Studies on the Rat Hepatic Glucagon Receptor

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Abbreviations

The abbreviations used in this thesis are in agreement with the recommendations of the editors of the Biochemical Journal ((1992) *Biochem. J.* **281**, 1-19) with the following additions:

CA	carrier ampholyte
CHAPS	3-[(cholamidopropyl) dimethylammonio]-1-propansulphonate
Gal	galactose
GlcNAc	*N*-acetylglucosamine
G_i	inhibitory guanine nucleotide binding regulatory protein
G_s	stimulatory guanine nucleotide binding regulatory protein
HBSS	Hanks' balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HSAB	hydroxysuccinimidyl-*p*-azidobenzoate
IEF	isoelectric focusing
IPG	immobilised pH gradient
MPI	mixture of protease inhibitors
NAPSCl	2-nitro-4-azidophenylsulphenyl chloride
PMSF	phenylmethylsulphonyl fluoride
R_s	Stokes radius
TEMED	*N,N,N',N'*-tetra methylethylenediamine
TH-glucagon	[1-*N*-α-trinitrophenylhistidine, 12-homoarginine] glucagon
TNP-glucagon	N^{α}-Trinitrophenyl glucagon
TPA	12-*o*-tetradecanoylphorbol 13-acetate
WGA	wheat germ agglutinin

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Summary

Treatment of liver plasma membranes with CHAPS was shown to yield glucagon receptor in a soluble, active form. Characterisation of the CHAPS-solubilised receptor using sucrose density gradient centrifugation and gel filtration on Sepharose CL-6B showed that it exists predominantly in a highly aggregated form, and that this aggregation is specific and unchanged by alterations to the detergent concentration or ionic strength. The smallest molecular weight species for the receptor in CHAPS was determined to be ~190 kDa and this is proposed to be a trimer of the hormone binding subunit.

Isolation of the active, CHAPS-solubilised receptor was attempted using affinity chromatography with immobilised glucagon, prepared by coupling bromoacetylated BioGel P-150 to the met-25 residue of glucagon. Specific binding of the receptor to the affinity gel was demonstrated. However, various elution methods (with glucagon, urea or low pH buffers) did not yield active receptor. Attempts to elute the receptor with SDS followed by characterisation on SDS-PAGE were unsuccessful due to background non-specific binding to the affinity matrix of the majority of proteins present in the CHAPS extract.

Thermostability studies on the CHAPS-solubilised receptor, showing biphasic decay at 35°C, provided further evidence of possible receptor heterogeneity.

It has been proposed that the desensitisation of adenylate cyclase after exposure of hepatocytes to glucagon, or other ligands which stimulate inositol phospholipid metabolism, arises as a result of phosphorylation of the glucagon receptor by protein kinase C. This was investigated by carrying out isoelectric focusing of samples of partially purified (by wheat germ agglutinin-agarose chromatography) receptor to which ^{125}I-glucagon had been directly cross-linked by UV irradiation, using immobilised pH gradient gels and agarose IEF gels. In both cases the procedure was optimised to allow good resolution of the components of

solubilised liver membranes, but focusing of the labelled receptor was not possible, presumably due to aggregation and/or precipitation at the site of sample application. An alternative approach was to look for a difference in the thermostability of the receptor in membrane preparations from control and desensitised hepatocytes. No difference was observed, even in the presence of phosphatase inhibitors, indicating either that desensitisation is not brought about by receptor phosphorylation, or that phosphorylation does occur but does not result in a change in the heat stability of the receptor.

Chapter 1 Introduction

1.1 Structure of Glucagon

Glucagon, a 29 amino acid polypeptide hormone with a molecular weight of 3485, has a highly conserved primary structure amongst mammals. The amino acid sequence of porcine (Bromer *et al.*, 1957), bovine (Bromer *et al.*, 1971), human (Thomsen *et al.*, 1972), rat (Sundby and Markussen, 1971a) and rabbit (Sundby and Markussen, 1971b) glucagons is identical (Figure 1.1). However, guinea pig glucagon is an exception among mammalian glucagons in that its sequence differs by five amino acid residues. Furthermore, duck (Sundby *et al.*, 1972) and turkey (Markussen *et al.*, 1972) glucagons differ from porcine glucagon by two and one amino acids respectively; however, the crystal forms of both of these avian glucagons are identical to that of porcine glucagon despite the difference in primary structure. Piscine glucagons appear to differ more substantially with anglerfish glucagon showing only 69% homology with porcine glucagon (Trakatellio *et al.*, 1975).

X-ray crystallography has shown that, in the crystalline state, glucagon molecules are self-associated into α-helical trimers. The helical region extends from residues 10 to 25 and results in the formation of two hydrophobic regions which are buried in the self-association process (Sasaki *et al.*, 1975). In concentrated (>1 mg/ml) basic solutions, glucagon molecules probably exist as trimers similar to those found in the crystal state (Gratzer *et al.*, 1972). A model for the structure of glucagon in dilute aqueous solution, i.e. the physiological situation, suggests a specific but flexible structure of low free energy with maximised hydrophobic close-packing, incorporating β-turns at positions 2-5, 10-13 and 15-18, an α-helical region between residues 19-27 and a β-sheet between residues 5-10 (Korn and Ottensmeyer, 1983).

Glucagon is a member of a multigenic family of several structurally related peptides that includes secretin (Mutt *et al.*, 1970), gastric inhibitory peptide (Brown and Dryburgh, 1971), vasoactive intestinal peptide (Mutt and Said, 1974), glicentin

His-Ser-Gln-Gly-Thr-Phe-Thr-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-

Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr

Figure 1.1 The Complete Amino Acid Sequence of Porcine Glucagon

(Thim and Moody, 1981), peptide HI-27 (Tatemoto and Mutt, 1981) and a growth hormone releasing factor (Spiess *et al.*, 1982). However, despite the startling similarities in the sequences of these peptides, each one couples to a specific receptor protein that will trigger distinct physiological events.

