incubation. Glucagon time courses in the presence of theophylline (1 mM) show similar characteristics with the exception that cAMP levels remain elevated for longer periods (Fig. 5.4). Basal levels of cAMP were not elevated by the phosphodiesterase inhibitor. At the highest glucagon concentration (50 ng/ml) cAMP did not decline at all but remained constant for up to 60 minutes incubation.

Insulin (250, 100 or 25 ng/ml), APP (100, 25 or 6.25 ng/ml) and calcium ionophore A23187 (5 or 1 $\mu\text{g/ml}$) failed to elevate basal cAMP levels over a sixty minute time period whether 1 mM theophylline was present in the incubation medium or not.

The distribution of hormone stimulated cAMP between intracellular and extracellular compartments is shown in Figs. 5.5 and 5.6. Cell suspension was incubated at 37^oC for the times shown with or without glucagon (12.5 or 25 ng/ml) and then samples (1 ml) were removed.

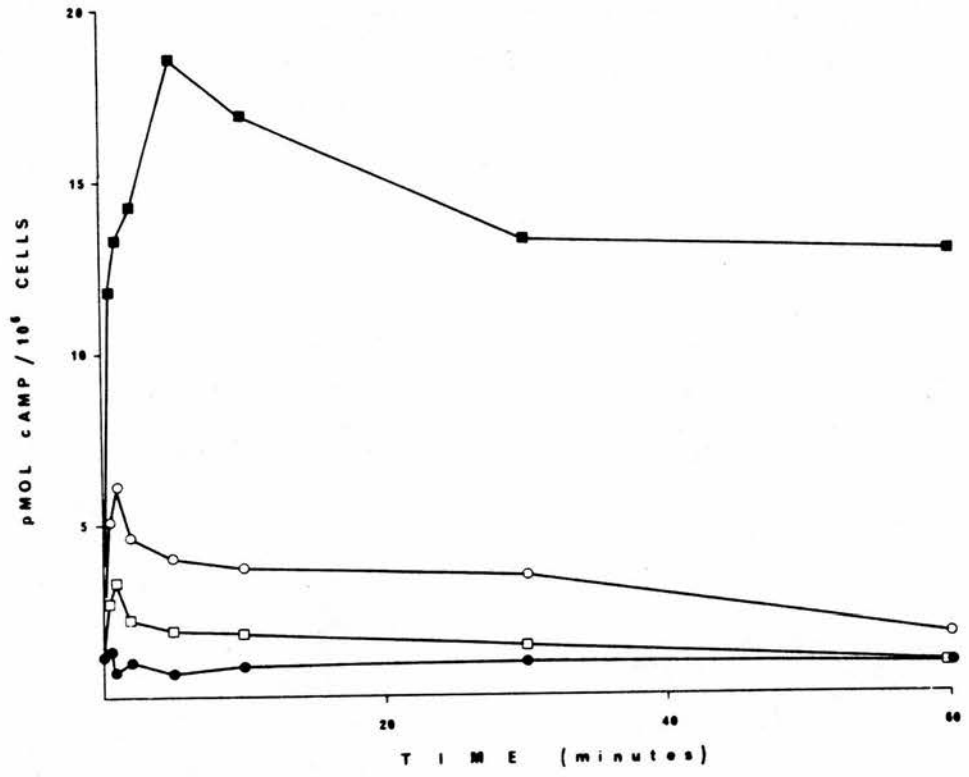


Fig. 5.2 Time course profile of basal (●-●) and glucagon 25 (■-■), 10 (○-○), 5 ng/ml (□-□), stimulated cAMP content.

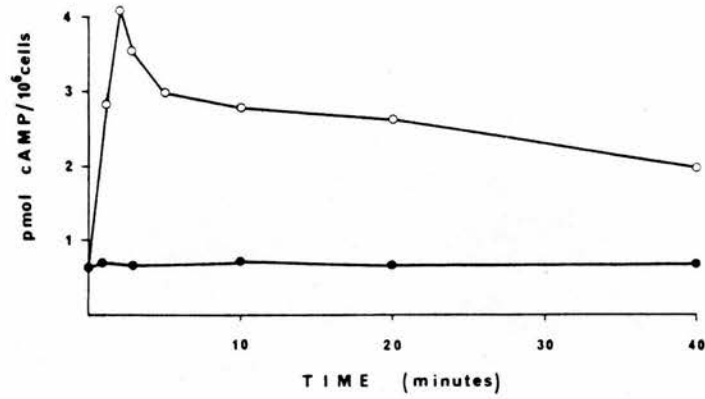


Fig. 5.3 Time course profile of basal (●-●) and adrenaline 1 μg/ml (O-O) stimulated cAMP content.

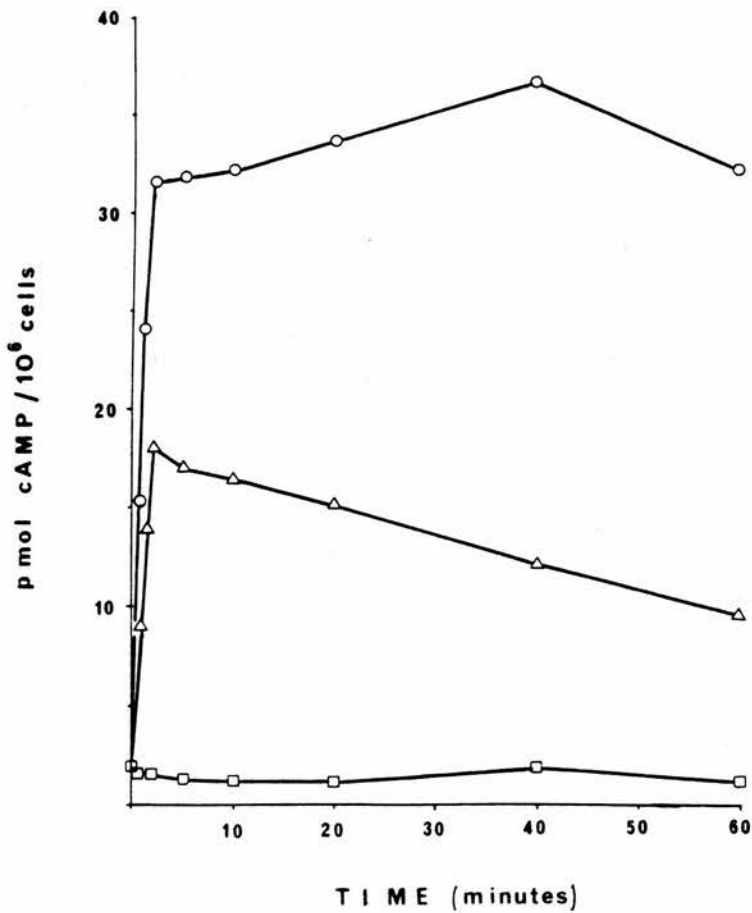
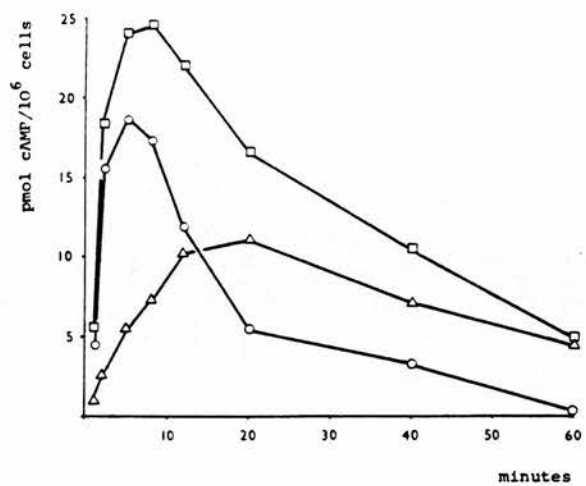
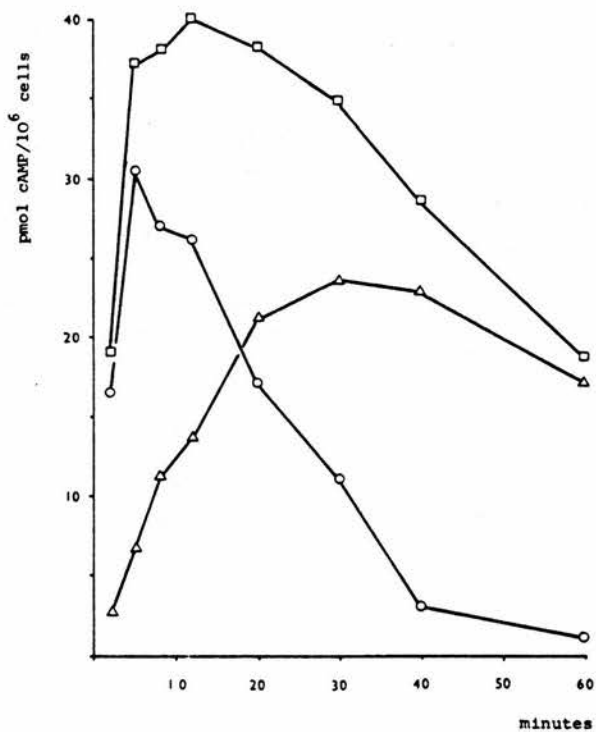


Fig. 5.4 Time course profile of basal (□-□) and glucagon 50 (O-O), 25 ng/ml (Δ-Δ) stimulated cAMP content in the presence of 1 mM theophylline.



EFFECT OF GLUCAGON (12.5 ng/ml) ON INTRACELLULAR (○—○) EXTRACELLULAR (△—△) AND TOTAL (□—□) cAMP

Fig. 5.5



EFFECT OF GLUCAGON (25 ng/ml) ON INTRACELLULAR (○—○) EXTRACELLULAR (△—△) AND TOTAL (□—□) cAMP

Fig. 5.6

The samples were either immediately added to an equal volume of ice cold 6% PCA to determine total cAMP in the suspension or centrifuged (2750 g; 30 seconds) at room temperature and a sample of supernatant added to an equal volume of ice cold 6% PCA to estimate extracellular cAMP. Lower centrifugation speeds (1400 g or 350 g; 30 sec) did not alter the amount of cAMP found in the supernatant. Intracellular cAMP was determined by difference. As shown in Figs. 5.5 and 5.6 there is a rapid accumulation of intracellular cAMP which reaches a peak level after 5 minutes for both glucagon concentrations used (12.5 and 25 ng/ml). Intracellular cAMP quickly falls however presumably due to intracellular degradation as well as loss to the external medium; even the highest stimulated intracellular levels return to basal after 60 minutes incubation. The nucleotide appears to be freely permeable through the plasma membrane, escaping to the extracellular medium. However this extracellular component of the total suspension cAMP is also degraded by some mechanism as peak concentrations are reached after 20 and 30 minutes using glucagon concentrations of 12.5 and 25 ng/ml respectively. This decrease in extracellular cAMP may be due to diesterases present in the external medium and/or diffusion of the nucleotide back into the cytoplasm for degradation by internal phosphodiesterase. At least 95% of the basal cAMP at any time point was found to be of intracellular origin.

In order to monitor the extracellular degradation of cAMP, a cell suspension (20.5×10^6 cells/ml) was incubated for 60 minutes at 37°C as previously described, the pots being regassed with O_2/CO_2 after 30 minutes. The suspension was then centrifuged once at 350 g for 2 minutes and then the supernatant was centrifuged once more at 2750 g for 2 minutes. The supernatant was then diluted with an equal volume of fresh KRB buffer containing 200 pmol/ml cAMP. The diluted supernatant containing

cAMP was then incubated for various times at 37°C in a reciprocal shaking water bath and samples (1 ml) withdrawn for estimation of the cAMP remaining. A parallel incubation of cAMP in fresh KRB buffer showed no decrease in the level of the nucleotide. However, incubations with the cell suspension supernatant resulted in marked degradation of cAMP indicating the presence of extracellular diesterases (Fig. 5.7). Cell suspensions did not show any marked signs of damage as judged by trypan blue exclusion. Increase in the extracellular factor capable of degrading cAMP was not associated with gross morphological damage to the hepatocytes.

c) Effect of Insulin on Glucagon stimulated cAMP content. As reported above insulin at any concentration failed to show an effect on basal cAMP levels. However addition of pharmacological levels of the hormone (500 ng/ml) to subsaturating levels of glucagon (10 ng/ml) showed reproducible changes in the time course of total cAMP production. Fig. 5.8 shows the effect of the simultaneous addition of glucagon (10 ng/ml) and insulin (500 ng/ml) or glucagon (10 ng/ml) alone, to cell suspension at time zero. Initial rates of production of cAMP were identical in both cases but the cAMP degradation rate was decreased in the presence of insulin. Preincubation of the cell suspension for 10 or 20 minutes with insulin (500 ng/ml) before addition of glucagon (10 ng/ml) consistently resulted in a slight decrease in the initial rate of cAMP production as well as decreasing the cAMP degradation rate (Fig. 5.9, 5.10). Preincubation also lowered the total amount of cAMP produced on addition of glucagon. The addition of high insulin concentrations failed to decrease the rate of glucagon (12.5 ng/ml) elevated intracellular cAMP degradation as shown in Fig. 5.6. Cell suspension was incubated in the presence of glucagon (12.5 ng/ml) or glucagon (12.5 ng/ml) plus insulin (500 ng/ml) for 5 minutes at 37°C.

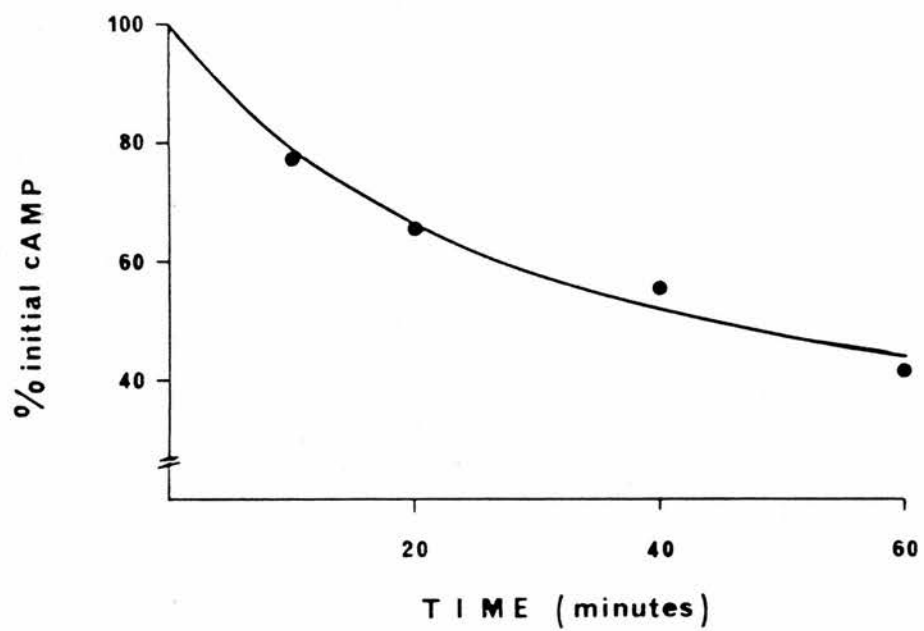


Fig. 5.7 Extracellular degradation of cAMP (100 pmol/ml). Diesterase activity in a cell suspension supernatant.

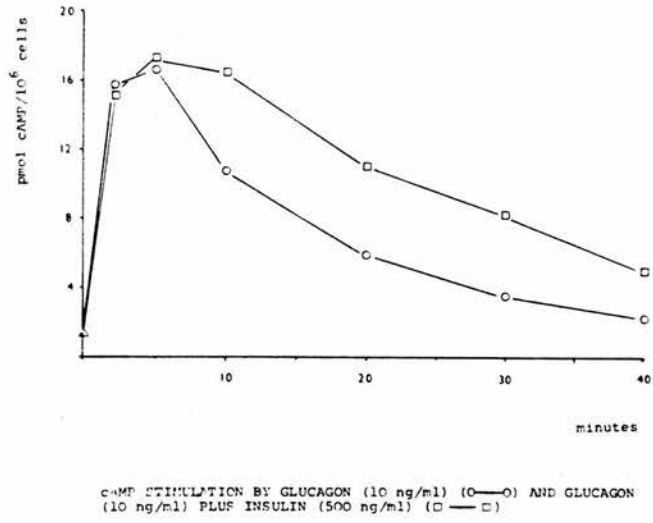


Fig. 5.8

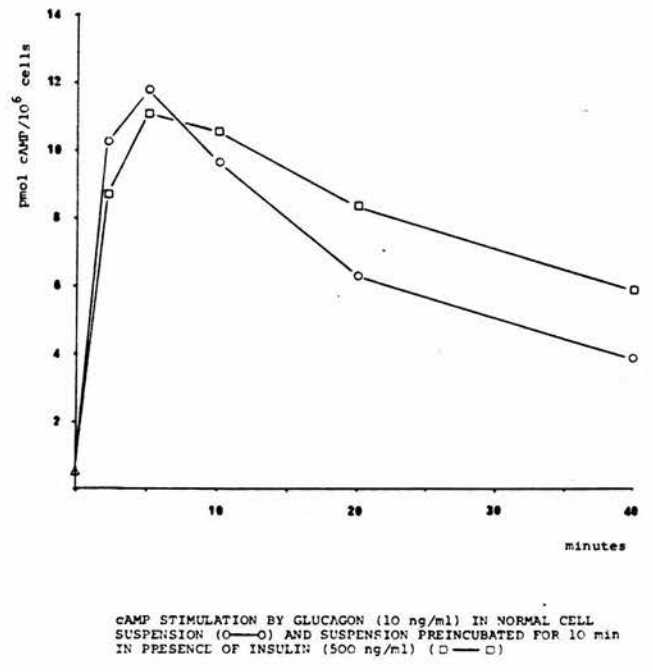


Fig. 5.9

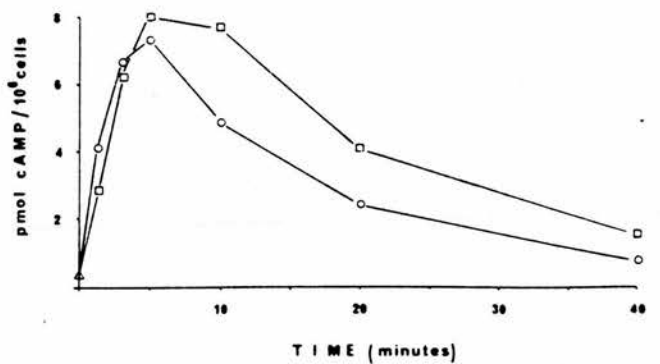


Fig. 5.10 cAMP stimulation by glucagon (10 ng/ml) in normal cell suspension (O-O) and suspension preincubated for 20 minutes in the presence of insulin (500 ng/ml) (□-□).

Cells were pelleted by centrifugation (350 g; 1 min) and resuspended to the same volume with fresh KRB buffer. The decrease in suspension cAMP was observed with time (Fig. 5.11). There was no significant difference in cAMP degradation rate between the two cell suspensions. Insulin effect on total cAMP degradation was not observed at concentrations of 100 ng/ml or below, even when lower glucagon concentrations (5 ng/ml) were employed.

d) Effects of Glucagon plus Adrenaline. The maximum stimulation of cAMP production by adrenaline (10 µg/ml) could be increased by up to 5 fold by glucagon (100 ng/ml) but adrenaline could not potentiate maximum effects by glucagon. In the presence of theophylline (1 mM) addition of glucagon (100 ng/ml) after preincubation for 5 minutes with adrenaline (10 µg/ml) induced only a slow increase in cAMP content (Fig. 5.12). The production of cAMP in response to glucagon was much slower than when fresh cells were exposed to glucagon alone.

e) Subsequent additions of Glucagon. Addition of glucagon in stages resulted in additive effects and rapid responses were found with later additions of glucagon. Fig. 5.13 shows the stimulation of total cAMP by cell suspension treated with 200 ng/ml glucagon at zero time or 100 ng/ml glucagon at zero time followed by a further addition giving 200 ng/ml after 15 minutes of incubation. Glucagon concentrations of 100 ng/ml appear to produce a maximum initial rate of cAMP production. Higher concentrations maintain the level of cAMP for longer periods. The further addition of glucagon after 15 minutes incubation giving 200 ng/ml produces a rapid increase in the declining cAMP level to values similar to when glucagon at 200 ng/ml was administered at zero time.

f) Effect of Bacitracin. The effect of the protease inhibitor bacitracin, on cAMP accumulate was also investigated. Incorporation of bacitracin

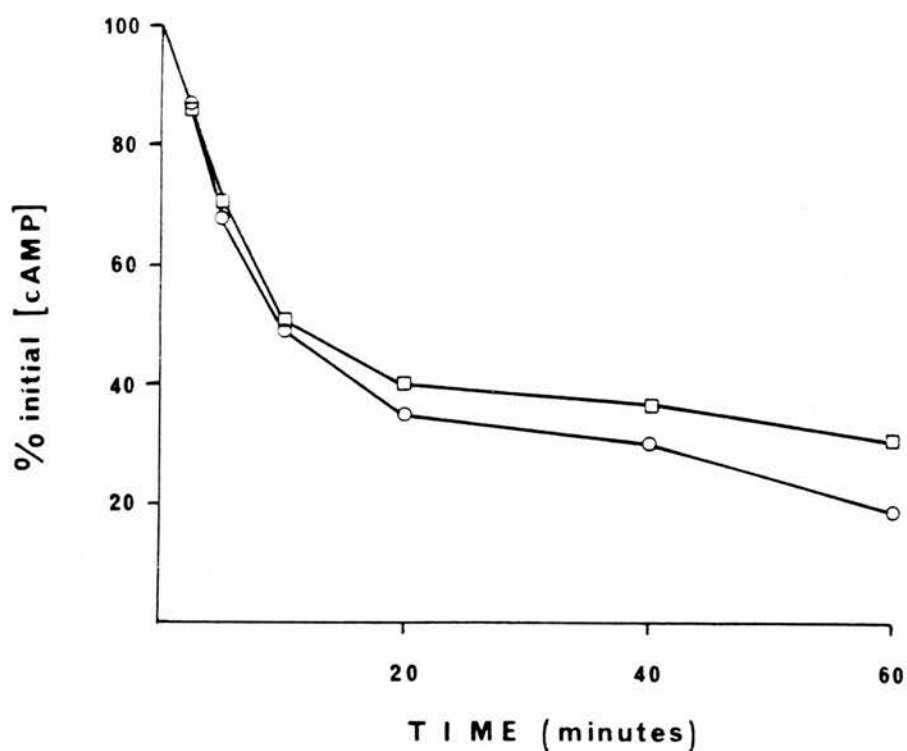


Fig. 5.11 Degradation of intracellular cAMP stimulated by glucagon (12.5 ng/ml) (O-O) or glucagon (12.5 ng/ml) plus insulin (500 ng/ml) (□-□).

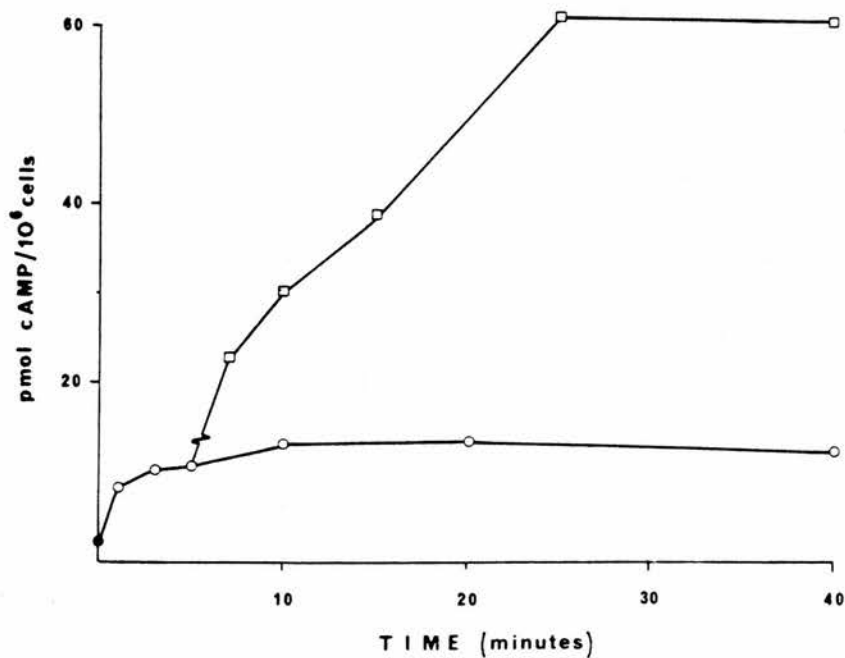


Fig. 5.12 Cell suspension cAMP in response to adrenaline (10 µg/ml) (O-O) and adrenaline (10 µg/ml) plus addition of glucagon (100 ng/ml) (□-□) at 5 minutes. Suspensions contained 1 mM theophylline. Basal cAMP indicated ●.

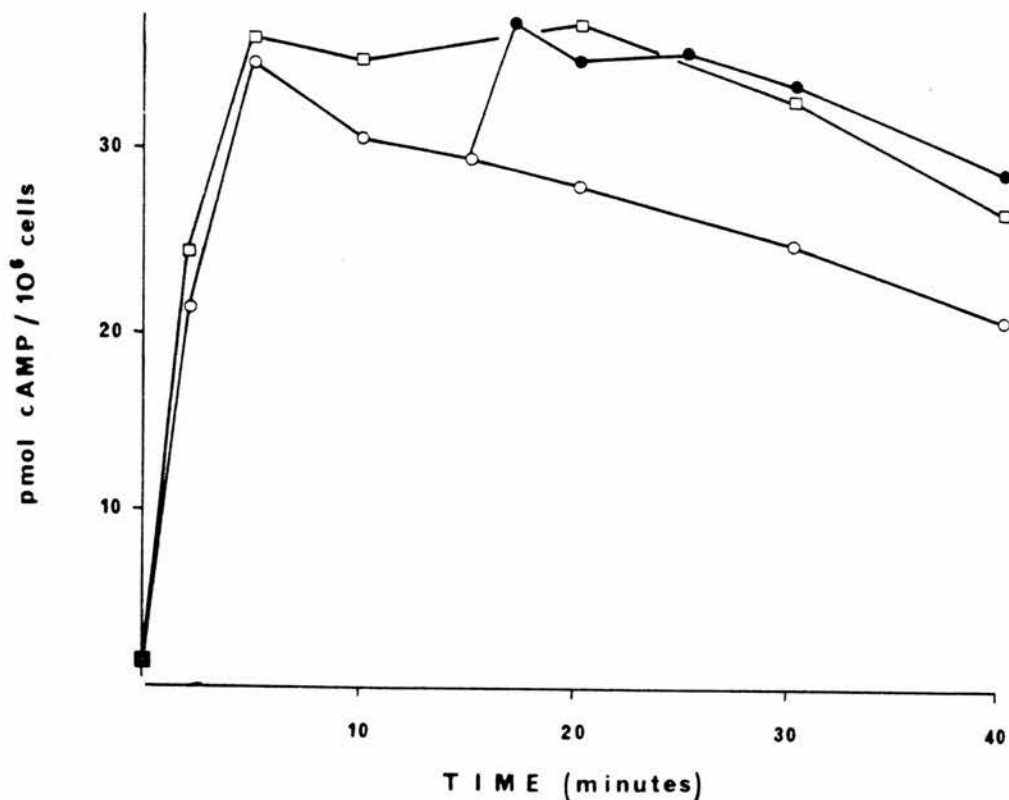


Fig. 5.13 cAMP production in response to glucagon (200 ng/ml) (□-□) or 100 ng/ml (O-O). Also increasing glucagon concentration from 100 to 200 ng/ml after 15 minutes of incubation (●-●).

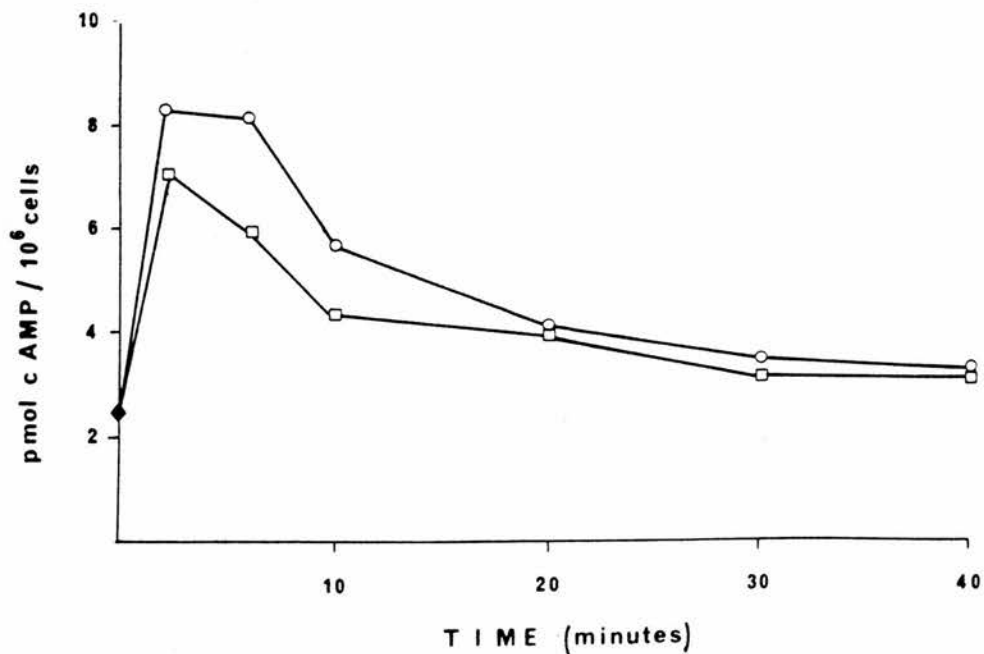


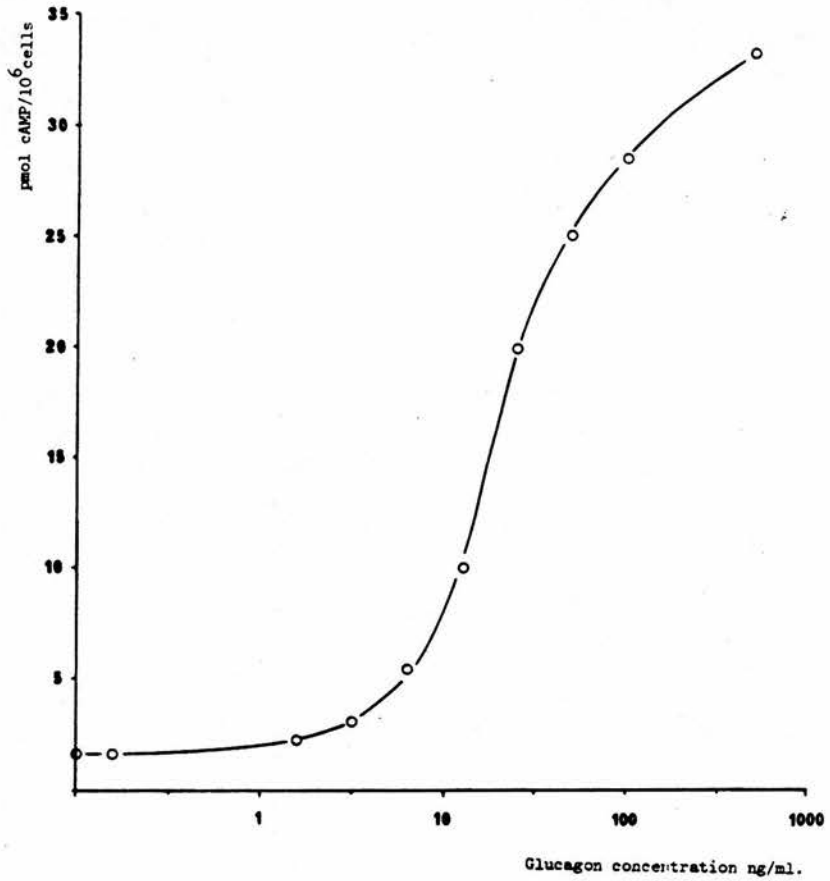
Fig. 5.14 cAMP production in response to glucagon (10 ng/ml) (O-O) or glucagon (10 ng/ml) in the presence of bacitracin (0.75 mg/ml) (□-□).

(0.75 mg/ml) into the incubation medium did not potentiate the effects of subsaturating glucagon concentrations but in fact tended to depress cAMP levels at all time points (Fig. 5.14). (Batch 25 collagenase used to prepare cells for this experiment.)

g) Dose response for Glucagon and Adrenaline. Cell suspensions, prepared using batch 25 collagenase, were incubated in the presence of varying concentrations of glucagon or adrenaline as described previously. After 2 minutes at 37°C, incubations were terminated by direct addition of an equal volume of 6% PCA to the incubation pots. Samples were processed for total cAMP content. The dose response curves of glucagon and adrenaline are shown in Figs. 5.15 and 5.16 respectively. The first significant increase in cAMP level was found with glucagon concentrations of 10 ng/ml and at adrenaline concentrations of 50 ng/ml with half maximum stimulations at approximately 45 ng/ml (13 nM) and 300 ng/ml (1.7 μM) respectively. Maximum levels of adrenaline stimulated cAMP were only one third of that produced by maximum glucagon concentrations. Closer examination of cAMP levels at low glucagon concentrations is shown in Fig. 5.17. Cell suspension (13.8×10^6 cells/ml) was incubated for 2 minutes with various glucagon concentrations in the presence of 5 mM theophylline. Basal cAMP levels of 1.96 ± 0.21 pmol/ 10^6 cells were elevated by approximately 30% to 2.51 ± 0.35 pmol/ 10^6 cells in the presence of 0.31 ng/ml glucagon - the limits correspond to standard deviations for three observations.

2) Cyclic GMP

Levels of cGMP tended to vary considerably between cell preparations. Basal nucleotide values varied over a six fold range with values from 10×10^{-15} to 60×10^{-15} mol/ 10^6 cells. Average basal levels cGMP were found to be $31.3 \pm 24.8 \times 10^{-15}$ mol/ 10^6 cells (standard deviation - n = 4). Besides the high variability between cell preparations the duplicate



GLUCAGON DOSE RESPONSE FOR cAMP PRODUCTION.

Fig. 5.15

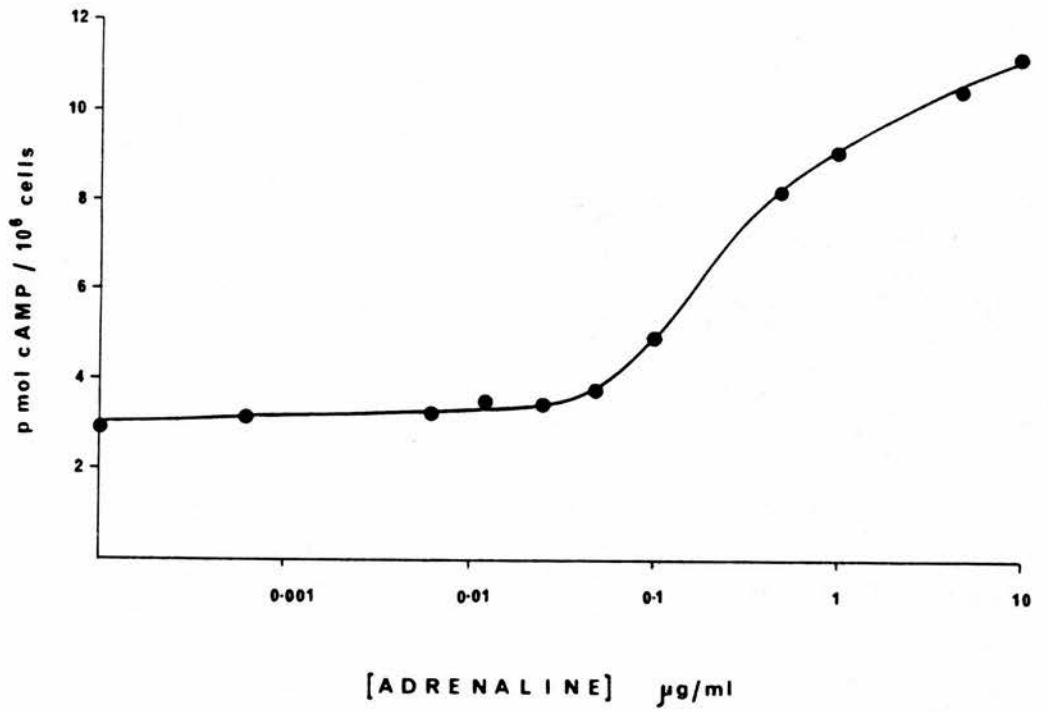


Fig. 5.16 Adrenaline dose response for cAMP production.

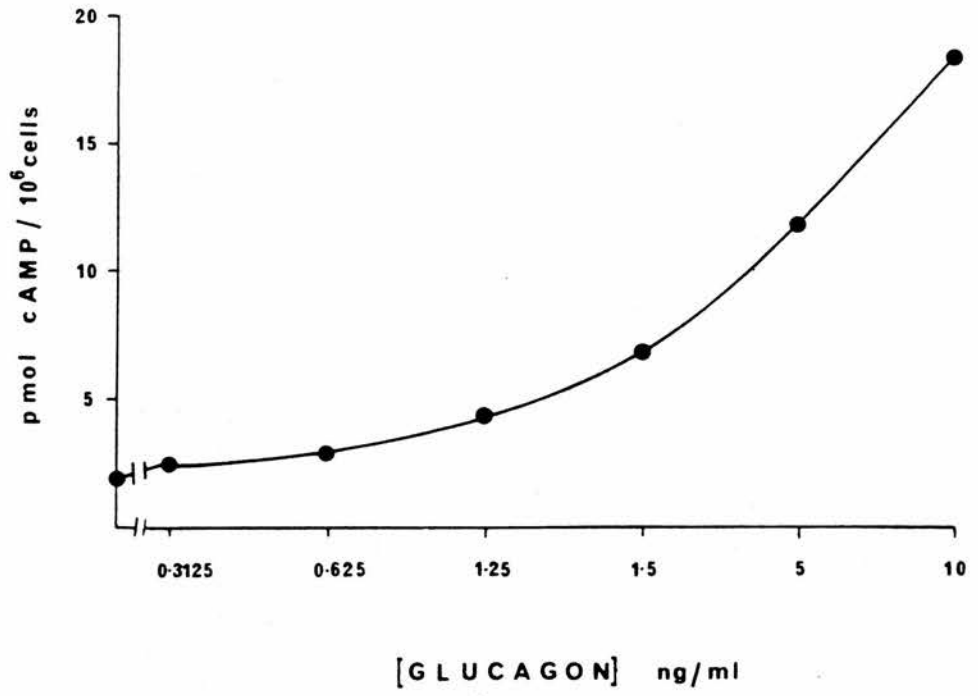


Fig. 5.17 Glucagon dose response for cAMP production in cell suspension containing 5 mM theophylline.

values obtained from individual experiments from cells incubated under identical conditions were also highly variable with values often fluctuating by up to 25%. This suggested variability in the assay method for cGMP, yet standard samples processed in an identical manner to the unknowns showed remarkable consistency (see Fig. 2, Chapter 2). The reasons for the observed variability are therefore unknown. The average percentage changes noted with various effectors are shown in Table 5.1. Values are calculated from experiments using four different cell preparations.

Due to the large fluctuations found when estimating cGMP levels any small changes in the concentration of this nucleotide would not be noticed under these conditions. It is however concluded that none of the effectors used (insulin, glucagon, APP, ionophore A23187, adrenaline) induced any large changes in total cGMP in the cell suspension comparable with that found for maximum stimulation of cAMP by glucagon. After centrifugation of the cell suspension less than 15% of the total cGMP immunoreactivity was found in the supernatant.

3) Adenylate Cyclase Activity

Adenylate cyclase activity was measured in crude homogenates of isolated cell suspension by the method outlined in Chapter 2. Activity of the cyclase was found to be highly sensitive to cell homogenate concentration and temperature. High concentrations of cell homogenate (176×10^6 cells/ml) showed rapid inactivation of the cyclase during incubations (Fig. 5.18). After 2 minutes there was a decrease in the glucagon stimulated cyclase rate and the cAMP already produced was rapidly lost even though the reaction medium contained 10 mM theophylline. No significant stimulation of activity could be demonstrated with 10 mM NaF using these high cell concentrations. Using lower cell homogenate concentrations (26.8 and 13.4×10^6 cells/ml), linear enzyme rates were

Table 5.1: Cell cGMP levels in cells incubated with various effectors.

Effector	Incubation time (minutes)	cGMP x 10 ⁻¹⁵ mol/10 ⁶ cells	
		Average	Standard deviation (n=4)
-	0	31.3	+25.0
-	10	28.5	+21.8
Insulin (100 ng/ml)	10	29.1	+22.2
Glucagon (100 ng/ml)	10	24.1	+14.4
APP (100 ng/ml)	10	30.0	+20.1
Ionophore A23187 (5 µg/ml)	10	20.4	+9.3
Adrenaline (1 µg/ml)	10	30.4	+19.7

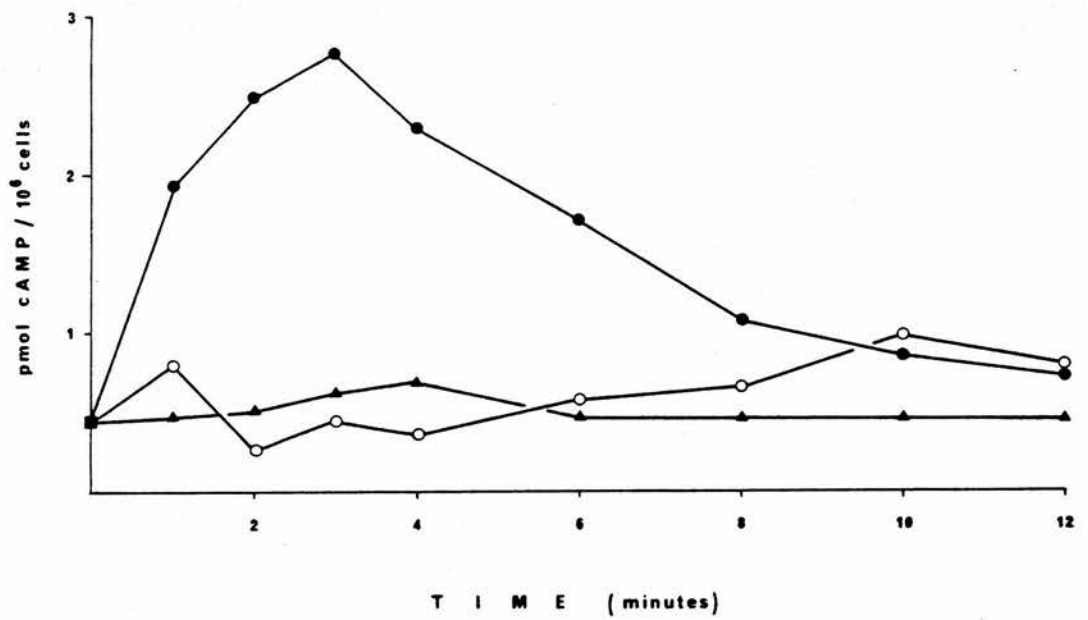


Fig. 5.18 Time course of basal (▲-▲), glucagon (10 ng/ml) (●-●) and sodium fluoride (10 mM) (O-O) stimulated adenylate cyclase production of cAMP using a cell homogenate of 176×10^6 cells/ml.

only found for the first 10 minutes of incubation after which activity suddenly stopped (Fig. 5.19). Levels of cAMP in the medium remained relatively constant indicating that theophylline was successfully inhibiting the endogenous phosphodiesterase. Glucagon directly stimulated adenylate cyclase by approximately 10 to 12 fold when added to these homogenates. Basal rates of nucleotide production increased from an average of 0.06 pmol/min/ 10^6 cells to 0.68 pmol/min/ 10^6 cells. Neither insulin nor APP (both at 100 ng/ml) had any direct effect on basal or glucagon stimulated cyclase activity under these conditions.