1.2 Biosynthesis of Glucagon

Glucagon is produced and secreted by the A cells of the islets of Langerhans. As with other polypeptide hormones, the synthesis of glucagon involves a larger precursor, preproglucagon, which is then enzymatically cleaved to give the functional form. Cloning and sequencing of mammalian pancreatic glucagon cDNAs from hamster (Bell *et al.*, 1983), bovine (Lopez *et al.*, 1983) and rat (Heinrich *et al.*, 1984) showed that pancreatic preproglucagon is a 180-amino acid polypeptide containing the sequences of three glucagon-related peptides in tandem, separated by intervening peptides. One of these peptides, glicentin, is a 69-amino acid polypeptide which has been isolated from intestine and shown to contain the entire sequence of glucagon in residues 33-61 (Thim and Moody, 1981) while

residues 1-30 represent the glicentin-related pancreatic peptide (GRPP) isolated from porcine pancreas (Thim and Moody, 1982). The two remaining peptides, termed glucagon-like peptides (GLPs) I and II, contain 37 and 33 amino acid respectively. The gene encoding preproglucagon is also expressed in the L cells of the gastrointestinal tract and in the brainstem and hypothalamic neurons, although the products of its expression are highly tissue-specific and differ from the pancreatic products. It has been shown that transcription of the gene in these tissues gives rise to preproglucagon mRNA transcripts which are identical to those produced in the pancreas (Mojsov *et al.*, 1986; Drucker and Asa, 1988). Therefore, diversification of preproglucagon gene expression leading to the appearance of different and highly specific peptides in pancreas, gastrointestinal tract and brain is brought about by tissue-specific post-translational modification of a common preproglucagon precursor (Mojsov *et al.*, 1986; Ørskov *et al.*, 1987). Proteolytic processing takes place as the preproglucagon migrates through the secretory pathway from the endoplasmic reticulum via the Golgi apparatus to the secretory granules. Figure 1.2 shows a scheme for the processing of preproglucagon in pancreas and intestine. The predominant pancreatic products are glucagon, GRPP, GLP-I and a large form of GLP-II whereas in the intestine glicentin, oxyntomodulin, GLP-I, GLP-II and intervening peptide II-amide predominate.

Transcription of the pancreatic preproglucagon gene has been shown to be regulated by a number of factors. Specific cis-acting DNA sequences in the 5'-flanking region of the rat glucagon gene direct islet-cell specific glucagon gene transcription (Drucker *et al.*, 1987) and it has been shown that one of these sequences, the proximal promoter element (G1), interacts with regulatory DNA binding proteins only in A cells, thus restricting glucagon gene expression to these cells (Philippe *et al.*, 1988). Transcription of the glucagon gene is negatively regulated by insulin (Philippe, 1989), explaining in part the high glucagon levels that are observed in states of insulin deficiency such as untreated Type 1 (insulin-dependent) diabetes mellitus (Muller *et al.*, 1971). The regulatory action of insulin

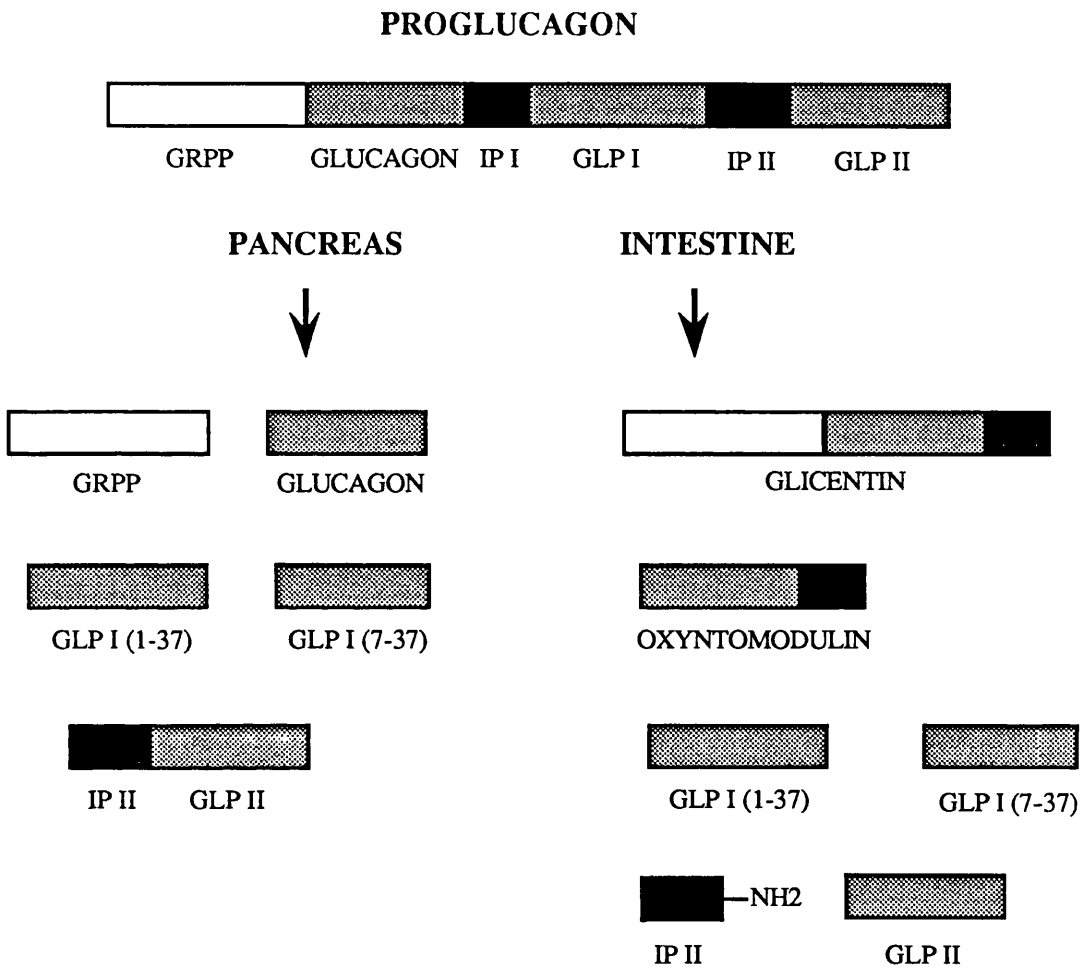


Figure 1.2 Scheme for the processing of proglucagon to specific peptides in the pancreas and intestine

is mediated by an insulin-responsive element (IRE) in the 5'-flanking region of the gene, probably *via* interactions with phosphorylated trans-acting factors whose phosphorylation state is modified by a cascade of phosphorylation-dephosphorylation reactions induced by insulin (Philippe, 1991).