The rapid inactivation of the cyclase is shown in Fig. 5.20. Cell homogenates were preincubated for various times at 30°C and then added to the cyclase incubation medium containing glucagon (100 ng/ml). Cyclic AMP production after 2, 5 and 10 minutes was determined. Only the cell homogenate which was precubated for one minute showed any glucagon stimulated cyclase activity. Homogenates preincubated for 11, 21 and 31 minutes showed no cyclase activity. Basal cyclase activity was also measured over 40 minutes of incubation. A low rate was noted over the first 10 minutes incubation at 30°C but thereafter no increase in medium cAMP was observed.

4) Phosphodiesterase Activity

High and low K_m phosphodiesterase activities were measured in crude cell homogenates after preincubation of the cells for 20 minutes with or without various effectors. Table 5.2 shows the result of changes in cell homogenate concentration on low and high K_m phosphodiesterase activities which were measured at cAMP concentrations of 5 and 250 μ M respectively. Cell homogenates ranging from 133×10^6 cells/ml to 4.2×10^6 cells/ml were added to the assay medium and incubated for 1 hour at 37°C. Cell suspension homogenates of under 10×10^6 cells/ml were used in subsequent incubations. Cell suspensions were preincubated

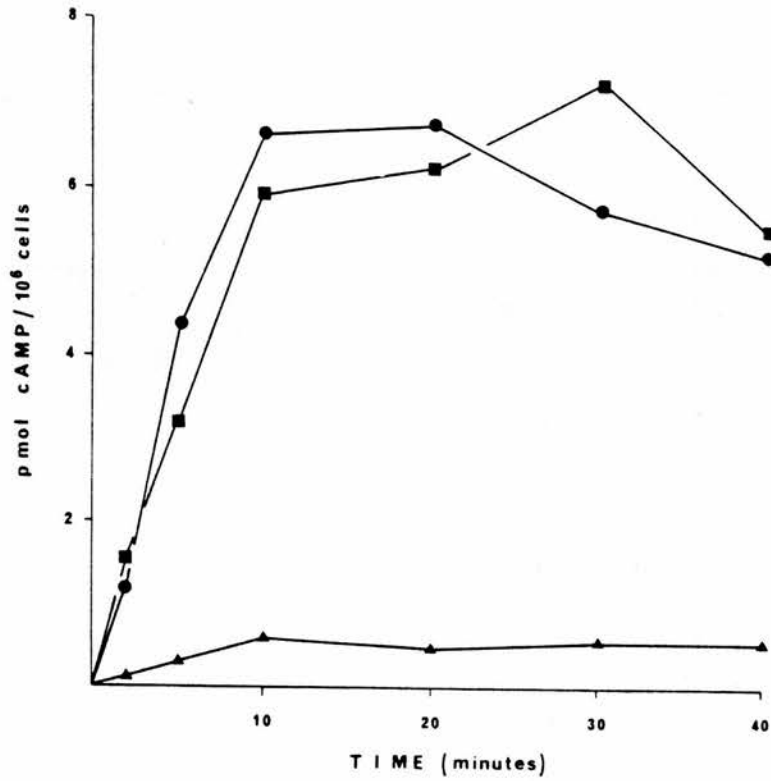


Fig. 5.19 Glucagon stimulated (●-●; 26.8×10^6 cells/ml; ■-■ 13.4×10^6 cells/ml) and basal (▲-▲ 13.4×10^6 cells/ml) adenylate cyclase activity in cell homogenates.

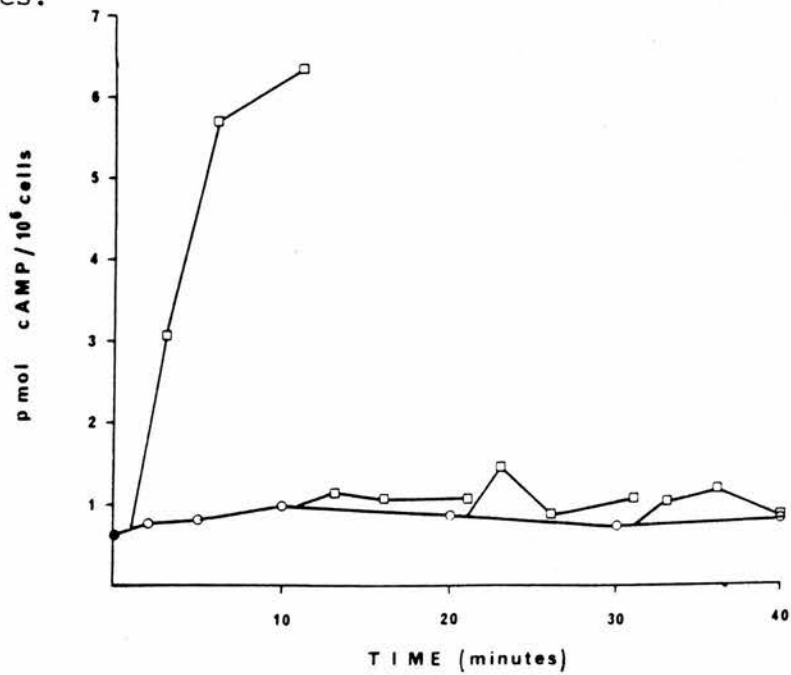


Fig. 5.20 Inactivation of adenylate cyclase, basal (O-O) and glucagon (100 ng/ml) stimulated (□-□), on incubation of homogenates at 30°C.

Table 5.2: Effect of cell homogenate concentration on phosphodiesterase activity.

Values given are averages of duplicate observations. High K_m enzyme assayed at 250 μM cAMP; low K_m enzyme assayed at 5 μM cAMP.

Homogenate concentration ($\times 10^6$ cells/ml)	Enzyme activity pmol cAMP/min/ 10^6 cells	
	High K_m	Low K_m
133	3.59	0.65
66	5.95	1.19
33	10.23	1.75
16	8.18	1.95
8	7.69	1.95
4	7.17	2.35

with or without insulin, glucagon, or APP (all at 100 ng/ml) for 20 minutes at 37°C. Cells were pelleted by centrifugation (2750 g; 1 min) and homogenised in extraction buffer as outlined in Chapter 2 and phosphodiesterase activity determined. Activities found for two different experiments are shown in Table 5.3. The second experiment was carried out using an extraction buffer containing 0.2% v/v triton X-100 which resulted in higher activities of both K_m enzymes. No significant increase in high or low K_m activity was noted with any of the effectors used at 5 or 250 μM cAMP respectively. Activity of the low K_m enzyme was also assayed using 0.5 μM cAMP as substrate after preincubation of the cells with the hormones for either 10 or 40 minutes (Table 5.4). Again no significant change in phosphodiesterase activity was found.

Discussion

The sensitivity of the cell preparations, with respect to cAMP production, to low concentrations of glucagon was found to vary depending on the particular batch of collagenase used to prepare the cells. Initial experiments on time course of cAMP production were carried out using cells prepared by batch 17 collagenase obtained from Boehringer Corporation Ltd. and were found to show significant stimulations in basal cAMP levels at glucagon concentrations as low as 2.5 ng/ml ($0.72 \times 10^{-9} \text{ M}$). More recent experiments including the dose response curves were carried out with cells isolated using batches 25 or 27 collagenase and consistently yielded less sensitive cells. Concentrations of nearly 10 ng/ml ($2.9 \times 10^{-9} \text{ M}$) glucagon were required to produce measurable effects on basal cAMP levels in the absence of theophylline, with half maximum stimulations obtained at approximately 45 ng/ml ($12.9 \times 10^{-9} \text{ M}$). These hormone levels are higher than those reported for rat hepatocytes of $1.5 \times 10^{-9} \text{ M}$ (Pilkis *et al.* 1975), 1 to $2 \times 10^{-9} \text{ M}$ (Sonne *et al.* 1978) and $6.0 \times 10^{-9} \text{ M}$ (Rosselin *et al.* 1974). However in the presence of 5 mM

Table 5.3: Effect of Insulin, Glucagon and APP on cAMP phosphodiesterase activity.

Activities of high and low K_m enzymes measured at 250 μM and 5 μM cAMP respectively. Cell suspension preincubated with effector for 20 minutes before assay of activity. Standard deviations given are based on four observations.

Experiment A - normal extraction buffer

Effector	Enzyme activity pmol cAMP/min/ 10^6 cells	
	High K_m	Low K_m
-	6.75 $^{\pm}$ 0.59	2.43 $^{\pm}$ 0.19
Insulin (100 ng/ml)	6.49 $^{\pm}$ 0.21	2.58 $^{\pm}$ 0.12
Glucagon (100 ng/ml)	6.34 $^{\pm}$ 0.31	2.27 $^{\pm}$ 0.15
APP (100 ng/ml)	7.09 $^{\pm}$ 0.68	2.21 $^{\pm}$ 0.06

Experiment B - extraction buffer containing 0.2% v/v triton X-100

Effector	Enzyme activity pmol cAMP/min/ 10^6 cells	
	High K_m	Low K_m
-	11.19 $^{\pm}$ 0.57	3.58 $^{\pm}$ 0.13
Insulin (100 ng/ml)	11.82 $^{\pm}$ 1.02	3.60 $^{\pm}$ 0.10
Glucagon (100 ng/ml)	10.41 $^{\pm}$ 0.49	3.81 $^{\pm}$ 0.25
APP (100 ng/ml)	12.15 $^{\pm}$ 0.60	3.57 $^{\pm}$ 0.10

Table 5.4: Effect of Insulin, Glucagon and APP on low K_m cAMP phosphodiesterase activity.

Activity of the low K_m enzyme was measured using $0.5 \mu\text{M}$ cAMP as substrate after preincubation of cell suspension for 10 or 40 minutes with effector. Extraction buffer contained 0.2% v/v triton X-100. Standard deviations based on four observations.

Effector	Preincubation time (minutes)	Enzyme activity pmol cAMP/min/ 10^6 cells
-	10	0.759 ± 0.052
-	40	0.734 ± 0.058
Insulin (100 ng/ml)	10	0.758 ± 0.013
Insulin (100 ng/ml)	40	0.770 ± 0.016
Glucagon (100 ng/ml)	10	0.762 ± 0.026
Glucagon (100 ng/ml)	40	0.801 ± 0.018
APP (100 ng/ml)	10	0.745 ± 0.037
APP (100 ng/ml)	40	0.767 ± 0.044

theophylline, substantial stimulation of basal cAMP (approximately 30%) could be found with concentrations of 0.3 ng/ml (8.6×10^{-11} M) (cells prepared with Batch 25 collagenase). These small increases in cAMP level were not detected under the conditions used to monitor the larger increases in the nucleotide level but are most likely to be more important in the physiological control of metabolism. Due to this and the fact of the reduced sensitivity found with batches 25 and 27 collagenase the figure reported for half maximum responses will be overestimated. Half maximal activation of the cyclase by adrenaline was found at about 300 μ g/ml (1.7×10^{-6} M) which again will be an overestimate of the true value. This is almost identical to the half maximal value for adrenaline stimulation of cAMP in rat hepatocytes (Tolbert et al. 1973).

Basal levels of the cyclic nucleotide varied considerably between cell preparations isolated with different collagenase batches. High levels of the nucleotide, 1 to 4 pmols cAMP/ 10^6 cells, were found immediately after the isolation procedure, but these generally stabilised to lower values (0.5 to 1.5 pmols/ 10^6 cells) within 10 to 15 minutes. Similar nucleotide levels were found for rat hepatocytes by Rosselin et al. (1974) (0.7 to 1.4 pmol/ 10^6 cells). Assuming that the nucleotide is evenly distributed throughout a hypothetically spherical cell, then the chicken hepatocyte would contain approximately a 8 fold higher basal concentration of cAMP than the rat (diameter of chicken and rat hepatocytes are 12 μ m and 24 μ m respectively - Capuzzi et al. 1974, Dickson 1977). However Tolbert et al. (1973) have reported basal levels of cAMP in rat hepatocytes of 3 pmol/ 10^6 cells; likewise Sonne et al. (1978) report basal levels in the region of 5 pmol/ 10^6 cells which correspond well with the theoretical basal concentrations as found for the chicken hepatocyte. Rosselin et al. (1974) also reported that basal levels of cAMP were stimulated up to ten fold in the presence of (1 mM) theophylline alone.

However Pilkis et al. (1975) found only minor increases in the basal level with theophylline.

When preparing the hepatocytes using collagenase batches 25 and 27, much higher basal levels of cAMP were noted with values ranging from 1.5 to sometimes 5 pmols/ 10^6 cells, after preincubation of the cells for up to 40 minutes. The increased basal concentration of the nucleotide may be due to activation of adenylate cyclase by various contaminants present in certain batches of collagenase. Hanoune et al. (1977) have previously shown that very low concentrations of crude collagenase are capable of stimulating adenylate cyclase in rat liver plasma membranes. The stimulatory factor was found to be a protein which could be separated from the main collagenase component of the preparation and had a molecular weight on gel chromatography of 30,000. The maintenance of the elevated cAMP levels in the chicken hepatocytes is also consistent with the apparently irreversible effect of this protein stimulator in the rat liver plasma membranes. This activation may be due to certain endogenous serine proteases in the crude collagenase preparation. Although Rosselin et al. (1974) reported no effect of trypsin on basal cAMP concentrations other workers have reported that various serine proteases like trypsin are capable of activating adenylate cyclase in cultured fibroblasts (Wallach et al. 1978) and in membrane fractions from rat ovary (Richert and Ryan 1977). If collagenase contaminants are responsible for these elevations in cAMP, direct comparison of basal and perhaps hormone stimulated levels of the cyclic nucleotide will be critically dependent on the extent of activator contamination of individual collagenase preparations, the length of time and the concentration of collagenase used during the perfusion. Even the lowest values found for the chicken hepatocytes (0.5 to 1.5 pmoles/ 10^6 cells) and indeed by other workers using rat hepatocytes may be elevated above the natural basal concentration due to the collagenase digest.

Time courses of cAMP production stimulated by glucagon or adrenaline resulted in an initial rapid accumulation of the nucleotide generally reaching a peak between 2 (low hormone concentrations) and 10 minutes (high hormone concentrations) of incubation. The nucleotide was rapidly lost to the external medium and was degraded in both intracellular and extracellular compartments. Similar time course profiles have been reported for rat hepatocytes (Pilkis et al. 1975). After the initial rapid accumulation of cAMP, levels quickly (low hormone concentrations) or gradually (high hormone concentrations) returned to basal values. The brief increase in production of cAMP must be controlled mainly by the cyclase and not the cAMP phosphodiesterase since inhibition of the latter with theophylline or caffeine does not prevent the abrupt termination of nucleotide production although degradation is inhibited.

Maximum stimulation of the cyclic nucleotide by glucagon varied from 10 to 60 fold and by adrenaline by 5 to 10 fold depending mostly on the initial basal levels. Maximum stimulation by glucagon in the absence of theophylline consistently yielded concentrations of 30 to 40 pmoles/ 10^6 cells after 10 minutes of incubation. Theophylline did not increase maximum content of the cells but maintained the cAMP level for longer periods. Results reported for rat hepatocytes have shown considerable variation. Rosselin et al. (1974) reported maximum stimulated levels of only 10 to 15 pmols/ 10^6 cells for rat hepatocytes although this could be increased to 60 to 90 pmols/ 10^6 cells in the presence of theophylline. Pilkis et al. (1975) reported a 10 fold increase in the nucleotide which was doubled on addition of theophylline whereas Sonne et al. (1978) found over a 40 fold stimulation with cAMP levels reaching 200 pmol/ 10^6 cells. The maximum theoretical intracellular concentrations found in chicken hepatocytes are very similar to those reported by Sonne et al. (1978) for the rat but the results obtained by Rosselin et al. (1974)

would indicate an approximate two fold higher nucleotide level in the hormone stimulated chicken hepatocyte compared with the rat hepatocyte. These variations are most likely due to the use of different collagenase types and methods to isolate the cells as explained previously, making direct comparisons very difficult if not impossible.

Concentrations of glucagon well above those needed to produce maximum effects are capable of maintaining the cAMP levels for longer periods than lower hormone concentrations that also initially produce maximum effect. This was also found with rat hepatocytes (Pilkis et al. 1975). Decrease in the cAMP level on incubation must therefore be due to hormone degradation. Hormone degradation is not accompanied by receptor or cyclase degradation since after a fall in a once maximal cAMP level the hepatocytes respond rapidly to further increases in glucagon concentration although cAMP levels cannot be elevated above the initial maximal values. High glucagon concentrations however only induce a slow increase in intracellular cAMP in cells preincubated with adrenaline suggesting the initial catecholamine enhanced nucleotide accumulation antagonises further activation by glucagon.

Although insulin has been shown to considerably decrease submaximal glucagon stimulated levels of cAMP in isolated rat hepatocytes (Pilkis et al. 1975; Westwood and Siddle 1979) as well as perfused rat liver (Exton and Park 1972) preincubation of chicken hepatocytes for 10 minutes with pharmacological concentrations of insulin only slightly inhibited the peak of cAMP production. Simultaneous addition of insulin and glucagon to cell suspension failed to depress the peak of nucleotide production. The reasons for this are unclear. In all cases however high insulin concentrations markedly prevented total cAMP degradation in the suspensions. This was only observed at pharmacological levels of the hormone. This effect of insulin has also been reported for rat adipose

tissue. Concentrations of insulin above 10^{-4} M have been shown to potentiate adrenaline stimulated cAMP and lipolysis in rat adipose tissue (Desai et al. 1973; Hales et al. 1978). The reason for this response was not known.

Intracellular cAMP degradation was not inhibited by insulin suggesting that the rate of extracellular cAMP degradation must be decreased. Extracellular degradation was due to a soluble factor and was not associated with the exterior surface of the hepatocyte. The method by which insulin suppresses the rate of cAMP degradation is not known but may be due to one of the following:

- 1) Insulin, or a degradative product, directly inhibits the factor(s) present in the extracellular medium which degrades cAMP.
- 2) Insulin, or a degradative product, binds extracellular cAMP preventing degradation by the active factor(s).
- 3) Insulin inhibits the release of the degradative factor(s) from the cell or plasma membrane.
- 4) Insulin inhibits re-entry of extracellular cAMP for degradation by the intracellular phosphodiesterase.

Whatever the reason for the decreased cAMP loss from the suspension it is unlikely that this phenomenon will have any physiological importance.

Neither APP nor ionophore A23187 when incubated with the hepatocytes for varying periods, altered basal or hormone-stimulated cAMP levels. This is in contrast to the decrease in glucagon stimulated cAMP response by ionophore A23187 reported for rat hepatocytes (Westwood and Siddle 1979). It is possible that insulin lowering of cAMP levels in rat hepatocytes involves increase in intracellular calcium and that some component of the interdependent action of these two effectors is not present in the chicken hepatocyte.

Adenylate cyclase activity in cell homogenates was stimulated by glucagon but no direct effect on the enzyme was noted on addition of insulin or APP. This absence of a direct effect by insulin has been reported by others (Pohl et al. 1971; Thompson et al. 1973; Pilakis et al. 1974). The loss of cyclase activity during preincubation may be as a result of endogenous GTP hydrolysis which is known to stimulate the enzyme (Rodbell 1971b,c; Lad et al. 1977).

Despite reports of insulin stimulated phosphodiesterase activity in rat hepatocytes (Loten et al. 1978) no effect was noted when using the chicken hepatocytes. This was supported by the unchanged degradation rates of intracellular cAMP in hepatocytes incubated with insulin.

Inconclusive results were obtained for the effects of the hormones and ionophore A23187 on cellular cGMP. Due to the high variability encountered in basal cGMP between and within individual experiments, changes in cGMP concentration by up to 100% would not be detected. However no gross changes in the basal nucleotide level similar to those seen with cAMP were observed after incubation with various effectors including insulin. A lack of effect by insulin on cGMP levels after short (5 minutes) or long (60 minutes) term incubations was also reported for rat hepatocytes (Flockhart and Siddle 1979), with basal concentrations of the guanine nucleotide in the rat of 20 pmol/g wet wt.

Summary

The chicken hepatocyte adenylate cyclase responds to glucagon with up to ten fold lower sensitivity than that found with isolated rat hepatocytes. Sensitivity to adrenaline, however, appears to be similar to that reported for rat hepatocytes. Hepatocytes responded by large increases in cellular cAMP of up to 60 times basal which are similar if not higher in magnitude than those observed with rat hepatocytes. As previously found for mammalian liver, saturating concentrations of

adrenaline fail to activate the cyclase population to the extent of that seen with saturating glucagon concentrations, resulting in lower cellular cAMP levels. It appears that all the adenylate cyclase is activated by glucagon, since adrenaline has no additional effect; presumably only a small proportion of cyclase molecules is activated when all adrenaline receptors are fully occupied. Insulin does not appear to significantly lower submaximally stimulated cAMP levels as reported for rat hepatocytes. Pharmacological insulin concentrations in some unknown way maintain the level of extracellular cAMP for longer periods although this is not thought to have any physiological importance. Insulin was also found to have no effect on adenylate cyclase or phosphodiesterase activities. Basal cGMP levels in the cell are approximately 100 fold lower than those of cAMP and are not influenced by any of the hormones mentioned nor by ionophore A23187.

CHAPTER 6

HORMONE CONTROL OF CARBOHYDRATE

AND LIPID METABOLISM

Introduction

Both carbohydrate and lipid metabolism in the mammalian liver can be directly regulated by the pancreatic hormones, insulin and glucagon (Exton and Park 1972; McGarry et al. 1975). Great interest has been shown in the antagonistic actions of insulin and glucagon on the rat liver and hence a substantial literature has accumulated on this subject. Although adrenaline is also capable of stimulating glucose release from the liver in times of stress, glucagon is considered to be the most physiologically important hormone for the production of glucose via hepatic glycogenolysis and gluconeogenesis.

There have been many reports on the hormonal control of glycogenolysis and gluconeogenesis in rat hepatocytes (Johnson et al. 1972; Garrison and Haynes 1973; Tolbert and Fain 1974; Wagle 1975; Hue et al. 1978; Pilkis et al. 1978) but studies of the control of lipid metabolism in rat liver have been less frequent with fewer reports on perfused rat liver, and liver slices (Meikle et al. 1973; Raskin et al. 1974; Harris et al. 1978) or hepatocytes (Geelan and Gibson 1975). Despite the lack of interest in lipogenesis in the rat hepatocyte however, Goodridge and co-workers have carried out numerous investigations into the high rates of lipid synthesis found in neonatal chicken hepatocytes (Goodridge 1973, 1975; Goodridge et al. 1974; Goodridge and Adelman 1976); and it is only recently that any detailed study on the hormonal control of carbohydrate metabolism in chicken hepatocytes has been reported (Anderson and Langslow 1975; Dickson 1977; Dickson et al. 1978; Dickson and Langslow 1978).

Both glucagon and adrenaline stimulate glucose release from rat hepatocytes isolated from fed rats and also stimulate gluconeogenesis from a number of different precursors in hepatocytes isolated from starved rats (Johnson et al. 1972; Garrison and Haynes 1973; Tolbert

and Fain 1974; Pilkis et al. 1975). Garrison and Haynes (1973) reported half maximum stimulation of glucose production (ED_{50}) from rat hepatocytes (glycogenolysis or gluconeogenesis) with glucagon concentrations around 1.5×10^{-7} M. However if incubations were carried out for shorter time intervals and if the oxidised β -chain of insulin was incorporated into the medium as a protective agent against glucagon degradation, values as low as 1.0×10^{-10} M were found. Even under the latter conditions the glucagon dose response curve covered glucagon concentrations of over three orders of magnitude. The lower value for half maximum stimulation reported by Garrison and Haynes (1973) compares well with the figures reported for glucagon infusion in the perfused rat liver where ED_{50} values in the range 10^{-10} to 10^{-9} M were found (Exton and Park 1968; Lewis et al. 1970; Exton et al. 1971a,b). Hue et al. (1978) reported a half maximal stimulation of gluconeogenesis by glucagon in rat liver cells at 1×10^{-9} M with the dose response range over one order of magnitude indicating a very sensitive cell preparation. Likewise Pilkis et al. (1975) have reported ED_{50} values for gluconeogenesis from lactate at glucagon concentrations of 3×10^{-10} M. Again the dose response curve covers a very narrow range of glucagon concentrations.

Adrenaline has a half maximal response in the perfused rat liver at concentrations around 10^{-8} M (Exton and Park 1968) with reported ED_{50} values for glucose release in rat hepatocytes varying from 2.0×10^{-7} M (Tolbert et al. 1973; Pilkis et al. 1975) to 2.5×10^{-6} M (Garrison and Haynes 1973). Again reported ED_{50} values for glycogenolysis and gluconeogenesis are similar with values of 1.3×10^{-6} M and 2.5×10^{-6} M respectively (Garrison and Haynes 1973). Reports by Pilkis et al. (1975) and Claus and Pilkis (1976) suggest that adrenaline is not capable of stimulating gluconeogenesis to the same extent as glucagon in rat hepatocytes. However this has not been found by others using perfused rat liver (Exton and Park 1968; Exton et al. 1971a,b) or rat hepatocytes (Garrison and Haynes 1973).

Higher values reported for half maximum stimulation of glucose release are probably induced by a loss in sensitivity due to the collagenase digest (Garrison and Haynes 1973; Pilkis et al. 1975). Generally the effects of glucagon are noticeable at very low concentrations (10^{-10} M and below); however, the concentration of hormone needed to produce maximum metabolic effect shows considerable variation. The higher the concentration of hormone necessary to produce the maximum response the higher the reported value for ED_{50} and the lower is the sensitivity of the cell preparation.

Maximum stimulatory effects of glucagon and adrenaline on gluconeogenesis in rat hepatocytes have been reported to be additive (Tolbert and Fain 1974) indicating two independent stimulatory mechanisms which both fail to saturate the metabolic effect. However this finding has not been supported by other workers using rat hepatocytes (Pilkis et al. 1975; Hue et al. 1978) or chicken hepatocytes (Anderson and Langslow 1975), where the maximum effect by glucagon could not be potentiated by addition of adrenaline. However it is becoming increasingly apparent that the actions of the catecholamines on the liver takes place mainly in the rat via the cAMP-independent α -receptor (see Chapter 1 and review by Pilkis et al. 1978).

Chicken hepatocytes have been reported to show similar responses to rat hepatocytes with respect to glucagon stimulated gluconeogenesis (Dickson and Langslow 1978) and glycogenolysis (Dickson et al. 1978). Although the dose response range of glucagon in perfused rat liver and isolated rat hepatocytes was found to be the same for both glycogenolysis and gluconeogenesis with similar concentrations inducing half maximal response (Lewis et al. 1970; Garrison and Haynes 1973), the ED_{50} values for these metabolic pathways differ by an order of magnitude in chicken hepatocytes. A glucagon concentration of 9.0×10^{-9} M was required to

produce half maximal stimulation of glycogenolysis with the effect saturating at 10^{-7} M (Dickson 1977; Dickson et al. 1978) whereas glucagon at 7.7×10^{-10} M was sufficient to produce half maximal stimulation of gluconeogenesis (Dickson 1977; Dickson and Langslow 1978). However, if the stimulation of gluconeogenesis failed to reach saturation, a lower ED_{50} value would result. If indeed a difference in half maximal stimulation does exist between the two pathways, this suggests that the rate limiting step for stimulation of gluconeogenesis in chicken hepatocytes lies beyond the level of the hormone receptor.

Dickson et al. (1978) have also shown that adrenaline stimulates glycogenolysis in the chicken hepatocyte with an ED_{50} at 3.5×10^{-9} M and saturation at 10^{-7} M. Low levels of adrenaline therefore appear to be much more effective in chicken hepatocytes than in rat hepatocytes, with concentrations as low as 1×10^{-9} M showing a considerable stimulation of glucose release.

Dibutyryl cAMP (Bt_2 cAMP) has been shown to stimulate glycogenolysis and gluconeogenesis in rat hepatocytes (ED_{50} at 4.5×10^{-7} M and 7.0×10^{-7} M respectively - Garrison and Haynes 1973) and chicken hepatocytes (ED_{50} approximately 2×10^{-6} M - Dickson 1977). In both systems the cyclic nucleotide was capable of giving a maximum stimulation comparable with glucagon. The dose response curves for Bt_2 cAMP are almost identical for glycogenolysis or gluconeogenesis in the chicken hepatocyte (Dickson 1977) in spite of their ten fold difference in sensitivity to glucagon as reported above. This indicates that some other glucagon stimulated factor besides cAMP may be responsible for the enhanced gluconeogenic rate at low hormone concentrations.

Insulin antagonises the effects of glucagon and adrenaline on glycogenolysis and gluconeogenesis in the rat liver (Exton and Park 1972) and rat hepatocytes (Johnson et al. 1972; Wagle 1975; Hue et al. 1978).

However insulin has been reported not to effect glycogen deposition nor basal or hormone stimulated glucose release in hepatocytes isolated from fed or starved chickens (Anderson and Langslow 1975). This lack of effect of insulin on the hepatocyte is in agreement with the apparent insensitivity of the chicken to intravenous injections of the hormone (see Chapter 1).

There have been numerous reports on the control of lipogenesis in the chicken liver (Allred and Roehrig 1972; Goodridge 1973; 1975; Capuzzi et al. 1971; 1974; Watkins and Lane 1976). Glucagon inhibits lipogenesis in the chicken hepatocyte with an ED_{50} of 2×10^{-9} M (Goodridge 1973) as does Bt_2cAMP with an ED_{50} of 1×10^{-4} M (Capuzzi et al. 1974). Watkins and Lane (1976) suggested that the inhibition of lipogenesis by glucagon or Bt_2cAMP was due to a decrease in intracellular citrate, the allosteric activator of acetylCoA carboxylase. These workers concluded that the actions of glucagon and Bt_2cAMP were due to inhibition of the glycolytic pathway resulting in a decrease by up to 90% in the intracellular citrate concentration.

Insulin has been reported to increase basal lipid synthesis by approximately 15% in chicken hepatocytes and also to depress the inhibition imposed by cAMP (Capuzzi et al. 1971). This appears to be the only report suggesting a direct action of insulin on the isolated chicken hepatocyte.

Methods

Hepatocytes isolated from fed chickens were used in investigations of glycogenolysis and lipogenesis. Hepatocytes isolated from 24 hour starved birds were used in the gluconeogenesis experiments. All isolations were carried out using a high potassium content KRB buffer (see Chapter 3) to prevent glycogenolysis and loss of glucose during preparation (Dickson 1977).

Immediately after isolation, the cell suspension was diluted with fresh normal KRB buffer to give concentrations in the range 6 to 10×10^6 cells/ml, and dispensed into plastic incubation pots (25 x 75 mm). If required, hormones or effectors were added to the pots in small volumes (10 to 25 μ l) before addition of the cell suspension. Pots were gassed for 10 to 20 seconds with 95% O₂/5% CO₂ and incubated at 37°C in a reciprocal shaking waterbath (120-140 strokes/minute). After the required incubation times cells were pelleted by centrifugation (2750 g; 1 min) and processed for the determination of glycogen or incorporation of [¹⁴C]-acetate into lipid as outlined in Chapter 2. The supernatant was also sampled for estimations of glucose released to the external medium (see Chapter 2).

Results

1) Glycogenolysis

As reported in the results for glucagon induced cAMP accumulation in hepatocyte suspensions (Chapter 5), the sensitivity of the cell suspension was dependent on which batch of collagenase was used to prepare the cells. However only slight variations in the concentration range of hormone stimulation was found using cells prepared using the same batch of collagenase with dose response curves giving virtually identical plots. Batch 17 collagenase again produced the most sensitive cell preparation compared to the other batches tested (25, 29 and 30).

a) Time course of glucose release. The average glycogen content of hepatocytes isolated from fed birds was 30.32 ± 5.02 μ g glucose equivalents/ 10^6 cells (standard deviation: n = 7). The basal rate of glucose release and glycogen content of the hepatocytes is shown in Fig. 6.1. An approximately linear rate of glucose accumulation in the external medium was found with about 13 μ g glucose released per 10^6 cells per hour. This was accompanied by a parallel linear decrease in glycogen

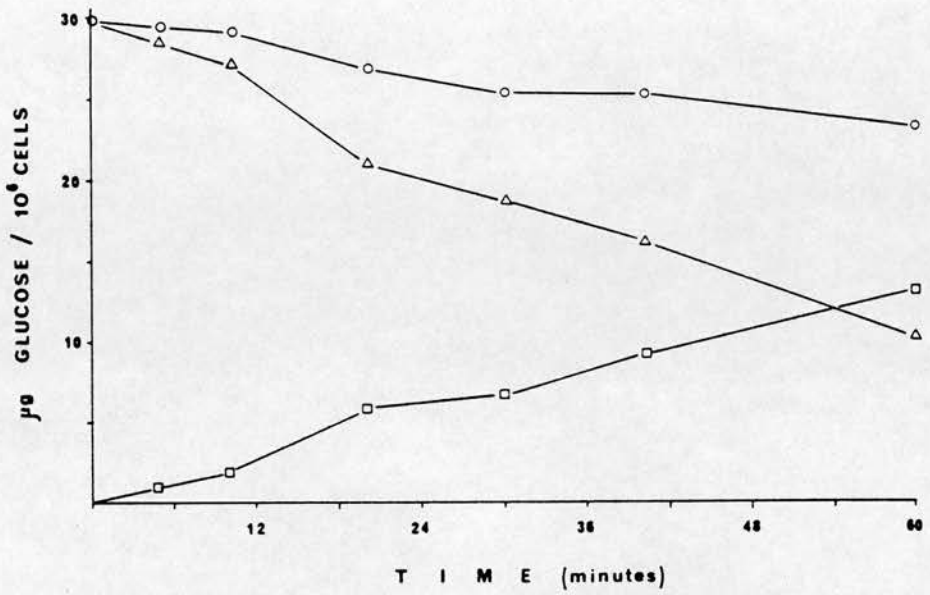


Fig. 6.1 Basal glucose release ($\square-\square$), glycogen content ($\Delta-\Delta$) and total carbohydrate (O-O) in cell suspensions incubated at 37°C .

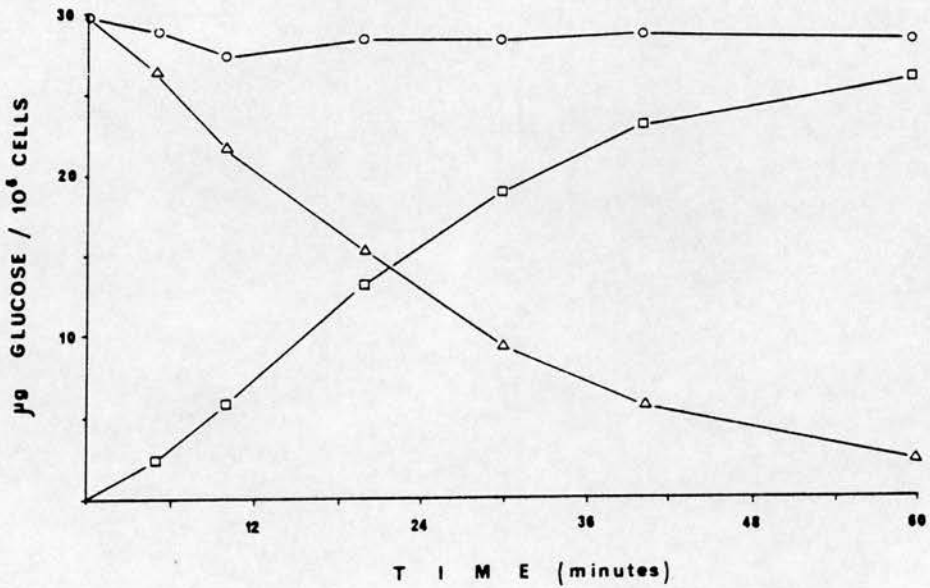


Fig. 6.2 Glucagon (10 ng/ml) stimulated glucose release ($\square-\square$) with resulting glycogen content ($\Delta-\Delta$) and total carbohydrate (O-O) in cell suspensions incubated at 37°C .

content of the cells although nearly 20 μg of glycogen, measured as glucose equivalents (see Chapter 2) was lost in one hour.

In the presence of saturating concentrations of glucagon (10 ng/ml; $2.9 \times 10^{-9}\text{M}$) the rate of accumulation of glucose in the external medium was approximately doubled and loss of cellular glycogen was greatly accelerated (Fig. 6.2). After only 20 minutes incubation, 13 μg of glucose was released per 10^6 cells, and within 30 minutes the glycogen content was depleted to the same level found for one hours incubation in the absence of glucagon. In the presence of glucagon the total carbohydrate content (glucose plus glycogen) of the cell suspension remained relatively constant for 60 minutes. Although there was an initial decrease in total carbohydrate content up to 10 minutes of incubation this was gradually replenished with longer incubation.

b) Glucagon dose response curve for glucose release. As mentioned above, the sensitivity of the cells varied depending on the batch of collagenase used during the initial preparative stages of isolation. The most sensitive preparations were consistently found using batch 17. Fig. 6.3 shows the release of glucose and loss of glycogen from cells incubated in the presence of varying concentrations of glucagon for 30 minutes at 37°C . Concentrations of glucagon at 0.15 ng/ml ($4.4 \times 10^{-11}\text{M}$) showed significant elevations in glucose release and glycogen breakdown. Half maximal effect was found with glucagon concentrations of 0.6 ng/ml ($1.7 \times 10^{-10}\text{M}$) and maximum effects with 5 ng/ml ($1.44 \times 10^{-9}\text{M}$). However all the later batches of collagenase used (25, 29 and 30) showed much lower sensitivities (Figs. 6.4, 6.5 and 6.6 respectively). Half maximal stimulations were found at concentrations of 2.6 ng/ml ($7.5 \times 10^{-10}\text{M}$) - batch 29; 3.8 ng/ml ($1.09 \times 10^{-9}\text{M}$) - batch 25; and 9.6 ng/ml ($2.8 \times 10^{-9}\text{M}$) - batch 30. Values reported are averages for two experiments using individual cell preparations isolated with the same batch of collagenase.

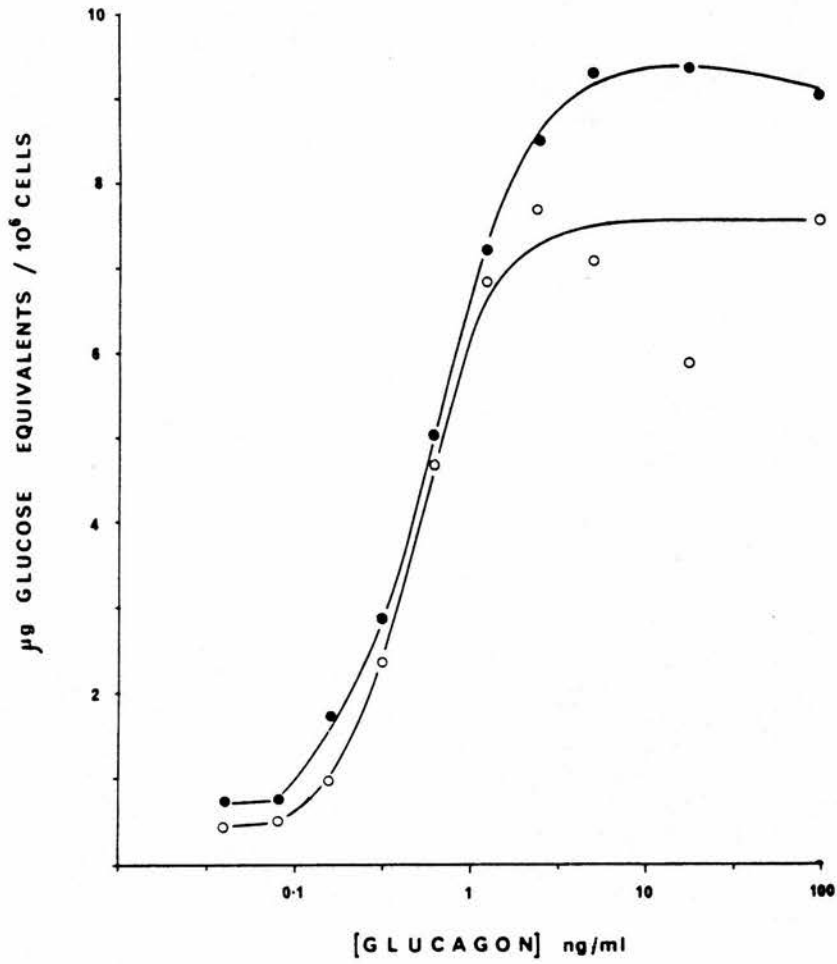


Fig. 6.3 Glucagon dose response for glucose release (●-●) and glycogen loss (O-O) from cell suspensions incubated for 30 minutes at 37°C. Values obtained in the absence of hormone subtracted from all points.