Expression of the glucagon gene is increased by the action of both protein kinase C (Philippe *et al.*, 1987; Yamato *et al.*, 1990) and cAMP-dependent protein kinase A (Knepel *et al.*, 1990; Drucker *et al.*, 1991). This shows the involvement of both cAMP and inositol phospholipid pathways in the regulation of transcription of the gene. Indeed, the rat glucagon gene has been shown to contain a cAMP response element (CRE) in the 5'-flanking region which is proposed to interact with a protein kinase A-phosphorylated activating transcription factor (Drucker *et al.*, 1991).

1.3 Secretion of Glucagon

It is well established that glucose inhibits the release of glucagon (Ohneda *et al.*, 1969) while, conversely, low glucose concentration stimulates its release (Gerich *et al.*, 1973). A model has been proposed (Rorsman *et al.*, 1991) whereby an increase in glucose concentration leads to stimulation of insulin and GABA release from B cells. GABA binds to the $GABA_A$-receptor Cl^- channels of A cells and increases the Cl^- permeability of the A cells. This results in hyperpolarisation, inhibition of electrical activity, reduction of Ca^{2+} influx, decrease in intracellular $[Ca^{2+}]$ and suppression of glucagon secretion, as it has been shown that glucagon secretion increases with increasing intracellular $[Ca^{2+}]$ (Niki *et al.*, 1986). However, as described in section 1.2, insulin itself also has a negative effect on glucagon secretion by inhibiting transcription of the glucagon gene. Furthermore, somatostatin, another pancreatic hormone which is released in parallel with insulin, also inhibits glucagon release.

The effect of other stimulators or inhibitors of glucagon secretion is highly dependent on the concentration of glucose so that substances which would normally stimulate the release of glucagon fail to elicit such a response in the hyperglycemic state (Unger *et al.*, 1969). Secretion is stimulated by amino acids, with the exception of the branch chain amino acids (Rocha *et al.*, 1972). Arginine is a particularly potent stimulator and it has been proposed that its effect is mediated by protein kinase C-activated increase of glucagon gene expression (Yamato *et al.*, 1990). Conversely, high concentrations of free fatty acids inhibit glucagon secretion (Madison *et al.*, 1968), thus reducing the rate of lipolysis and ketogenesis and preventing the development of progressive ketoacidosis during prolonged starvation.

Catecholamines stimulate glucagon secretion through both α_2-adrenoceptors (Iguchi *et al.*, 1989) and β_2-adrenoceptors (Lacey *et al.*, 1991). This stimulation can be elicited not only by circulating catecholamines but by direct sympathetic neural innervation of the pancreas (Iguchi *et al.*, 1989). Cholinergic stimulation also increases the secretion of glucagon.

1.4 Physiological and Biochemical Effects of Glucagon

The major target site for glucagon action is the liver and it has been long recognised that glucagon is an important regulator of hepatic fuel metabolism. The hyperglycemic effect of the hormone is brought about by its activation of glycogenolysis and gluconeogenesis in the liver. Glycogenolysis is stimulated by glucagon-dependent activation of phosphorylase *a* and inactivation of glycogen synthetase (Unger *et al.*, 1978) while gluconeogenesis is stimulated by a glucagon-dependent increase in the expression of the phospho*enol*pyruvate carboxykinase gene (Christ *et al.*, 1988) and by increased hepatic uptake of gluconeogenic precursors such as alanine and glutamine (Brockman *et al.*, 1975). Additionally,

glucagon also activates glucose-6-phosphatase, enabling ready conversion of glucose-6-phosphate resulting from glycogenolysis and gluconeogenesis to free glucose which may then be exported from the liver.

Glucagon inhibits hepatic fatty acid synthesis by decreasing the activity of acetyl CoA carboxylase (McGarry *et al.*, 1975). The reduced levels of insulin which accompany increased levels of glucagon lead to an increase in delivery to the liver of free fatty acids which are directed towards ketogenesis by glucagon-stimulated activation of both carnitine palmitoyl transferase I (McGarry *et al.*, 1978) and 3-hydroxy-3-methylglutaryl coenzyme A synthase (Quant *et al.*, 1987).

Additional effects of glucagon on the liver include stimulation of urea synthesis (Petersen *et al.*, 1987), reduction of steroid metabolism (Hussin *et al.*, 1988) and stimulation of tyrosine aminotransferase during embryonic development (Onoagbe and Dickson, 1992).

In addition to the liver, glucagon has been shown to exert important physiological effects on other tissues. These include lipolysis in adipose tissue (Hagen, 1961), stimulation of renal bicarbonate excretion (Paillard and Bichara, 1989), inhibition of gastric acid secretion (Bataille *et al.*, 1988), stimulation of insulin secretion by pancreatic B cells (Freychet, 1990) and regulation of branch-chain amino acid metabolism in the heart (Hildebrandt *et al.*, 1988).

It has long been known that the physiological effects of glucagon are brought about by the elevation of intracellular cAMP concentration. However, more recently it has been shown that glucagon can activate cAMP-independent processes through the stimulation of phospholipid metabolism. This is discussed in detail in section 1.6.4.

1.5 Glucagon and Diabetes

Hyperglucagonemia is a common characteristic of both Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus. The presence of this state in untreated Type 1 diabetics is caused in part by a lack of an inhibitory effect of insulin on glucagon biosynthesis and secretion. However, the development of hyperglycemia in both Type 1 and Type 2 diabetes would be expected to inhibit glucagon secretion (section 1.3), but this is often not the case, suggesting insensitivity of A cells to glucose in the diabetic state (Freychet, 1990). It has been shown that hepatic response to glucagon is decreased in both Type 1 (Ørskov *et al.*, 1991) and Type 2 (Arner *et al.*, 1987) diabetes mellitus, resulting in decreased hepatic glucose production, and it is suggested that this is possibly a mechanism for reducing the effect of hyperglycemia.

1.6 Glucagon Receptor

1.6.1 Cellular and Subcellular Distribution of Glucagon Receptors

Specific glucagon-binding sites have been described in a number of mammalian and avian tissues, including liver (Rodbell *et al.* , 1971), adipose tissue (Desbuquois *et al.*, 1973), heart (Klein *et al.*, 1973), pancreas (Goldfine *et al.*, 1972), brain (Hoosein and Gurd, 1984) and retina (Sanchez *et al.*, 1988). Studies with separate liver cell populations have shown that only hepatocytes (parenchymal cells) contain glucagon binding sites (Wincek *et al.*, 1975).