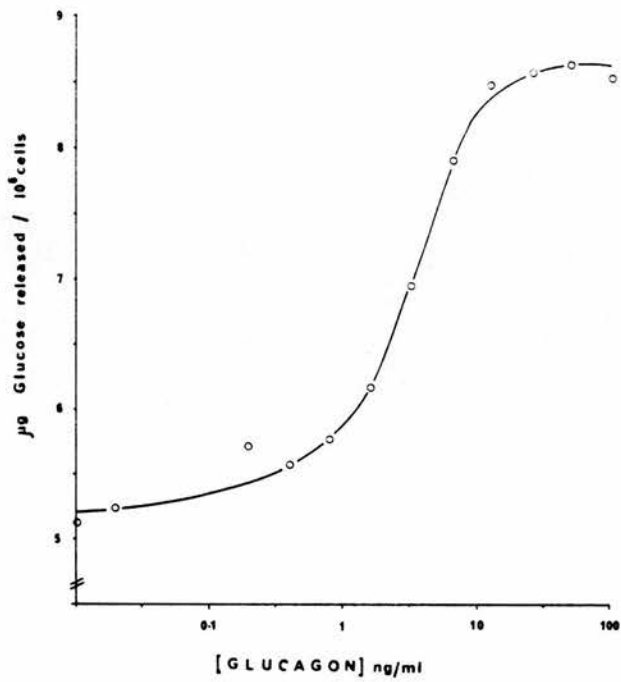


Fig. 6.4 Glucagon dose response for glucose release. Cells prepared using batch 25 collagenase.

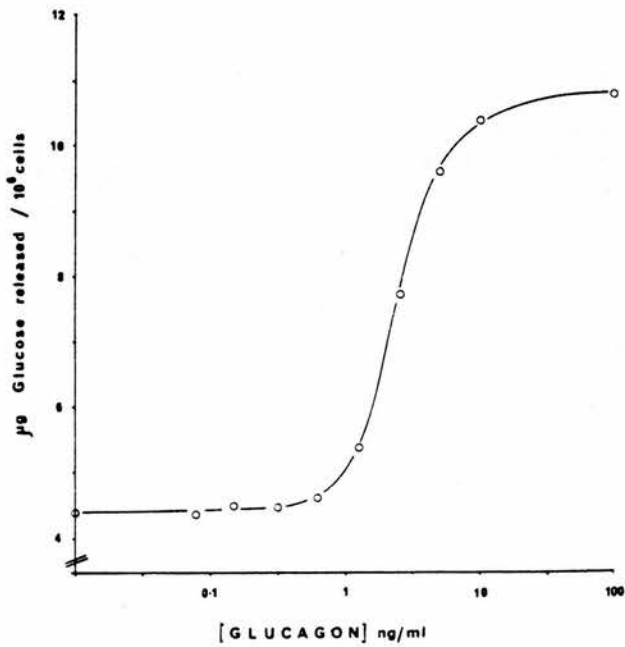


Fig. 6.5 Glucagon dose response for glucose release. Cells prepared using batch 29 collagenase.

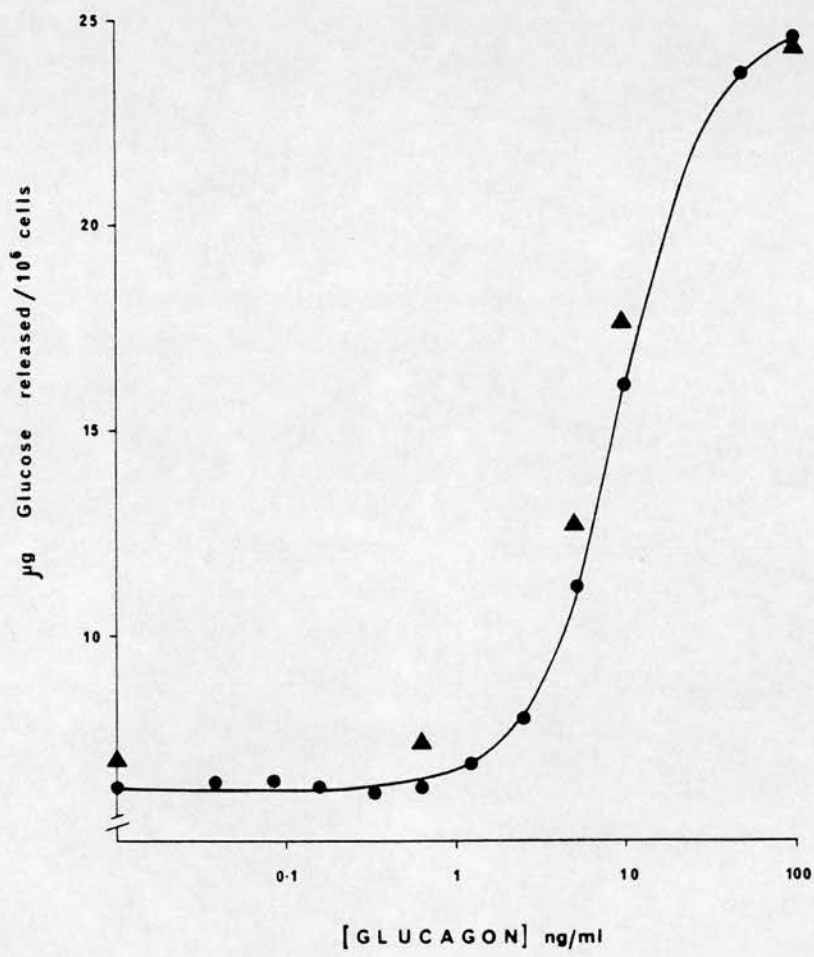


Fig. 6.6 Glucagon dose response for glucose release. Cells prepared by batch 30 collagenase incubated with (▲-▲) or without (●-●) Bt₂cGMP (0.2 µM).

Incorporation of soya bean trypsin inhibitor (20 $\mu\text{g/ml}$) throughout the preparative stages of cell isolation did not increase cell sensitivity. Fig. 6.7 shows the glucagon dose response curve for glucose release using cells prepared by batch 25 collagenase with added trypsin inhibitor. Half maximal effect was found at 3.2 ng/ml ($9.1 \times 10^{-10}\text{M}$) which compares well with cell preparations without the inhibitor. The inhibitor did not effect cell yield nor the percentage of cells which stained with trypan blue.

Addition of bacitracin (0.5 mg/ml) to the 30 minute incubations with glucagon did not alter the shape or position of the dose response curve (Fig. 6.7). Half maximum stimulations of glucose release were found at the same glucagon concentration and low concentrations of the hormone produced identical effects under both conditions.

Addition of Bt_2cGMP (0.2 μM) induced a slight increase in basal or submaximally glucagon stimulated glucose release (Fig. 6.6).

c) Adrenaline dose response. The chicken hepatocyte also showed a high affinity for adrenaline. Cells prepared by batch 17 collagenase produced half maximal stimulation with adrenaline concentrations of 17.4 ng/ml ($9.5 \times 10^{-8}\text{M}$) with maximal effect at concentrations at 100 ng/ml ($5.3 \times 10^{-7}\text{M}$) or above (Fig. 6.9). Using batch 25 collagenase to prepare the cells the dose response curve was not as symmetrical and half maximal stimulation was found at adrenaline concentrations of approximately 25 ng/ml ($1.36 \times 10^{-7}\text{M}$) with maximum effect at 1000 ng/ml ($5.5 \times 10^{-6}\text{M}$) (Fig. 6.8).

d) Glucagon-adrenaline relationship (Table 6.1). Maximal effects of glucagon and adrenaline on glucose release were not additive. Adrenaline (1 $\mu\text{g/ml}$) was capable of producing a maximum stimulation of glucose release from the hepatocytes similar to that found using saturating glucagon concentrations.

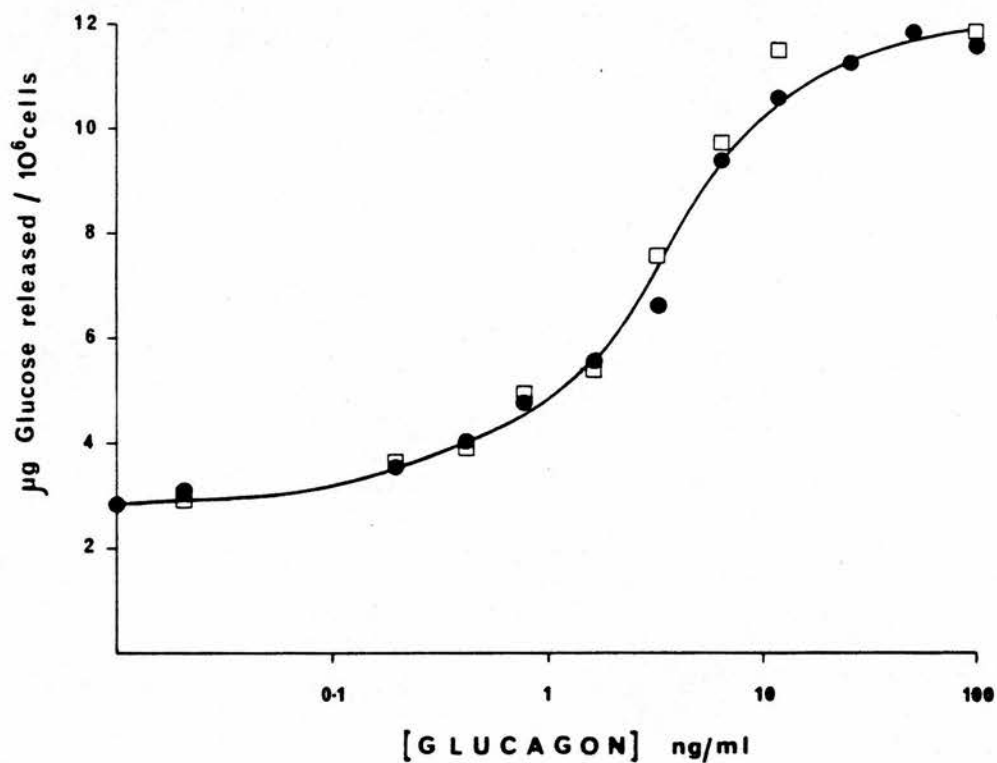


Fig. 6.7 Glucagon dose response for glucose release. Cells prepared using batch 25 collagenase containing added soya bean trypsin inhibitor. Cells incubated with (□-□) or without (●-●) bacitracin (0.5 mg/ml).

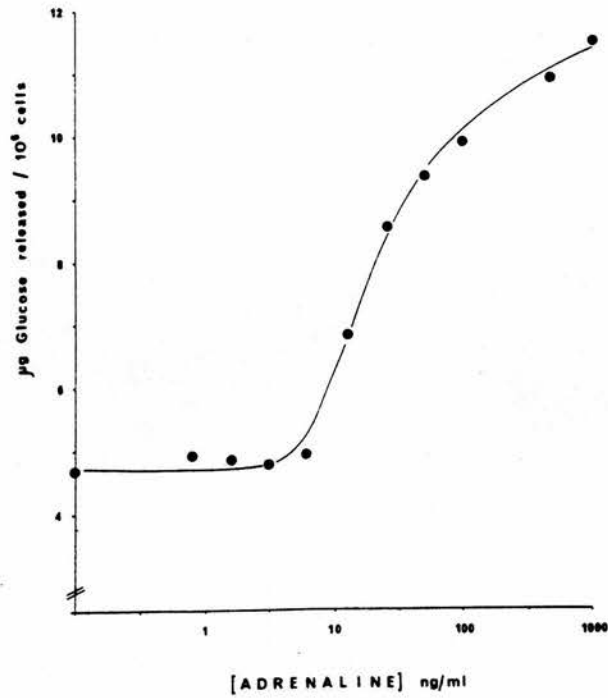


Fig. 6.8 Adrenaline dose response for glucose release. Cells prepared by batch 25 collagenase.

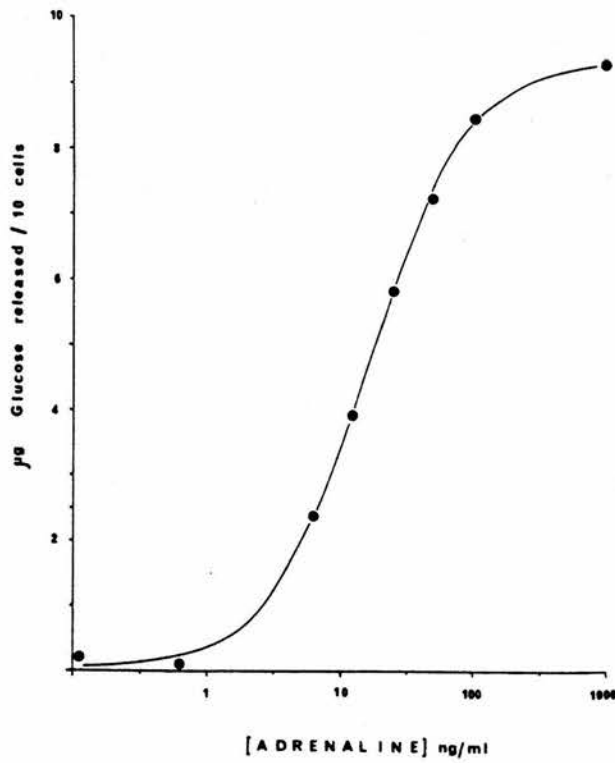


Fig. 6.9 Adrenaline dose response for glucose release. Cells prepared by batch 17 collagenase. Glucose release in absence of hormone subtracted from all points.

Table 6.1: Glucose release - Effect of maximum concentrations of
Glucagon and Adrenaline.

Cell suspension incubated for 30 minutes at 37°C with or without hormone and glucose release determined. Batch 17 collagenase used to prepare cells. Standard deviations are based on three observations.

Condition	μg glucose released/ 10^6 cells
Basal	5.13 ⁺ 0.22
Glucagon (10 ng/ml)	14.82 ⁺ 0.54
Glucagon (100 ng/ml)	14.50 ⁺ 0.63
Glucagon (10 ng/ml) + Adrenaline (1 $\mu\text{g}/\text{ml}$)	14.14 ⁺ 0.96
Adrenaline (1 $\mu\text{g}/\text{ml}$)	14.66 ⁺ 0.38
Adrenaline (5 $\mu\text{g}/\text{ml}$)	14.11 ⁺ 0.25
Adrenaline (1 $\mu\text{g}/\text{ml}$) + Glucagon (100 ng/ml)	14.62 ⁺ 0.91

e) Insulin, APP and ionophore A23187. Neither porcine or chicken insulin (10 or 500 ng/ml) nor APP (10 or 500 ng/ml) showed any significant effect on basal glucose release from hepatocytes from fed birds (Figs. 6.10 and 6.11). However ionophore A23187 (5 $\mu\text{g/ml}$) showed a substantial increase in glucose accumulation in the medium (Fig. 6.10). Although insulin (10, 100 or 500 ng/ml) again showed no significant effect on submaximally stimulated glucagon levels, increasing APP concentrations (10 to 500 ng/ml) resulted in a small progressive increase in glucose release. Ionophore A23187 exhibited a biphasic effect on hormone stimulated glucose release. Low concentrations of ionophore (0.1 and 0.5 $\mu\text{g/ml}$) showed significant inhibition of glucose release whereas high concentrations (1 or 5 $\mu\text{g/ml}$) gave marked stimulations (Figs. 6.10 and 6.11). However, higher concentrations of the ionophore (10 $\mu\text{g/ml}$) produced a marked inhibition in glucose release. The inhibition at lower concentrations is most probably due to the ethanol in which the stock ionophore is dissolved. Fig. 6.12 shows the inhibition of glucose release by various ethanol concentrations. The ethanol content at different ionophore concentrations is also given and it can be seen that glucose release is inhibited to approximately the same extent at nearly all ethanol concentrations.

Incubation of cells results in a progressive decrease in glycogen content (Fig. 6.1). Addition of physiological glucose concentrations (15 mM) to cell incubations reduced the glycogen loss after one hour by 25 to 30%. Further addition of either porcine or chicken insulin (0.001 to 100 ng/ml) to the medium did not increase glycogen content of the cells. Incubation of cells with increasing APP concentrations showed a slight preservation of glycogen content although glucose output remained constant (Fig. 6.11).

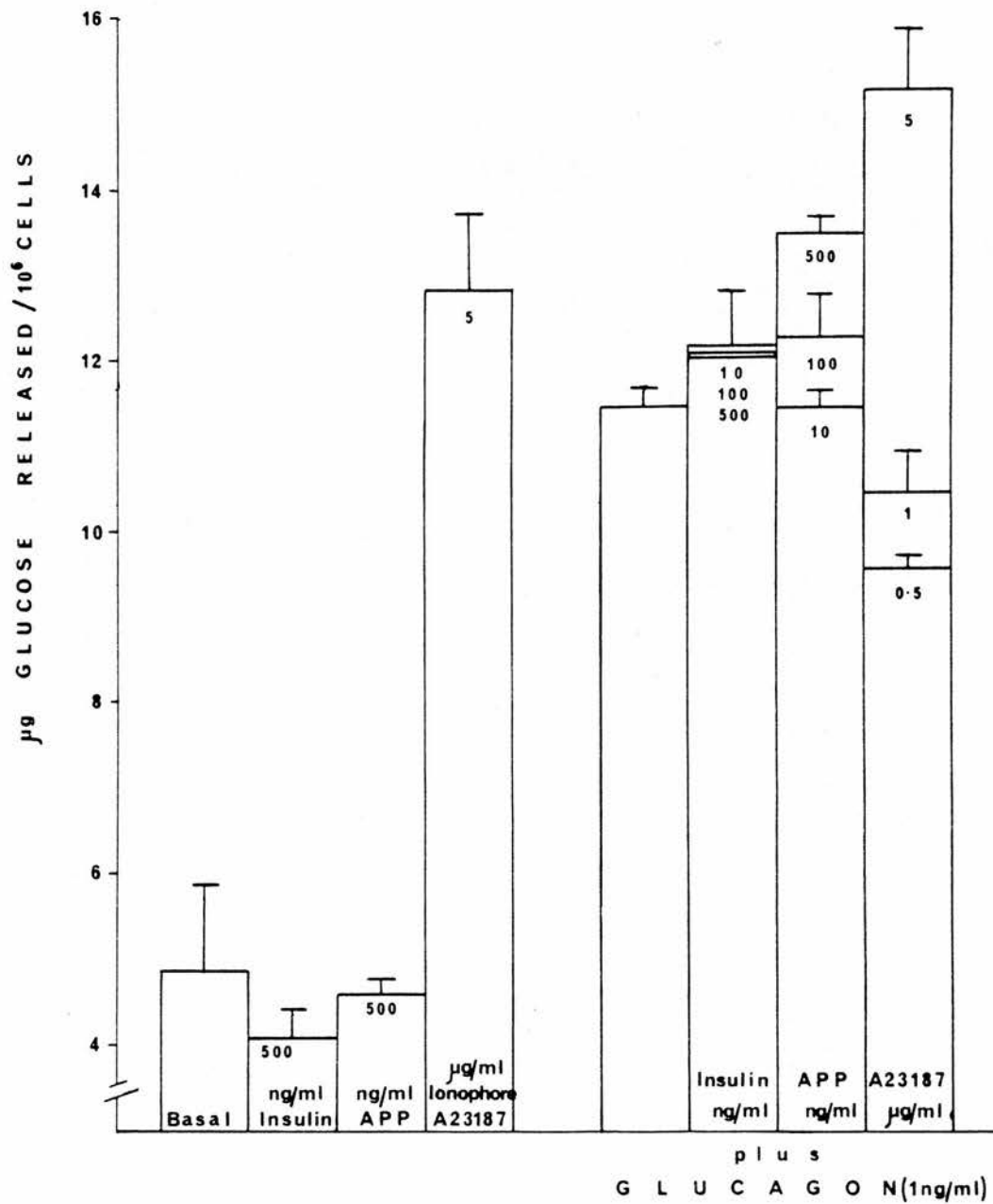


Fig. 6.10 Effect of insulin, APP and ionophore A23187 on basal and submaximally glucagon stimulated glucose release.

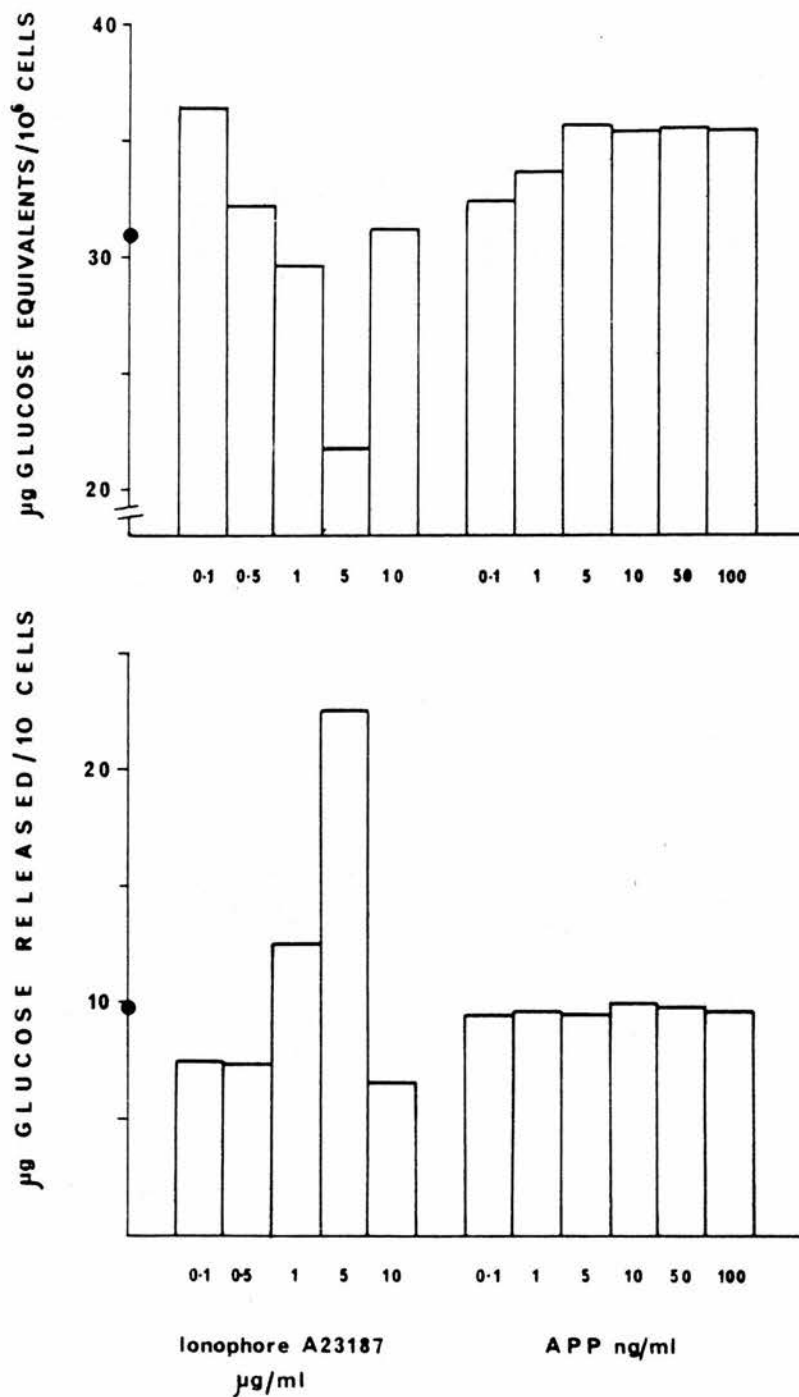


Fig. 6.11 Effect of ionophore A23187 and APP on basal glucose release and glycogen content after 30 minutes incubation. (●) denotes the values in the absence of effector.