In hepatocytes, specific glucagon binding sites are predominantly associated with plasma membranes although minor amounts have been identified in endoplasmic reticulum and Golgi membranes (Desbuquois, 1983).

1.6.2 Glucagon-Receptor Binding

The binding of glucagon to hepatic plasma membranes and isolated hepatocytes does not conform to a single binding equilibrium. As much as a 50,000-fold increase in the concentration of hormone is required to cause a reduction from 90% to 10% of maximal binding of ^{125}I-labelled glucagon whereas theory predicts that a 100-fold increase should be sufficient for a single binding equilibrium (Mason and Tager, 1985). This complex binding has been modelled in terms of a time-dependent, temperature-dependent, non-cooperative interconversion of two states of a single receptor population (Horwitz *et al.*, 1985). In this model, binding of glucagon to the receptor yields a low affinity complex which converts with time to a high affinity complex, the conversion step being independent of the degree of fractional receptor occupancy and occurring at 37°C but not at 0°C. The presence of low and high affinity states and their differing dissociation rates account for the kinetic complexity of binding. Horwitz *et al.* (1986) proposed that the conversion from low to high affinity complexes is required for stimulation of adenylate cyclase, either through direct activation by the high affinity complex coupling to the stimulatory guanine nucleotide binding protein or by energetic coupling of the conversion process with adenylate cyclase activation.

Alternatively, the observed complexity of glucagon-receptor interactions has been modelled in terms of two non-interacting populations of receptor, one with a high affinity and the other with a low affinity for glucagon (Bonnevie-Nielsen and Tager, 1983; Mason and Tager, 1985), and it has been proposed that occupancy of the high affinity sites is required to elicit a biological response (Hagopian *et al.*, 1987). This model was modified further by Bharucha and Tager (1990) to account for the demonstration of the conversion of reversibly bound glucagon to a nondissociable state in which glucagon remains irreversibly bound even after acid or protease treatment. Hence, four glucagon-receptor complexes were proposed to account for dissociable and non-dissociable interactions with both high and low

affinity binding sites. The ratio of high to low affinity sites was shown to be approximately 1:10.

1.6.2.1 Effect of Guanine Nucleotides on Binding

Guanine nucleotides decrease the affinity of glucagon for its receptor in liver membranes (Rodbell *et al.*, 1971). The effect of GTP on receptor binding affinity arises as a result of binding of GTP to the stimulatory G protein. High affinity binding of glucagon to its receptor is proposed to arise from binding to a receptor associated with a stimulatory G protein. Binding of GTP to the G protein results in its dissociation from the receptor and the subsequent conformational change in the receptor results in low affinity binding of glucagon.

Wyborski *et al.* (1988) have proposed that the effect is due to guanine nucleotides slowing the conversion of the receptor from a low affinity to high affinity state by stabilising the low affinity conformation. The rank order among naturally occurring guanine nucleotides for stabilising the low affinity state is GTP > GDP >> GMP = no nucleotides. Hence it was proposed that while GTP is required for coupling of the glucagon receptor to adenylate cyclase, it also regulates activation of adenylate cyclase by slowing the conversion of "inactive" low affinity glucagon-receptor complexes to "active" high affinity complexes.

Binding of GTP to a cytoplasmic site on the glucagon receptor has also been proposed (Iyengar *et al.*, 1979; McVittie and Gurd, 1989).

1.6.2.2 Effect of Ions on Binding

A number of salts inhibit the binding of glucagon to its receptors in liver membranes (Desbuquois, 1983). With monovalent ions, inhibition is demonstrable at 0.1 M and is virtually complete at 1 M. Divalent cations decrease glucagon binding at lower concentrations. However, in the presence of GTP, maximal

binding occurs in the presence of 2.5 mM Mg^{2+}, suggesting a rôle for this cation in the regulation of glucagon receptor affinity and the presence of a cation binding site on the receptor (Lipson *et al.*, 1988) or the stimulatory G protein (Birnbaumer *et al.*, 1990).

1.6.3 Glucagon Receptor Structure

In the absence of a successful procedure for the purification of the glucagon receptor, much of the known information on its size and structure has been obtained from the techniques of target size analysis and photoaffinity cross-linking. Early work using target size analysis by irradiation inactivation gave conflicting information about the size and association state of the glucagon receptor in the membrane environment. Houslay *et al.* (1977) proposed an independent, mobile receptor of 217 kDa, although this work did not include analysis of the possible contribution of regulatory guanine nucleotide binding proteins. Martin *et al.* (1979) proposed that in the absence of glucagon the receptor exists in a free form but that binding of glucagon to receptor results in the association of the receptor with G protein and adenylate cyclase in a transient complex which is dissociated by GTP. The existence of the receptor in the membrane in the form of large (670 kDa) multimeric complexes associated with G proteins was proposed by Schlegel *et al.* (1979).

Covalent cross-linking of labelled hormone to its receptor has been an important method in the identification and characterisation of peptide hormone receptors, e.g. the insulin receptor (Pilch and Czech, 1980) and indeed has been used to identify the glucagon receptor. Cross-linking has been demonstrated using heterobifunctional cross-linking reagents such as 2-nitro-4-azidophenylsulphenyl chloride (NAPSCl) (Demoliou and Epand, 1980; Demoliou-Mason and Epand, 1982) and hydroxysuccinimidyl-*p*-azidobenzoate (HSAB) (Johnson *et al.*, 1981;

Iyengar and Herberg, 1984; Herberg *et al.*, 1984). The use of NAPSCl involves the synthesis of a photoreactive radiolabelled glucagon (^{125}I-glucagon-NAPS) which is incubated with liver plasma membranes. UV irradiation is then used to form a covalent cross-link between the glucagon derivative and the receptor. However, using HSAB no glucagon derivative is synthesised; instead, radiolabelled glucagon is incubated with membranes, HSAB is added and UV irradiation is carried out. Direct cross-linking by UV irradiation, without the use of photoaffinity reagents, was later reported (Iwanij and Hur, 1985).

The method of photoaffinity cross-linking has resulted in conflicting information about the molecular structure of the glucagon receptor. Johnson *et al.* (1981), using HSAB for cross-linking, identified an ^{125}I-labelled glucagon binding rat liver plasma membrane protein of 53 kDa. Later, Demoliou-Mason and Epand (1982), using ^{125}I-glucagon-NAPS for cross-linking, reported a receptor complex of 200-250 kDa from Ultrogel chromatography of photolabelled Lubrol-solubilised membranes and, on the basis of results from gel electrophoresis of those fractions containing the complex, proposed that the glucagon receptor in Lubrol-PX is an oligomer composed of at least two subunits in the molecular weight range 52-70 kDa that are linked together or greatly stabilised by disulphide bonds.

Iyengar and Herberg (1984) reported the labelling of a 63 kDa peptide by ^{125}I-glucagon using HSAB as cross-linker. This labelling was inhibited by low concentrations of unlabelled glucagon but unaffected by other peptide hormones, indicating that it was specific for glucagon. Labelling was also shown to be sensitive to GTP, indicating that the 63 kDa peptide had the capacity to interact with the stimulatory guanine nucleotide binding protein. The localisation of the hormone-binding region within this 63 kDa peptide was studied by comparing the effect of limited elastase treatment of membranes that had ^{125}I-glucagon specifically but not covalently bound to treatment of membranes prior to exposure to the labelled hormone. Bound label was then cross-linked and the membranes analysed by SDS-PAGE. It was shown that elastase treatment of the unoccupied receptor yielded a 24

kDa peptide that bound ^{125}I-glucagon specifically. Labelling of this peptide was sensitive to GTP, indicating that the fragment retained the capacity to interact with the stimulatory guanine nucleotide binding protein. However, treatment of the hormone occupied receptor with elastase resulted in the identification of a 33 kDa peptide which, like the 24 kDa peptide, was shown to retain the capacity to interact with the stimulatory guanine nucleotide binding protein. The 33 kDa protein was also given by the action of other proteases such as subtilisin, protease V8 and trypsin, and is in fact the most commonly observed naturally occurring fragment of the receptor when the hormone-binding reaction is carried out in the absence of protease inhibitors. Elastase treatment of the 33 kDa peptide resulted in the formation the 24 kDa peptide and a 15 kDa peptide, indicating that the 24 kDa peptide is contained within the 33 kDa peptide.

Treatment of the labelled 63 kDa peptide with endo-β-N-acetyl-glucosaminidase F (Endo F) resulted in the appearance of four discrete bands on SDS-PAGE gels, suggesting the presence of at least four N-linked glycans and a peptide 'backbone' of 45 kDa. However, treatment of the 24 kDa and 33 kDa peptides with Endo F indicated that neither of these fragments contain N-linked glycans.

Iyengar and Herberg concluded that the glucagon receptor is a glycoprotein of ~60 kDa which contains at least four N-linked glycans accounting for 18 kDa, with the hormone-binding function and capacity to interact with the stimulatory regulator contained within a fragment of ~21 kDa. They proposed that hormone occupancy of the receptor results in the exposure of a proteolytically sensitive region which can be acted upon by proteases to form a peptide of ~30 kDa which does not contain any N-linked glycans but still contains the hormone-binding and stimulatory regulator-interacting regions. Since deglycosylated membrane proteins turn over more rapidly than their glycosylated counterparts (Olden *et al.*, 1982), it was proposed that the formation of the 33 kDa peptide plays a rôle in the down-regulation of the receptor.

Herberg *et al.* (1984) later reported the identification of a 119 kDa species after solubilisation of cross-linked membranes with Lubrol-PX and proposed that the hepatic glucagon receptor exists in the membrane and in non-denaturing detergent solution as a dimer of the 60 kDa hormone-binding subunit.

Direct cross-linking by UV irradiation, without the use of any cross-linking reagents, was used by Iwanij and Hur (1985) to identify a glucagon-receptor complex of 62 kDa and they proposed the presence of intramolecular disulphide bonds since reduction of the complex with dithiothreitol resulted in a decrease in the electrophoretic mobility. The presence of four N-linked glycans on the receptor was again demonstrated.

Proteolytic mapping studies using monoclonal antibodies prepared against partially purified directly cross-linked rat hepatic glucagon receptor (Iwanij and Vincent, 1990) have shown that the hormone binding site of the receptor is located on a 25 kDa fragment containing the intact COOH-terminal domain and the structure of the receptor has been proposed to be similar to the structure of the rhodopsin molecule, as has been shown for other receptors which transduce signals via guanine nucleotide binding proteins, including muscarinic acetylcholine receptors (Peralta *et al.*, 1987) and adrenergic receptors (O' Dowd *et al.*, 1987). This structure consists of seven transmembrane domains with three extracellular and three cytoplasmic loops. The extracellular amino terminal domain usually contains one or more sites for N-linked glycosylation, while the cytoplasmic domains often contain potential sites for phosphorylation by one or more cytosolic kinases. Not all receptors which interact with G proteins have this structure, e.g the receptor for insulin-like growth factor II has a single membrane-spanning domain (Okamoto *et al.*, 1990). However, the predicted amino acid sequence obtained from the recent isolation of a cDNA clone for the glucagon receptor (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993) has suggested that the seven transmembrane domain model is indeed applicable to the glucagon receptor (see section 5.3).

Species specific structural differences among glucagon receptors have been suggested by the failure of antibodies raised against the rat receptor to recognise the chicken or canine forms of the receptor (Iwanij and Vincent, 1990).

1.6.4 Glucagon Receptor and Signal Transduction

It is well established that treatment of hepatocytes with glucagon causes an elevation of the intracellular concentration of cyclic AMP. This process involves the binding of glucagon to its receptor followed by a conformational change which results in coupling of the receptor to the stimulatory G protein, G_s. This causes the α subunit of G_s to exchange a bound GDP for GTP and to dissociate from the $\beta\gamma$ subunits. The GTP-bound form of the α subunit then activates the catalytic subunit of adenylate cyclase, resulting in the production of cAMP. The increase in intracellular cAMP is rapid but transient due to its degradation by specific phosphodiesterases, at least one of which is activated by protein kinase A-activated phosphorylation (Kilgour *et al.*, 1989), and the desensitisation of glucagon-stimulated adenylate cyclase (see section 1.7). Glucagon has also been shown to exert cAMP-independent actions on hepatocytes. Early evidence showed that low concentrations of glucagon could exert metabolic effects while giving no discernible increase in either cAMP concentration or protein kinase A activity (Birnbaum and Fain, 1977; Okajima and Ui, 1976). Later work showed that the glucagon analogues TH-glucagon and TNP-glucagon were unable to activate adenylate cyclase or increase the intracellular concentration of cAMP yet brought about the full stimulation of glycogenolysis, gluconeogenesis and urea synthesis in hepatocytes (Corvera *et al.*, 1984; Cote and Epand, 1979). These results suggest the involvement of glucagon in the activation of other signal transduction pathway(s) in addition to stimulation of adenylate cyclase. Both glucagon and TH-glucagon have been shown to stimulate inositol phospholipid metabolism (Wakelam