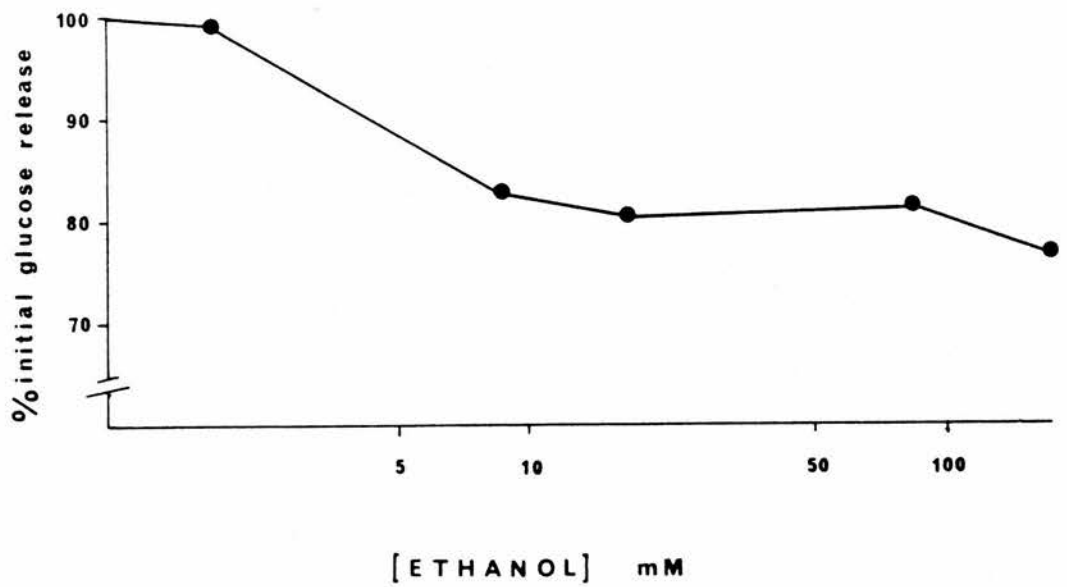


Fig. 6.12 Inhibition of basal glucose release in the presence of ethanol-Ionophore concentrations in cell suspensions necessitated the following ethanol concentrations.

Ionophore concentration	Resulting ethanol
$\mu\text{g/ml}$	mM
10	200
5	100
1	20
0.5	10
0.1	2

f) Cyclic AMP and Dibutyryl cAMP. Cyclic AMP and the synthetic derivative dibutyryl cAMP (Bt_2cAMP) also stimulated glucose release (Figs. 6.13 and 6.14 respectively). Bt_2cAMP was much more effective with a maximum stimulation of glucose release over basal of over 200% at a concentration of 5×10^{-5} M. Half maximal response was found at 1.58×10^{-6} M. Relatively higher concentrations of cAMP were required to produce similar effects on glucose release. Half maximal stimulation was found at 5.0×10^{-5} M with minimum response at 6.0×10^{-4} M. Cyclic AMP also failed to stimulate glucose release to the same extent as Bt_2cAMP with only a 170% stimulation being found.

2) Gluconeogenesis

The control of gluconeogenesis was studied by monitoring the rate of incorporation of lactate into glucose in hepatocytes isolated from 24 hour starved birds. The glycogen content of these hepatocytes was always below measurable amounts under the conditions used (less than $0.5 \mu\text{g}$ glucose equivalents/ 10^6 cells).

a) Time course. Fig. 6.15 shows the time course of glucose output of the cells incubated in the presence of lactate (1.25 or 2.5 mM) with or without glucagon (100 ng/ml). Only small stimulations of glucose release were found which were more apparent at shorter time intervals.

b) Dose response to lactate. Fig. 6.16 shows the production of glucose after 30 minutes incubation with various lactate concentrations.

A linear response was only found with concentrations up to 1.5 mM. Higher concentrations, up to 10 mM, gradually saturated the rate of production of glucose from lactate resulting in maximum rates around $10 \mu\text{g}$ glucose per 10^6 cells per 30 minutes.

c) Glucagon and adrenaline. Cell suspensions prepared using batch 25 collagenase, were incubated in the presence of 1.5 mM lactate for 20 minutes in the presence of varying concentrations of glucagon (Fig. 6.17) or adrenaline (Fig. 6.18). Both hormones induced maximal increases of around 30% over basal. Half maximum activation of gluconeogenesis under

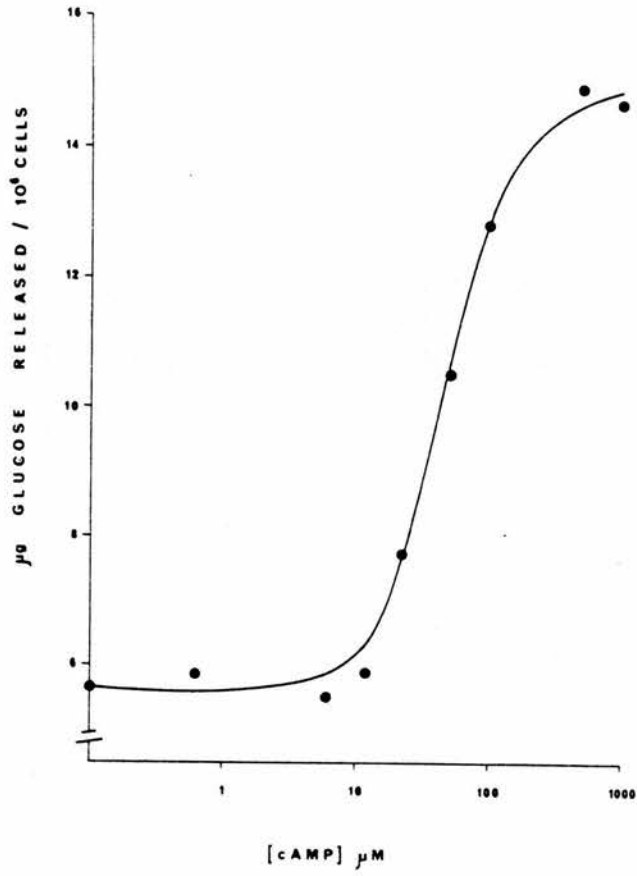


Fig. 6.13 cAMP dose response for glucose release.

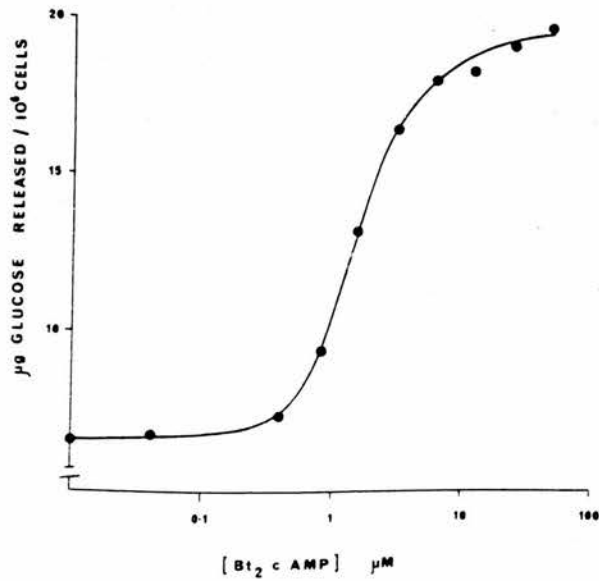


Fig. 6.14 Bt₂cAMP dose response for glucose release.

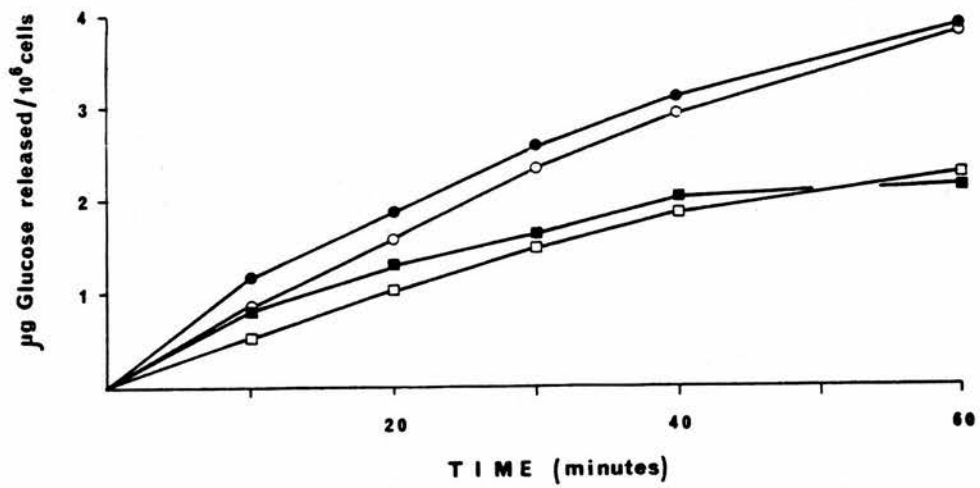


Fig. 6.15 Gluconeogenesis from lactate in the absence, □-□, 1.25 mM; ○-○ 2.5 mM; or the presence ■-■ 1.25 mM, ●-● 2.5 mM, of glucagon (100 ng/ml). Glucose release in the absence of lactate subtracted from all points.

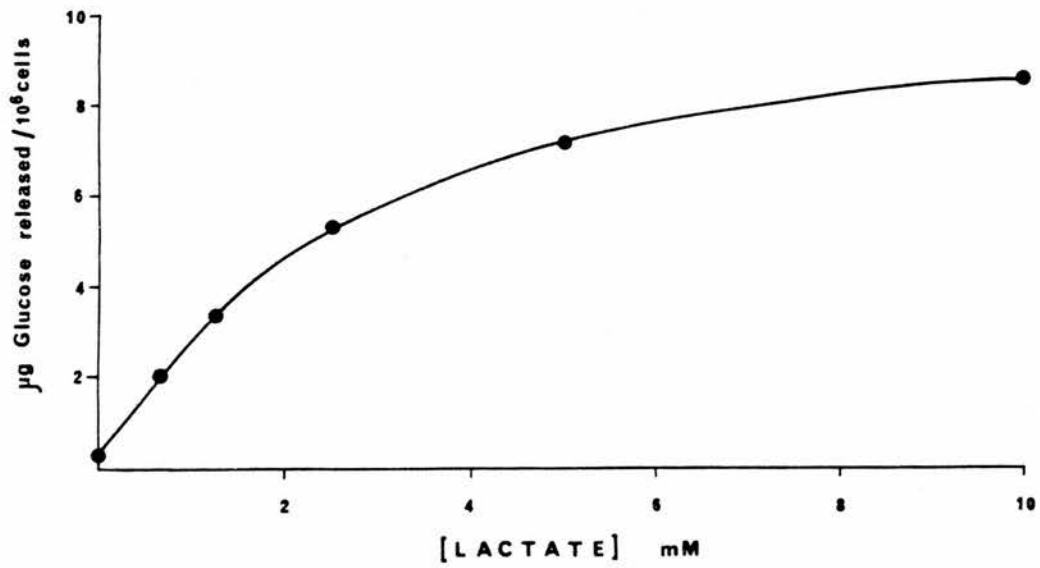
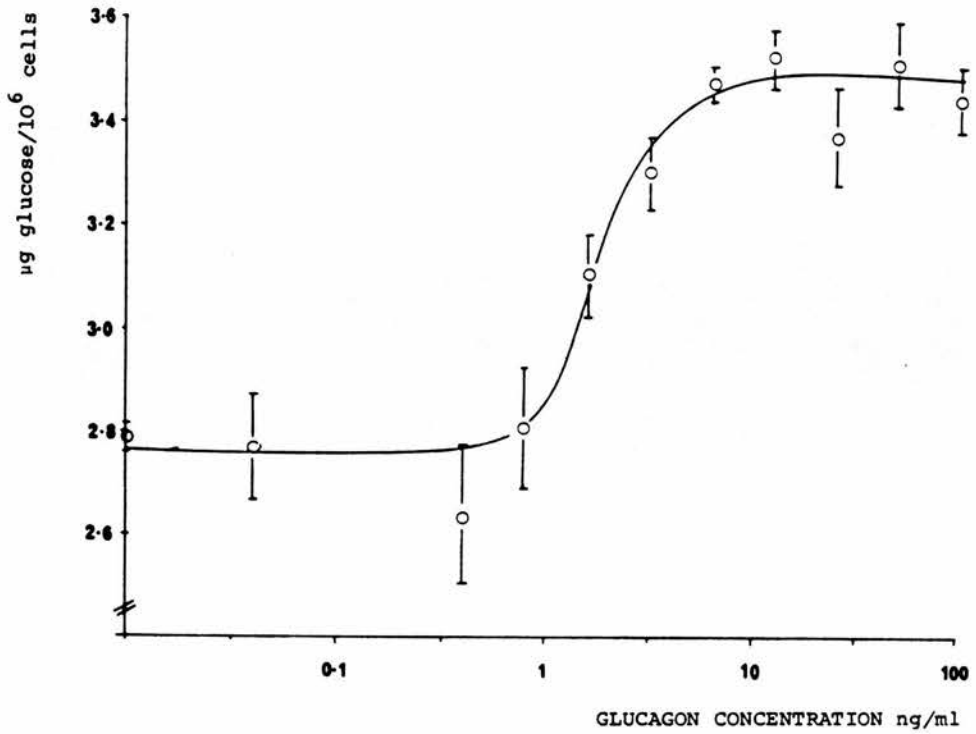
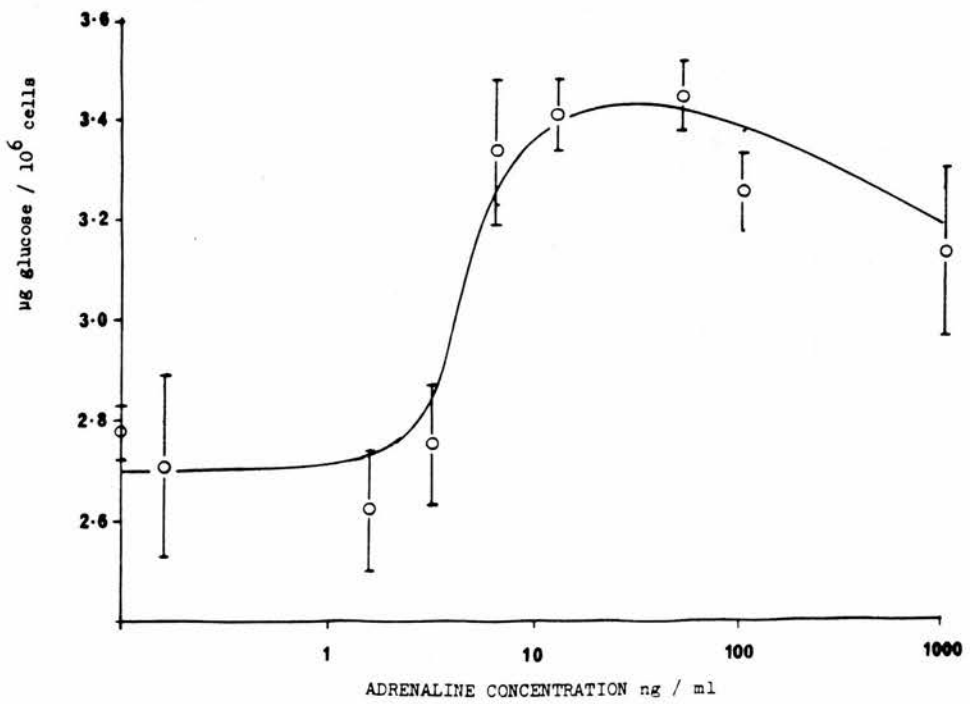


Fig. 6.16 Gluconeogenesis - glucose production from cells incubated for 30 minutes in the presence of various lactate concentrations.



STIMULATION OF GLUCONEOGENESIS FROM LACTATE (1.5 mM) BY GLUCAGON

Fig. 6.17



STIMULATION OF GLUCONEOGENESIS FROM LACTATE (1.5 mM) BY ADRENALINE.

Fig. 6.18

these conditions was found at concentrations of 1.68 ng/ml (4.8×10^{-10} M) glucagon and 3.8 ng/ml (2.08×10^{-8} M) adrenaline. High concentrations of adrenaline (100 ng/ml) but not glucagon resulted in a slight reduction in the stimulation of gluconeogenesis (Fig. 6.18).

d) APP (10 or 100 ng/ml) did not effect basal or hormone stimulated gluconeogenesis from lactate (1.5 mM) under the conditions mentioned above.

3) Lipogenesis

Lipogenesis was studied by the incorporation of acetate into triglyceride using cell suspensions prepared using batch 25 collagenase. Hepatocytes isolated from fed birds were used in all experiments.

a) Time course. Fig. 6.19 shows the time course of acetate incorporation into cellular triglyceride. [14 C]-Acetate (2.5 mM) was incubated in the presence of cell suspension with or without insulin (100 ng/ml) and/or glucagon (5 ng/ml). Cells were reoxygenated after one hour's incubation. Linear rates of tracer incorporation into triglyceride were found with up to 90 minutes of incubation. Glucagon markedly decreased the rate of incorporation whereas insulin had no effect on basal or glucagon reduced rates.

b) Glucagon and adrenaline. Cells were incubated for one hour with 2.5 mM acetate with or without various concentrations of glucagon or adrenaline (Figs. 6.20 and 6.21). Basal rates of acetate incorporation under these conditions was generally in the range 20 to 25 nmol acetate incorporated per 10^6 cells per hour. Although both glucagon and adrenaline were capable of reducing acetate incorporation into triglyceride by as much as 90%, neither hormone induced complete inhibition of lipogenesis. Half maximum effect of glucagon ranged from 3.5 to 3.8 ng/ml (1.00 to 1.09×10^{-9} M) with maximum effect around 12 ng/ml (3.4×10^{-9} M). Adrenaline was half maximally effective at 12.6 ng/ml (6.9×10^{-8} M) with maximum effect at 30 ng/ml (1.6×10^{-7} M).

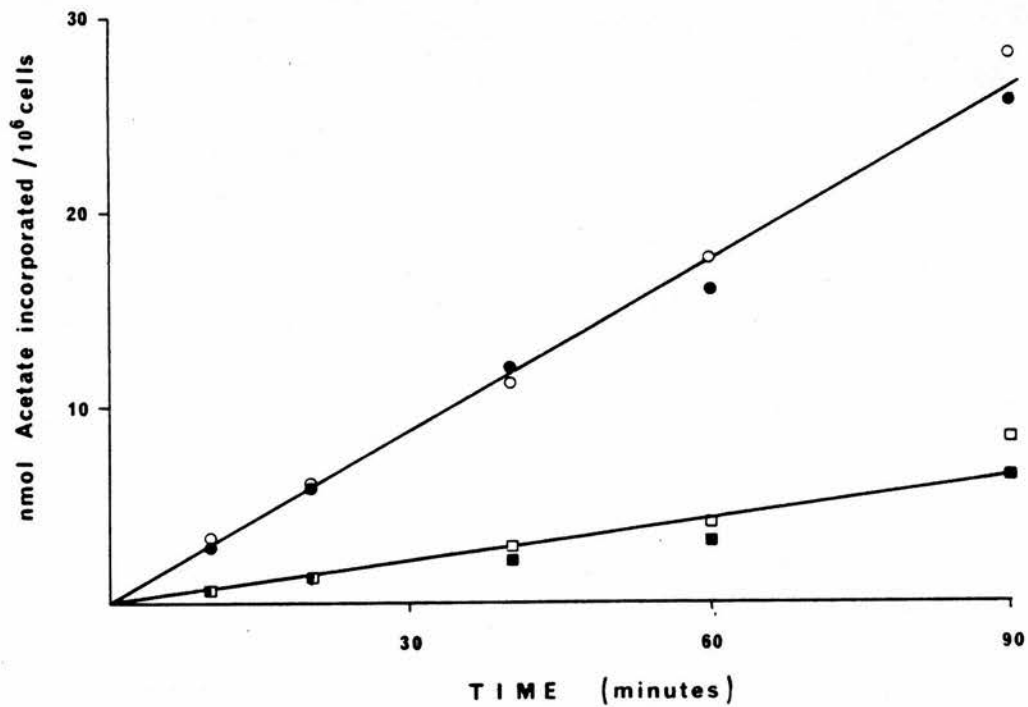


Fig. 6.19 Incorporation of acetate (2.5 mM) into cellular triglyceride in the absence of effector (O-O) or in the presence of glucagon (5 ng/ml) (□-□); insulin (500 ng/ml) (●-●) or insulin (500 ng/ml) plus glucagon (5 ng/ml) (■-■).