et al., 1986; Blackmore and Exton, 1986; Whipps *et al.*, 1987) and indeed glucagon can increase the intracellular concentrations of both diacylglycerol (Bocckino *et al.*, 1985) and free Ca^{2+} (Blackmore and Exton, 1986; Mine *et al.*, 1988), both of which could result from stimulation of inositol phospholipid metabolism, although the increase in free Ca^{2+} concentration is proposed to be a result of both cAMP-dependent and cAMP-independent processes (Mine *et al.*, 1988). It is also possible that diacylglycerol production can come from the breakdown of phosphatidylcholine (Muir and Murray, 1987). The involvement of glucagon in two different signal transduction pathways has led to the proposal of there being two different glucagon receptors, one coupled to adenylate cyclase and the other coupled to (inositol) phospholipid metabolism (see section 1.6.6.2).

1.6.5 Receptor Solubilisation and Activity

The choice of detergent for the solubilisation of native membrane proteins is often critical. Conformational denaturation of a protein upon solubilisation may arise as a result of changes in the environmental fluidity upon incorporation of the protein into micelles (Milder *et al.*, 1991), binding of detergent to the protein at sites not normally occupied by lipid (Simmonds *et al.*, 1982) or displacement from the protein surface of lipids needed to stabilise the protein structure (Robinson *et al.*, 1980). In addition, loss of activity can result from detergent-induced dissociation of cofactors or associated proteins, as well as specific interference by the detergent in the binding of substrates or ligands to the protein (Hjelmeland, 1990).

Both ionic and non-ionic detergents have been successfully used in the solubilisation and subsequent purification of membrane proteins. However, there appears to be no wholly rational basis for the selection of a particular detergent or class of detergents for a given application. In some cases homologous membrane proteins can be successfully solubilised using similar conditions, e.g. solubilisation

of the β_2-adrenergic receptor and its homologues with digitonin (Schorr *et al.*, 1981; Niznik *et al.*, 1986; Regan *et al.*, 1986) whereas in other cases preparations of the same protein from different sources can show different patterns of stability in the same detergent, e.g. $(Na^+ + K^+)$ - ATPases in $C_{12}E_8$ (Esmann, 1986).

The glucagon receptor behaves as a typical integral membrane protein in that treatment of labelled liver plasma membranes with lithium diiodosalicylate does not result in extraction of the cross-linked receptor (Iwanij and Hur, 1985) whereas it is solubilised by treatment with detergents such as Lubrol-PX (Demoliou-Mason and Epand, 1982; Herberg *et al.*, 1984; Iwanij and Vincent, 1990) and digitonin (Iwanij and Hur, 1985). However, detergent treatment of unlabelled membranes predominantly yields solubilised glucagon receptors that do not retain hormone-binding activity. Herberg *et al.* (1984) showed that while some activity is retained after extraction with deoxycholate, cholate and octylglucoside, the most effective detergent for solubilisation of active glucagon receptor is CHAPS. However, CHAPS-solubilised receptor is unstable, showing an almost total loss of activity after just 8 hours at 4°C. The presence of 137 mM NaCl in the extraction solution increases the stability of the receptor so that 50% of binding activity remains after 24 hours, but traditional stabilising agents such as glycerol, sucrose, dithiothreitol and supplemental phospholipid are ineffective in stabilising the soluble receptor (McVittie and Gurd, 1989).

The CHAPS-solubilised glucagon receptor was shown by competition binding assays to bind hormone with low affinity in a guanine nucleotide-insensitive manner (Herberg *et al.*, 1984). McVittie and Gurd (1989) used dissociation assays to show that the solubilised receptor is capable of interconversion from the low affinity to the high affinity state in a guanine nucleotide-sensitive manner as observed for the membrane-bound receptor, GTP favouring the low affinity form and requiring the presence of Mg^{2+} for its effect to be seen.

1.6.6 Heterogeneity of the Glucagon Receptor

Evidence of glucagon receptors with different affinity to the hormone and different glycosylation, and the observation that glucagon activates two separate signalling pathways, has led to proposals that there is a heterogeneous population of glucagon receptors in liver.

1.6.6.1 Differential Receptor Affinity and Glycosylation

As described in section 1.6.2, the complex binding of glucagon to its receptor has been modelled both in terms of a single population of receptors which interconvert between low and high affinity forms, and two non-interacting receptor populations of high and low affinity. A correlation between affinity and glycosylation has been made by Mason and Tager (1985). They used gel filtration and affinity chromatography of digitonin-solubilised non-cross-linked glucagon-receptor complexes from canine hepatic plasma membranes to identify two forms of the receptor, one of which was found to bind to wheat germ agglutinin and was specifically eluted by N-acetylglucosamine and the other of which was found not to bind. Analysis of material from membranes incubated with ^{125}I-glucagon and with different concentrations of unlabelled glucagon showed that low concentrations of the competitor caused radioactivity in the fraction specifically bound to wheat germ agglutinin to decrease markedly whereas higher concentrations of competitor were required to decrease radioactivity in the unbound fraction. Indeed, the values for concentrations of unlabelled glucagon required to give half-maximal inhibition of ^{125}I-glucagon association with receptor agreed well with the dissociation constants derived mathematically from the overall interaction of glucagon with soluble receptors. Thus it was shown that high affinity receptors bind to wheat germ agglutinin whereas low affinity receptors do not bind to the agglutinin and hence it was proposed that separate populations of glucagon receptors with different

affinities for hormone arise as a result of differential glycosylation. However, wheat germ agglutinin chromatography of Lubrol-solubilised cross-linked glucagon-receptor complexes has yielded conflicting results. Herberg *et al.* (1984) found no labelled receptor remaining in solution after exposure to wheat germ agglutinin-Sepharose, whereas Iwanij and Vincent (1990) found that the flowthrough contained labelled material of a slightly lower molecular weight than that of the adsorbed receptor, and proposed that this represented a desialiated form of the receptor.

1.6.6.2 Signal Transduction Pathways and Receptor Heterogeneity

As described previously (section 1.6.4) glucagon can exert effects through the stimulation of both adenylate cyclase and inositol phospholipid metabolism. Therefore it has been proposed that glucagon exerts its effect on liver via two different receptors rather than via a single receptor coupled to two signalling pathways (Wakelam *et al.*, 1986). This hypothesis was supported by the observation that the K_a value for the action of glucagon in stimulating the inositol phospholipid response is lower than that for its ability to stimulate adenylate cyclase. Hence it was proposed that rat hepatocytes have two functionally distinct receptors with different affinities: GR1 receptors coupled to the stimulation of inositol phospholipid metabolism, and GR2 receptors coupled to the stimulation of adenylate cyclase.

Other receptor systems show that coupling to adenylate cyclase and phospholipid metabolism can occur through single or multiple receptor populations. For example, the effects of vasopressin are mediated through three different receptors (Birnbaumer *et al.*, 1990). V-1a receptors are coupled to stimulation of phospholipases C, D and A_2 and the inhibition of adenylate cyclase. V-1b receptors are coupled to the stimulation of phospholipase C, while V-2 receptors stimulate

adenylate cyclase. These effects are suggested to be mediated by the coupling of different receptors to different G proteins. Conversely, calcitonin stimulates both inositol phospholipid metabolism and adenylate cyclase via a single receptor (Chabre *et al.*, 1992). In this case, stimulation of two different signalling pathways is proposed to be brought about by the coupling of two different G proteins to a single receptor population.

1.7 Desensitisation of Glucagon-Stimulated Adenylate Cyclase

Glucagon treatment of intact hepatocytes causes a rapid time- and dose-dependent desensitisation of glucagon-stimulated adenylate cyclase activity (Murphy *et al.*, 1987). The total number of glucagon binding sites is unaltered over the period of desensitisation, indicating that internalisation of receptor does not occur during this period of time. TH-glucagon which, as mentioned previously, does not cause an increase in cAMP, also brings about desensitisation, demonstrating that the desensitisation of glucagon-stimulated adenylate cyclase is a cAMP-independent process. Since both glucagon and TH-glucagon have been shown to stimulate inositol phospholipid metabolism (Wakelam *et al.*, 1986), it has been proposed that glucagon brings about the desensitisation of the ability of GR-2 receptors to stimulate adenylate cyclase by stimulating inositol phospholipid metabolism through GR-1 receptors. This is supported by the observation that other ligands known to stimulate inositol phospholipid metabolism in hepatocytes such as angiotensin and vasopressin also elicit desensitisation of glucagon-stimulated adenylate cyclase. Since the phorbol ester TPA, a known stimulator of protein kinase C, mimicks this desensitisation, it is proposed that the stimulation of protein kinase C plays an important rôle in the molecular mechanism of desensitisation of glucagon-stimulated adenylate cyclase. Indeed, diacylglycerol, a product of inositol

phospholipid metabolism, is a stimulator of protein kinase C and treatment of hepatocytes with two synthetic diacylglycerols has been shown to mimic glucagon-mediated desensitisation of adenylate cyclase (Newlands and Houslay, 1991). The molecular mechanism of desensitisation is not due to the activation of the inhibitory guanine nucleotide regulatory protein G_i as this protein has been shown to be inactivated upon treatment of hepatocytes with glucagon (Murphy *et al.*, 1989). Hence it is proposed that desensitisation occurs as a result of phosphorylation of either the glucagon receptor or the stimulatory G protein followed by the uncoupling of the ability of these two proteins to interact (Murphy *et al.*, 1987). As treatment of isolated membranes with pure protein kinase C does not result in the phosphorylation of the stimulatory G protein (Bushfield *et al.*, 1990), phosphorylation of the glucagon receptor seems more likely.

Receptor phosphorylation and subsequent impairment of receptor-G_s coupling has been shown to play an important rôle in the desensitisation of adenylate cyclase in β_2-adrenergic receptor systems. This receptor is a target for three different kinases: protein kinase A (Benovic *et al.*, 1985; Bouvier *et al.*, 1987), β_2-adrenergic receptor kinase (Benovic *et al.*, 1987) and protein kinase C (Johnson *et al.*, 1990). However, a difference between the ability of the three kinases to phosphorylate the β_2-adrenergic receptor is that whilst the presence of agonist is essential for the action of β_2-adrenergic receptor kinase and enhances the rate of phosphorylation of the receptor by protein kinase A, there appears to be no requirement for agonist to be present for the action of protein kinase C. Phosphorylation by both protein kinase A and β_2-adrenergic receptor kinase has been shown to lead directly or indirectly to uncoupling of the receptor from G_s (Kobilka, 1992) and it is proposed that phosphorylation by protein kinase C brings about a similar action. Indeed, the treatment of cells with phorbol esters to activate protein kinase C has been shown to lead to the uncoupling of G_s from the receptor (Garte and Belman, 1980).

1.8 Aims Of Project

The elucidation of the mechanism of action of glucagon stimulation of adenylate cyclase and inositol phospholipid metabolism at the molecular level requires a detailed understanding of the structure and function of the glucagon receptor. Some information on the structure of the receptor has been obtained by the use of affinity cross-linking techniques and some progress has been made in the solubilisation and stabilisation of free, active receptor, an important initial step in its isolation. However, to date the receptor has not been purified to homogeneity. Therefore, full characterisation of its structure, including the possibility of there being two or more structurally distinct forms, and its rôle in the transduction of binding signal to intracellular action has not been achieved. The aims of this project were the isolation and further characterisation of the rat hepatic glucagon receptor, especially with regard to receptor heterogeneity and the possibility of receptor phosphorylation in the desensitisation of glucagon-stimulated adenylate cyclase. Two lines of investigation were undertaken:

i) studies on the CHAPS-solubilised receptor, including stabilisation, characterisation and attempted isolation using affinity chromatography on immobilised glucagon

ii) use of affinity cross-linking and thermostability studies to investigate the possibility of receptor phosphorylation.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Fine Chemicals

N-acetylglucosamine (GlcNAc), bovine serum albumin, Coomassie blue G and R, dextran, glucagon, hydrogen peroxide, leupeptin, Lubrol-PX, NADH, Nonidet P40, ovalbumin, oxaloacetic acid, pepstatin A, phenylmethylsulphonyl fluoride (PMSF), o-phospho-L-serine and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Sigma Chemical Company, Poole, Dorset, U.K.

Acrylamide, EDTA, Folin & Ciocalteau's reagent, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), *NN'*-methylenebisacrylamide and sodium dodecyl sulphate (SDS) were supplied by FSA Laboratory Supplies, Loughborough, U.K.