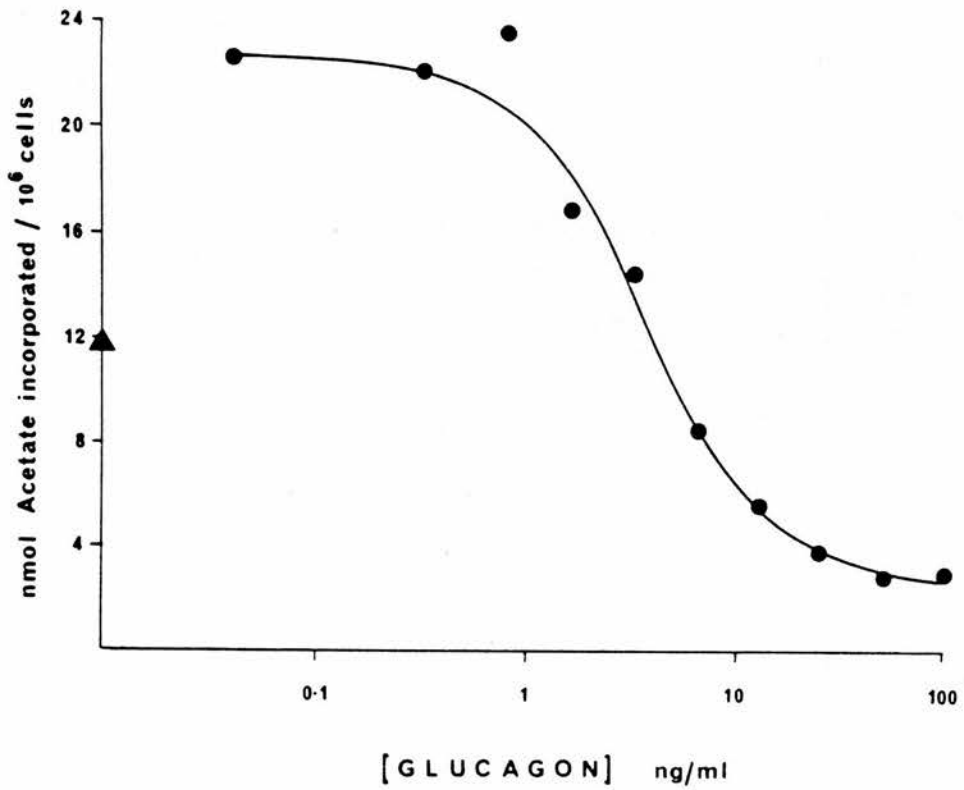


Fig. 6.20 Glucagon dose response for triglyceride synthesis from acetate (2.5 mM) (●-●). ▲ indicates the effect of theophylline (10 mM) on basal triglyceride synthesis.

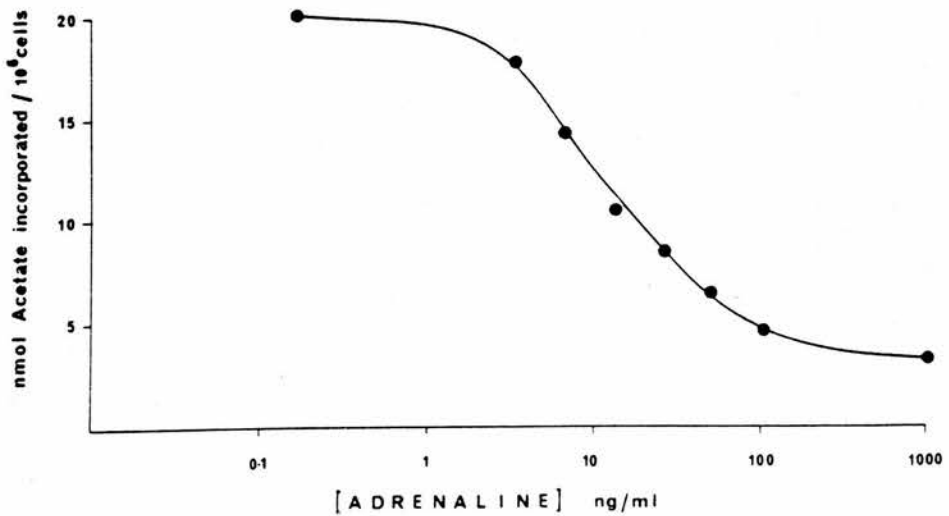


Fig. 6.21 Adrenaline dose response for triglyceride synthesis from acetate (2.5 mM).

c) APP. Basal or glucagon inhibited lipogenesis was not effected by APP (1 to 1000 ng/ml).

4) Effects of Caffeine and Theophylline

Cells isolated by method A (see Chapter 3) were used for the majority of the experiments incorporating these phosphodiesterase inhibitors. However similar if not identical characteristics were found when using cells prepared by method B (see Chapter 3). Although theophylline successfully maintained hormone stimulated intracellular cAMP levels in the hepatocytes (see Chapter 4), a wide range of concentrations of theophylline and caffeine inhibited glycogenolysis from hepatocytes isolated from fed birds (Figs. 6.22 and 6.23) and gluconeogenesis from lactate in hepatocytes isolated from starved birds (Fig. 6.24). The methylxanthines (10 mM) were found to inhibit basal, hormone or Bt_2 cAMP stimulated gluconeogenesis from lactate (2.5 mM) or fructose (2.5 mM) by 40 to 70% (Table 6.2). Incubation of cell suspension in the presence of theophylline or caffeine did not increase the percentage of cells which stained with trypan blue. The effects of the methylxanthines on glucose release could be reversed by washing the cells by three repetitive centrifugations (50 g; 2 min) and resuspension in fresh KRB. These cells, which were relieved of the inhibition by washing were still sensitive to the re-addition of caffeine or theophylline. Theophylline also decreased lipogenesis from acetate by 50% (Fig. 6.20) and decreased intracellular ATP content by nearly 30% (Fig. 6.25).

Discussion

The main difficulty in quantitatively assessing the results obtained in this work and the comparison with figures reported by others, has with the variability in cell sensitivity to the hormone studied. The apparent sensitivity does not only depend on the individual method used during the preparative procedure but is also dependent on the quality of collagenase used. Different batches of collagenase obtained from the same supplier

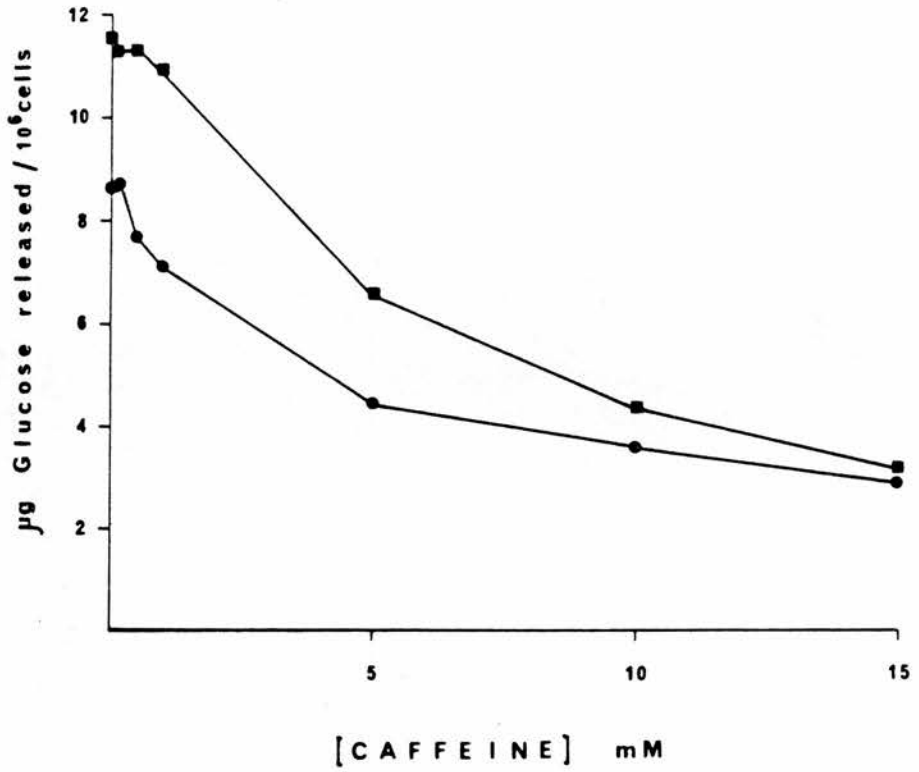


Fig. 6.22 Effect of caffeine on basal (●-●) and glucagon stimulated (10 ng/ml) (■-■) glucose release after 30 minutes incubation at 37°C.

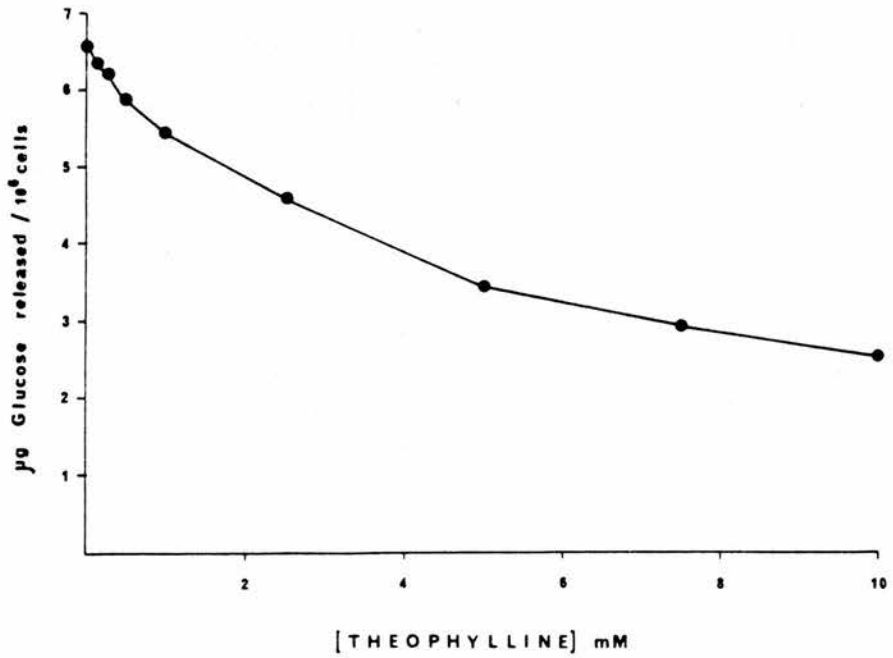


Fig. 6.23 Effect of theophylline on basal glucose release after 30 minutes incubation at 37°C.

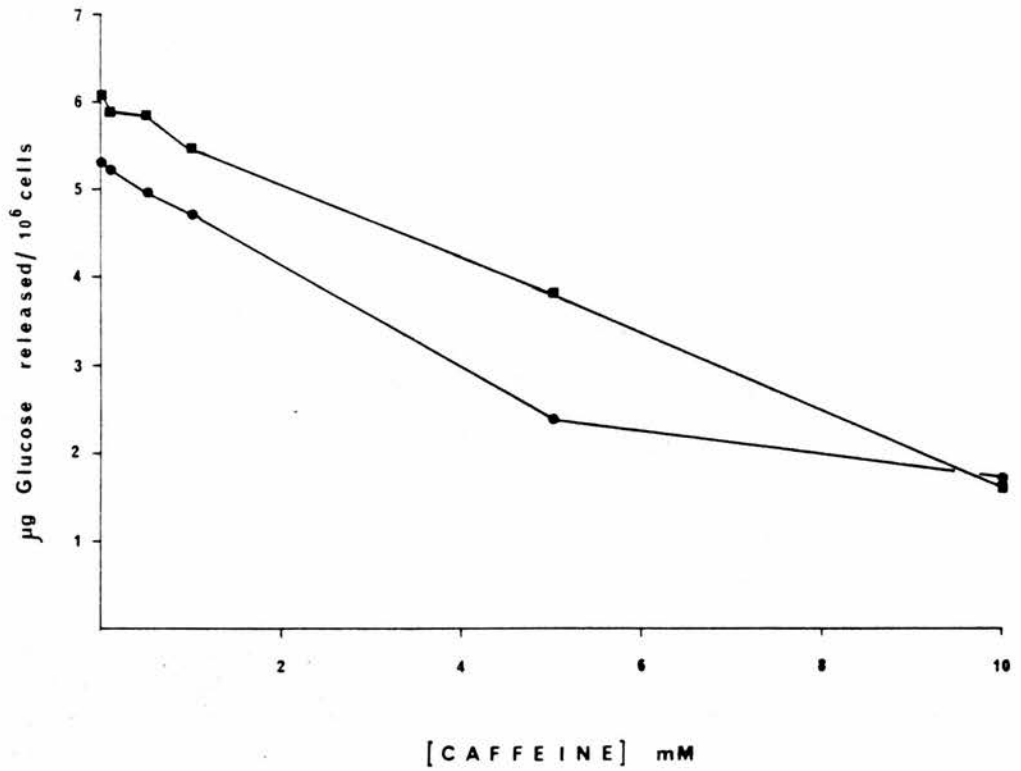


Fig. 6.24 Effect of caffeine on basal ●-● and glucagon (10 ng/nl) stimulated (■-■) gluconeogenesis from lactate (2 mM).

Table 6.2: Effect of Caffeine on Hepatocyte Gluconeogenesis from Lactate or Fructose.

Substrate	Glucose release - μg glucose/ 10^6 cells/30 minutes \pm standard deviation (no. of individual cell preparations)			
	Basal	plus Glucagon (10 ng/ml)	plus Adrenaline (100 ng/ml)	plus Bt ₂ cAMP (7.5 $\mu\text{g}/\text{ml}$)
2.5 mM Lactate	3.84 ⁺ -1.13(8)	4.75 ⁺ -1.15(3)	4.05 ⁺ -0.64(3)	7.76 ⁺ -0.46(3)
2.5 mM Lactate + 10 mM Caffeine	2.06 ⁺ -0.85(8)	2.73 ⁺ -0.76(3)	1.70 ⁺ -0.71(3)	2.67 ⁺ -0.55(3)
2.5 mM Fructose	4.75 ⁺ -0.63(3)	5.65 ⁺ -0.54(3)	-	-
2.5 mM Fructose + 10 mM Caffeine	2.40 ⁺ -0.28(3)	2.35 ⁺ -0.94(3)	-	-

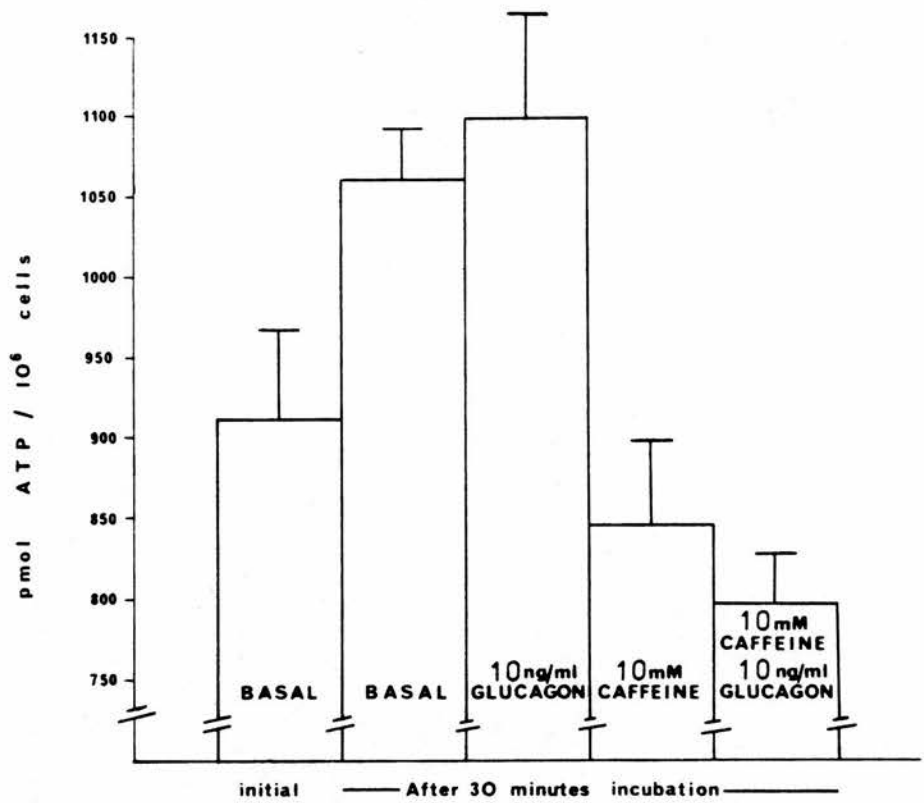


Fig. 6.25 Effect of caffeine on cellular ATP content. Error bars indicate standard deviation of 4 estimates from one representative experiment.

induced marked variations in the sensitivity of the isolated cells, therefore direct quantitative comparisons could only be made with cells prepared using the same collagenase batch number.

Cells isolated from fed birds contained glycogen stores comparable with those reported by Dickson (1977). Incubation of cells resulted in a gradual decrease in total carbohydrate in the cell suspension. Cellular glycogen was continually degraded to glucose and then either released to the external medium or, presumably, used for cell respiration. In the presence of glucagon, however, the total carbohydrate content of the cell suspension was conserved indicating that the hepatocytes were using some other energy source (protein and/or lipid deposits) for respiration: in the presence of glucagon total cell suspension carbohydrate was initially reduced and then re-elevated but never exceeded the initial glycogen content of the cells. This suggests that gluconeogenesis from various endogenous precursors is activated by glucagon to maintain the carbohydrate store of the cell.

Due to the difficulties in maintaining cell sensitivity as mentioned above, the affinity for glucagon varied over a ten fold range. The most sensitive preparations, with half maximum effect of glucagon on glycogenolysis at 1.7×10^{-10} M and adrenaline at 9.5×10^{-8} M probably gives the closest reflection of the in vivo sensitivity of the liver cell to the hormones. Immunoreactive glucagon present in peripheral blood of starved chickens has been reported to be up to 3 or 4×10^{-10} M (Samols et al. 1969; Krug et al. 1976) and presumably the glucagon immunoreactivity in the portal blood will be considerably higher. However, after pancreatectomy approximately half of the glucagon immunoreactivity still remains in the blood which indicates that only 50% of the circulating glucagon estimated by radioimmunoassay is derived from the pancreas (Krug et al. 1976). The extra pancreatic source of this glucagon-like

immunoreactivity (GLI) is the ileum and jejunum of the gut. Gut GLI has been suggested not to be active in carbohydrate metabolism in the chicken liver (Krug and Miahle 1971) suggesting that the liver is exposed only to low concentrations of active pancreatic glucagon (1 to 2×10^{-10} M). Obviously the extent of the pancreatectomy is very important (see Chapter 1).

Although the sensitivity to glucagon was over fifty fold greater than reported by Dickson et al. (1978) (ED_{50} of 9.3×10^{-9} M) the sensitivity of the cells to adrenaline was over one order of magnitude lower. Since no reports were made of different batches of collagenase being used to prepare the cells during their studies, it must be assumed that different hormone-effector systems are not desensitised to the same extent during cell isolation and that different types of collagenase may specifically affect certain hormone receptor systems to a greater extent than others.

Both glucagon and adrenaline stimulated glycogenolysis by nearly 200% and gluconeogenesis from lactate by 30%. These values are slightly lower than those reported for rat hepatocytes (Garrison and Haynes 1973) and perfused rat liver (Exton and Park 1968) but compare well with previous reports using chicken hepatocytes (Dickson 1977; Dickson et al. 1978).

Using cell preparations with lower affinity, half maximum stimulation of gluconeogenesis was found with much lower concentrations of glucagon and adrenaline than needed to produce half maximal stimulation of glycogenolysis. Glucagon produced an ED_{50} at 9.1×10^{-10} M for glycogenolysis and 4.8×10^{-10} M for gluconeogenesis (both batch 25 collagenase). Likewise adrenaline produced an ED_{50} at 1.36×10^{-7} M for glycogenolysis and 2.08×10^{-8} M for gluconeogenesis (again batch 25 collagenase). Although no differences in estimated ED_{50} values have been found for hormone stimulated glycogenolysis or gluconeogenesis in the rat (Garrison and Haynes 1973; Lewis et al. 1970) other workers using chicken hepatocytes also report similar variations in ED_{50} values for the two pathways (Dickson 1977; Dickson et al. 1978; Dickson and Langslow 1978).

Dibutyryl cAMP and cAMP produced similar stimulations of glycogenolysis to glucagon or adrenaline, indicating that both hormones stimulate this process via the cyclic nucleotide. Bt_2cAMP is much more effective at lower concentrations and also produces a slightly greater stimulation than cAMP, which is possibly due to both its enhanced solubility in the plasma membrane and a relatively lower affinity for phosphodiesterase. Concentrations producing half maximum stimulation of glycogenolysis ($1.5 \times 10^{-6} M$) are almost identical to those reported for chicken hepatocytes by Dickson (1977) ($2.0 \times 10^{-6} M$). Since Bt_2cGMP showed a small increase in basal or submaximally hormone stimulated values it is unlikely that this intracellular nucleotide is responsible for the antagonistic effects of insulin as found in the rat (Exton and Park 1972; Wagle 1975).

APP was found to have no effect on gluconeogenesis from lactate or lipogenesis from acetate. However weak reproducible effects were noted on glucagon stimulated glycogenolysis where APP slightly potentiated glucose release. This may be due to the protection of glucagon from degradation since APP alone had no effect on glucose release. However addition of bacitracin; a potent inhibitor of glucagon degradation, did not potentiate the glucagon response. There was also a small decrease in the basal rates of glycogen breakdown in the presence of APP but there was no simultaneous effect on glucose release. A more detailed study of these effects would have to be carried out before any valid conclusion could be made. However APP does not directly enhance glycogen breakdown or inhibit lipogenesis in the liver cell suggesting that the effects in vivo of this hormone (Chapter 1; Hazelwood et al. 1973) are not due to a direct action on the liver. McCumbee and Hazelwood (1976) have also reported a lack of effect of APP on isolated chicken hepatocytes but found that APP was capable of inhibiting lipolysis in chicken adipocytes.

The different ED_{50} values reported in this work were not due to a shift in the entire dose response curve to lower concentrations but mainly to a saturation of response at lower hormone concentrations. Therefore the hormonal effects on gluconeogenesis saturate at lower levels of hormone occupancy than are necessary to fully stimulate glycogenolysis.

Using batch 25 collagenase, half maximum inhibition of lipogenesis occurs with glucagon and adrenaline concentrations of 1.04×10^{-9} M and 6.9×10^{-8} M respectively. The dose response to glucagon compares well with that found for stimulation of glycogenolysis. However the concentration of adrenaline which induces half maximal inhibition of lipogenesis is intermediate between values obtained for gluconeogenesis and glycogenolysis. The low value reported for gluconeogenesis is again due to the effect saturating at low concentrations of adrenaline and not due to increased sensitivity at low hormone concentrations. The apparently high concentration of adrenaline necessary to half maximally stimulate glycogenolysis is due to the assymetric shape of the dose response curve (Fig. 6.8) which fails to completely saturate the effect at high hormone concentrations. Although the reason for this shape of the curve is unknown, it may result from altered adrenaline binding to the α and/or β type receptors induced by proteases in batch 25 collagenase. This skewed curve is not observed when studying inhibition of lipogenesis. This may be due to the hormonal effect being mediated by a different receptor population than that for glycogenolysis. The 90% maximum inhibition of lipogenesis by either glucagon or adrenaline compares well with the reported 90% maximum reduction in intracellular citrate concentrations in the presence of these hormones (Watkins and Lane 1976). This work therefore generally supports the theory that hormonal inhibition of lipogenesis is due to a decrease in intracellular citrate.

Insulin (porcine or chicken) was found to have no effect on glycogenolysis, glycogen synthesis or lipogenesis, alone or in the presence of glucagon. A lack of effect of insulin on glycogenolysis in the chicken hepatocyte had been previously reported (Anderson and Langslow 1975). The stimulation of hepatic lipogenesis reported by Capuzzi et al. (1971) could not be repeated. It is therefore concluded that under the conditions used, insulin has no direct biological effect on carbohydrate and lipid metabolism in the chicken hepatocyte.

The complex effect of ionophore A23187 on glycogenolysis was due both to the carrier ethanol and the ionophore itself. Ethanol reduces glucose output from hepatocytes isolated from fed birds by 20 to 30% over a wide range of concentrations. Similar reductions in gluconeogenesis in the presence of ethanol have been found previously (Dickson 1977). The ionophore however succeeds in stimulating glucose output in the presence of ethanol with a corresponding decrease in glycogen content. This glycogenolytic effect of the ionophore has been reported by others using rat hepatocytes (Westwood and Siddle 1979).

Theophylline and caffeine are both potent inhibitors of cAMP phosphodiesterase and have been widely used to potentiate the actions of glucagon on cAMP accumulation. Theophylline maintained hormone stimulated levels of cAMP in the chicken hepatocytes (see Chapter 4) and also potentiated glucagons effect in rat hepatocytes (Pilkis et al. 1975; Sonne et al. 1977; Westwood and Siddle 1979). However these methylxanthines were found to inhibit basal or glucagon stimulated effects on cell metabolism. Miller et al. (1974) have reported an inhibition of glycogen phosphorylase activity by theophylline which reduces basal or glucagon stimulated glucose release from rat liver slices. This would explain the decrease in glycogenolysis found in hepatocytes isolated from fed birds but would not however account for the decrease observed

in gluconeogenesis or lipogenesis. No gross morphological damage to the hepatocytes was detected and this was supported by the ease in reversing the effects of the methylxanthines by simply washing the cells. It is concluded that the methylxanthines, besides inhibiting phosphodiesterase, also markedly decrease the metabolic flux through the major carbohydrate and lipid pathways of the chicken liver. The method by which this is carried out is uncertain but may be due to a general decrease in cellular respiration since both ATP content and oxygen consumption of the cells is lowered (see Chapter 3). The site of action of the methylxanthines is unknown.

Summary

Both glucagon and adrenaline were capable of producing identical stimulations of glycogenolysis and gluconeogenesis while inhibiting lipogenesis in chicken hepatocytes. Concentrations of the hormones which induced these effects were similar to the physiological levels in the blood. Although insulin failed to show any direct effects on lipid or carbohydrate metabolism on the hepatocytes, APP tended to maintain the glycogen content of the cells during incubation and high concentrations slightly potentiated glucagon action on glucose release in hepatocytes isolated from fed birds. It is not known if this is of any physiological importance or if the effects reported are secondary to some non-physiological effect of the hormone. Ionophore A23187 is also capable of stimulating glycogenolysis in the hepatocytes although the effects were antagonised by the carrier ethanol. This suggests that increasing intracellular Ca^{2+} levels can also stimulate glycogen breakdown probably via direct activation of phosphorylase kinase (see Chapter 5). The methylxanthines although potentiating the actions of glucagon on cAMP production inhibit basal or hormone stimulated glucose release from glycogenolysis or gluconeogenesis and also inhibit lipogenesis in the

chicken hepatocyte. It is suggested that as well as inhibiting phosphodiesterase the methylxanthines also have an unknown non-specific inhibitory effect on cell metabolism leading to lowered cell respiration, ATP content and general metabolic flux.

CHAPTER 7

CONCLUDING DISCUSSION

Hepatocytes isolated from the liver of the domestic fowl have been used to study the direct effects of the pancreatic hormones. With incubation at 37°C, cells isolated using the recirculating perfusion method retained their cellular viability in cell suspensions for at least one (hepatocytes isolated from starved birds) to three hours (hepatocytes isolated from fed birds), provided the medium was adequately oxygenated. Preparation of cell suspensions at 37°C increased the likelihood of anoxia during isolation procedures, resulting in lower intracellular ATP and glycogen contents. However, the hepatocytes quickly recovered and displayed biological and physical characteristics similar to, if not better than, the original isolation method (see Chapter 3; Dickson 1977; Dickson et al. 1978). Although cell sensitivity to the pancreatic hormones and adrenaline was highly dependent on the extent of an endogenous protease contaminant of the collagenase, physiological concentrations of the hormones were shown to be effective in receptor binding and/or inducing a metabolic response.

Therefore the isolated chicken hepatocyte proved to be the ideal model for the study of the short term hormonal control of carbohydrate and lipid metabolism in the chicken liver and allowed a direct comparison to be made with the rat hepatocyte and also with the previous reports of studies in vivo carried out with the chicken. It must however be accepted that in preparation of the isolated hepatocytes it is possible to induce major changes in cell structure which ultimately may lead to an altered metabolic balance within the cell. Results which do not parallel well established responses in vivo must be carefully analysed and interpreted with respect to the conditions used for the study.

Birds show a relative imbalance in the sensitivities shown to intravenous injections of glucagon and insulin compared to the rat and most other mammals. In mammals, insulin is the most potent hormone,

physiologically regulating carbohydrate and lipid homeostasis. However in the bird, although insulin circulates in the blood at similar concentrations to those found in the mammal, glucagon is the most important pancreatic hormone. This imbalance is reflected in the apparent hyposensitivity in vivo to insulin and hypersensitivity to glucagon, which may be partially or entirely responsible for the higher blood glucose levels found in the bird even after prolonged starvation.

The hypersensitivity to glucagon found in vivo in the bird was also reflected in the response found with the isolated hepatocyte. Glucagon which circulates in the peripheral avian blood at concentrations up to 4×10^{-10} M in the starved state, binds specifically to a receptor molecule present in the isolated hepatocyte which has a dissociation constant for the hormone of 9.44×10^{-9} M. Even assuming there is a ten fold increase in glucagon concentration in the portal blood only a small population of the total receptor number would be occupied under the highest possible physiological concentrations of the hormone.

Glucagon also stimulated adenylate cyclase with a resultant increase in intracellular cAMP. Induction of this second messenger showed half maximal stimulation at a glucagon concentration of 13×10^{-9} M which compares favourably with the value found for the dissociation constant reported for hormone binding. A parallel relationship appears to exist between hormone binding and induction of cAMP accumulation suggesting that the high affinity glucagon receptor:adenylate cyclase ratio is approximately one to one. The degree of cAMP accumulation and the steady state occupancy of glucagon binding sites has also been shown to be near identical in rat hepatocytes (Soman and Felig 1978; Sonne et al. 1978). Others have not found a parallel relationship between cyclase activation and steady state binding (Birnbaumer and Pohl 1973; Rosselin et al. 1974). These latter workers however did not analyse the binding data

in terms of two components, therefore obtaining high values for K_d (see Chapter 4). Sonne et al. (1978), although finding that the dissociation constant for receptor binding to a high affinity component at the steady state (7.0×10^{-10} M) was almost identical to the ED_{50} value for cyclase activity after 3 minutes, the occupancy of glucagon binding within 3 minutes did not coincide with cyclase activation. Glucagon concentrations of 3×10^{-8} M was required to half saturate binding after 3 minutes whereas ED_{50} for cyclase activation was about 1×10^{-9} M.

If one relates my results to physiological levels of circulating glucagon, it appears that relatively small increases in the intracellular cAMP must be responsible for initiation of the metabolic effects of glucagon. Under optimum experimental conditions, hormone concentrations of 3×10^{-10} M (near physiological levels) induced an increase of approximately 30% in basal levels ^{to} (around $2 \text{ pmols cAMP}/10^6$ cells). Presumably these small increases in the cyclic nucleotide level are responsible for initiating the metabolic response. However, it was found that during a small number of cell isolations (less than 10%) the intracellular concentration of the nucleotide was elevated by as much as five fold during preparation, and this did not always decay back to basal levels on incubation. In fact, nearly all cell preparations showed a slight increase in cAMP immediately after isolation which decayed back to basal levels in ten to fifteen minutes. Assuming that this effect is not due to experimental artefacts, it must be accepted that varying degrees of nonspecific stimulation of the intracellular cyclic nucleotide pool failed to induce similar stimulations of metabolic responses to those observed with glucagon. If the cyclic nucleotide is evenly distributed throughout a spherical hepatocyte of $12 \mu\text{m}$ in diameter then basal intracellular cAMP concentrations immediately after isolation range from 5 to 30×10^{-6} M. Minimum glucagon stimulation of glycogenolysis

occurs at concentrations which elevate intracellular cAMP by a further 5 to 10×10^{-6} M over basal, with maximal stimulation at concentrations which elevate intracellular cAMP by only a further 50 to 100×10^{-6} M. Theoretically, it would seem that cells isolated with a cAMP content of 30×10^{-6} M should show a higher rate of glycogenolysis than cells with levels of 5×10^{-6} M. However this is not the case. Addition of extracellular Bt_2 cAMP at around 50×10^{-6} M saturates the glycogenolytic effect as does cAMP at 150×10^{-6} M. These figures compare well with the calculated intracellular levels of the nucleotide found after a hormone stimulation which just saturates the metabolic response. Therefore although the dose response range of the cyclic nucleotide is similar whether cells are incubated with glucagon or by direct addition of cAMP or Bt_2 cAMP, the apparent basal nucleotide levels show substantial variation without altering metabolic effect. Various other reports of basal nucleotide levels found in rat hepatocytes also show similar variations. The reason for this is unknown; however the explanation may lie in cAMP compartmentation within the cells which prevents activation of the protein kinase. As cAMP is generally considered to be uniformly distributed throughout the cell cytosol, compartmentation could be achieved by the presence of intracellular cAMP binding proteins other than the specific protein kinase. A closer investigation is required before any satisfactory conclusion can be reached.

The effects of glucagon on carbohydrate and lipid metabolism appear to be closely correlated with the physiological levels of the circulating hormone. Using cell preparations of high hormone sensitivity isolated by a collagenase batch containing little damaging proteolytic activity, half maximal effects of glucagon on glycogenolysis were found at 1.7×10^{-10} M with the smallest noticeable effect occurring at 4.4×10^{-11} M. These values are in the same concentration range as reported for glucagon

in the peripheral blood of starved chickens. Since portal blood glucagon concentrations will be elevated over values in peripheral blood the results suggest that glycogenolysis will be nearly maximally activated in the chicken liver during starvation. Direct comparisons with the figures already mentioned for binding and second messenger stimulation however must be made with lower affinity values due to the low sensitivity of the cells found with other batches of collagenase. Half maximum effective values for glucagon were found at 9.1×10^{-10} M for glycogenolysis; 4.8×10^{-10} M for gluconeogenesis and 1.05×10^{-9} M for lipogenesis. Both inhibition of lipogenesis and stimulation of glycogenolysis appear to have parallel dose response curves where effects on metabolism appear to saturate with approximately 10% of the total receptor sites occupied (1300 sites per cell) and 10% of the total possible cAMP produced (3 to 4 pmol/ 10^6 cells). The dose response to gluconeogenesis is over a much narrower range with the effect saturating at around half the values reported above for glycogenolysis.

Table 7.1 summarises the results for binding, ^{adenylate} cyclase activation and glycogenolytic effect to allow direct comparisons to be made. Clearly there are a large number of 'spare' glucagon receptors present on the chicken hepatocyte all of which appear to be associated with adenylyate cyclase. Only a small fraction of these receptor sites needs to be occupied for maximal effect of the hormone on the carbohydrate and lipid pathways mentioned. This supports the concept of the spare receptor theory found with mammalian tissues and may well increase the sensitivity of the chicken hepatocyte to small changes in circulating hormone concentration. It is also suggested that the calculated values reported for binding and adenylyate cyclase activation (Table 7.1) will almost certainly be of lower affinity than found in whole tissue due to the proteolytic degradation on cell isolation. One particular batch of

Table 7.1: Summary of results obtained for binding, cyclase activation and metabolic effect.

Binding results obtained using batch 17 or 25 collagenase. Adenylate cyclase activation results obtained using batch 25 collagenase.

Effect on carbohydrate and lipid metabolism using batch 17, 25, 29 or 30 collagenase. Average values given for all parameters.

Parameter	Glucagon	Adrenaline	Insulin	APP
1) Binding affinity (M)				
a) high affinity site	9.4×10^{-9}	-	3.0×10^{-10}	-
b) low affinity site	4.0×10^{-5}	-	1.95×10^{-7}	2.0×10^{-6}
2) Binding capacity (sites per cell)				
a) high affinity site	12,492	-	2178	-
b) low affinity site	1,500,000	-	713,000	300,000
3) cAMP production (M) half maximum activation	12.9×10^{-9}	1.7×10^{-6}	-	-
4) Metabolic effects ED ₅₀ value (M)				
a) glycogenolysis	1.7×10^{-10} to 2.8×10^{-9}	9.5×10^{-8} to 1.36×10^{-7}	-	-
b) gluconeogenesis	4.8×10^{-10}	2.08×10^{-8}	-	-
c) lipogenesis	$1.00-1.09 \times 10^{-9}$	6.9×10^{-8}	-	-

collagenase produced isolated hepatocytes with a five fold higher glycogenolytic sensitivity to the hormone and it is likely that similar increases in cell sensitivity to binding and cyclase activation would be found.

A high affinity receptor for insulin was found on the isolated hepatocyte with a dissociation constant of approximately 3×10^{-10} M. Physiological levels of insulin circulate in the peripheral blood within the range 8×10^{-11} M to 2×10^{-9} M and higher levels of the hormone are obviously found in the portal blood. Since the true affinity of the liver in vivo may be much higher, due to the possibility of partial degradation of the receptor on cell isolation and the relatively higher affinity of chicken insulin compared to the porcine insulin used in this study, it would appear that even at the lowest physiological levels of the circulatory hormone a large proportion of the insulin receptors would be occupied. Although this high affinity receptor is present on the chicken hepatocyte the binding of hormone failed to induce any measurable biological effect at physiological concentrations of the hormone. Pharmacological levels of the hormone (10^{-8} M) reduced the cAMP degradation rate in the external medium, but since this was only effective at concentrations well above those required to saturate all high affinity binding sites it is considered not to be of any physiological importance nor is it expected to illuminate the mode of hormone action in chicken liver.

Therefore in the chicken hepatocyte the receptor system for insulin remains intact although the effector system appears to be lacking. However an insulin requirement in the chicken has been shown in vivo. Insulin is capable of slightly lowering circulating glucose levels in the blood. Therefore, either the effect of insulin on the hepatocyte is lost on cell isolation or, more likely, insulin's regulatory effect

on circulating glucose levels in the bird is due to action on some other tissue, possibly muscle. Therefore it is suggested that the lack of sensitivity found in vivo to intravenous insulin is due to a lack of hormone effector system in the liver cell and that the low hypoglycaemic effect found, results from hormone stimulating glucose uptake in tissues other than the liver.

The third pancreatic hormone to be investigated, APP, induced only minor changes in carbohydrate metabolism. However one can only speculate if the effects noted are of any physiological significance and indeed if they are a direct result of hormone action. APP does not regulate basal or hormone stimulated cAMP levels which suggests that if the observed metabolic effects are a direct result of classical hormone action, some other intracellular mediator must be involved. The main factor against the direct action of APP on the liver cells was the failure to find a high affinity specific receptor on the hepatocyte plasma membrane. Even though APP circulates in peripheral blood at relatively high concentrations (1 to 3 x 10⁻⁹ M) compared to the other pancreatic hormones (and its levels are probably much higher in portal blood) it is unlikely that the low affinity receptors found in hepatocyte suspensions (K_d about 2 x 10⁻⁶ M) would be of any physiological importance. Although it is very unlikely it cannot be completely discounted that a small finite number of higher affinity sites are present on the liver cell which were not detected under the conditions used. Until further studies are carried out the only hypothesis that can be drawn is that APP does not bind to hepatocyte plasma membranes and the minor effects noted in carbohydrate flux are probably due to some nonspecific action of APP or a contaminating protein. This implies that the effects of APP found in vivo must result from actions on other tissues.

Although no binding studies were carried out using adrenaline it is obvious that high affinity receptors must exist for the catecholamine. Discussion of actions between α and β receptors as reported with various mammalian tissues cannot be made in this study. However since adrenaline elevated intracellular cAMP levels above that needed to induce maximum metabolic effect and was also capable of stimulating glycogenolysis to the same extent as glucagon, Bt_2 cAMP or cAMP it is suggested that the catecholamine may act predominantly through a β type receptor mechanism in the chicken hepatocyte in contrast to the predominant α type effect found for carbohydrate metabolism in rat hepatocytes. However it will be necessary to investigate catecholamine action further using pure α or β agonists and antagonists before any conclusive results can be obtained. Since adrenaline fails to elevate maximally glucagon stimulated cAMP levels it appears that there is no excess of adenylate cyclase molecules over glucagon receptors in the chicken hepatocyte and that adrenaline and glucagon receptors both share the cyclase population when activated by their respective hormones.

In summary, the isolated chicken hepatocyte proved to be a valuable model for analysing pancreatic polypeptide hormone action in the chicken liver. Glucagon appears to be the most important of the pancreatic hormones with respect to regulation of intracellular carbohydrate and lipid metabolism. Insulin, despite circulating in chicken blood at concentrations similar to those found in mammals and having a specific high affinity receptor present on the liver cell membrane does not have a biological action in the chicken hepatocyte suggesting a lack of the effector system that must be present in the mammalian hepatocyte. This apparent lack of the effector of hormone action could result in the chicken hepatocyte being the ideal model to study the mode of insulin action in the liver. Incorporation of various components of the rat liver cell

into the chicken hepatocyte may help to elucidate the membrane bound and/or intracellular factor(s) which mediate the hormone response in the mammal.

APP appears to be devoid of any effect on the chicken hepatocyte indicating that the site of action of this hormone lies elsewhere.

Under times of stress adrenaline can induce metabolic actions identical to those found with glucagon on carbohydrate and lipid metabolism in the hepatocyte. The high activities found with the catabolic hormones (glucagon and adrenaline) and the lack of effect of insulin in the chicken hepatocyte are consistent with the in vivo findings concerning these hormones in birds. However it has yet to be determined how avian carbohydrate and lipid metabolism can be so carefully regulated without the antagonistic actions of glucagon and insulin on the liver which is of paramount importance in the mammal.

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