Ammonium persulphate, bromophenol blue, citric acid, formaldehyde, 2-mercaptoethanol and sodium deoxycholate were obtained from BDH Chemicals Ltd., Poole, U.K.

Aprotinin and 3-[(cholamidopropyl)dimethylammonio]-1-propansulphonate (CHAPS) were obtained from Boehringer Mannheim, East Sussex, U.K.

Norit A activated charcoal was supplied by Aldrich Chemical Company Ltd., Gillingham, Dorset, U.K.

Ampholine, pH 3.5-10 was obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden.

Okadaic acid was supplied by Calbiochem, Nottingham, U.K.

o-nitrophenyl-β-D-galactopyranoside was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

All other chemicals used were of the highest grade available. All solutions were prepared, where appropriate, with deionised distilled water (dH_2O).

2.1.2 Radiochemicals

Iodine-125 (supplied as sodium iodide in dilute sodium hydroxide solution, pH 7-11, 15.2 Ci $^{125}I/\mu g$ iodine) and (3-[^{125}I]-iodotyrosyl^{10}) glucagon (~2000Ci/mmol) were obtained from Amersham International plc, Amersham, U.K

2.1.3 Enzymes

Catalase (18600 units/mg), β-galactosidase (315 units/mg) and malate dehydrogenase (2600 units/mg) were obtained from Sigma Chemical Company, Poole, Dorset, U.K.

2.1.4 Chromatographic and Electrophoretic Materials

Immobiline DryPlates (pH 4-7), GelBond film for agarose gels, agarose IEF, Sepharose CL-6B and gel filtration calibration standards were supplied by Pharmacia-LKB Biotechnology, Uppsala, Sweden.

Bio-Gel P-150 was obtained from Bio-Rad, Hemel Hempstead, Herts., U.K.

Wheat germ agglutinin-agarose (WGA-agarose) was obtained from Vector Laboratories, Peterborough, U.K.

"Rainbow markers", molecular weight range 14 300-200 000, for SDS-PAGE were obtained from Amersham International plc, Amersham, U.K.

2.1.5 Other Materials

Cellulose acetate filters (0.45μm pore size) were obtained from Schleicher & Schuell, Dassel, Germany. Centricon-10 microconcentrators were obtained from Amicon Ltd., Stonehouse, Glos., U.K.

2.2 Methods

2.2.1 Purification of Plasma Membranes from Whole Rat Liver

Liver plasma membranes were prepared using a modification (Marchmont *et al.*, 1981) of the method of Pilkis *et al.* (1974).

Three fed male Sprague-Dawley rats (~250 g) were stunned and decapitated and their livers were removed, washed and chopped in ice-cold 1 mM $KHCO_3$, pH 7.2. Using three volumes of liver : one volume of $KHCO_3$ solution, the livers were homogenised in a 50ml glass Potter-Elvejm homogeniser with six strokes of a motor-driven Teflon pestle. The homogenate was strained through two layers of muslin, diluted to 250 ml with $KHCO_3$ solution and centrifuged at 2 000 *g* for 10 minutes at 4°C. The resultant supernatant was discarded and the pellets poured onto sucrose (72 g) in a plastic beaker. The volume was made up to 120 ml with $KHCO_3$ solution and this suspension was stirred for 30 minutes at 4°C. Portions of 20 ml were then pipetted into clear centrifuge tubes and on top of each portion was carefully layered 48.2% (w/v) sucrose in 3 mM imidazole, pH 7.4 (12 ml), followed by 42.5% (w/v) sucrose in 3 mM imidazole, pH 7.4 (6.5 ml). These gradients were centrifuged at 100 000 *g* for 3 hours in a Beckman ultracentrifuge using an SW 28 rotor at 4°C. The plasma membranes collected as a fluffy brown band at the 42.5-48.2% sucrose interface were aspirated using a pasteur pipette, diluted with an equal volume of $KHCO_3$ solution and centrifuged at 25 000 *g* for 15 minutes at 4°C. The resultant pellet was resuspended in $KHCO_3$ solution (6 ml) and stored at -80°C in 0.5 ml aliquots (3-4 mg protein/ml).

2.2.2 Purification of Plasma Membranes from Isolated Rat Hepatocytes

Isolated hepatocytes from fed male Sprague-Dawley rats (~250 g) were prepared by Dr. A. Savage using the method of Berry and Friend (1969). The following protocol describes the preparation of plasma membranes from hepatocytes obtained from one liver.

Cells were suspended in 1 mM $KHCO_3$, pH 7.2 (20 ml) and homogenised in a 50 ml glass Potter-Elvejm homogeniser with six strokes of a motor-driven Teflon pestle. The homogenate was diluted to 40 ml with $KHCO_3$ solution and spun at 2 000 *g* for 10 minutes at 4°C. The resultant supernatant was discarded and the pellet poured onto sucrose (24 g). The volume was made up to 40 ml with $KHCO_3$ solution and the suspension was stirred for 30 minutes at 4°C. Purification of the plasma membranes on sucrose density gradients was then carried out as described in section 2.2.1, the final pellet being resuspended in $KHCO_3$ solution (1 ml) and stored at -80°C in 0.2 ml aliquots.

2.2.3 Estimation of Protein Concentration

Protein concentration was estimated using the method of Lowry *et al.* (1951) or Bradford (1976), using 1 mg/ml bovine serum albumin as the standard protein solution in each case.

2.2.4 Iodination of Glucagon

When possible, commercially obtained (3-[^{125}I]-iodotyrosyl^{10}) glucagon was used. However, due to the cost of this radiochemical it was occasionally necessary to prepare iodinated glucagon using a modification of the method of Giorgio *et al.* (1974).

Glucagon (5 µl of a 1 mM solution in 0.5M Tris-HCl, pH 8.5) was placed at the bottom of a plastic micro tube. On the sides of the tube was placed 6 mM chloramine T in 0.6 M phosphate buffer, pH 7.4 (5 µl) and Na^{125}I solution (20 µl, ~2mCi). The reaction was initiated by mixing and allowed to proceed for 10-15 seconds. Termination was achieved by the addition of 3mM sodium metabisulphite in 0.6 M phosphate buffer, pH 7.4 (50 µl). The reaction mixture was transferred to a 15 ml conical centrifuge tube with 1% (w/v) BSA in 10mM Tris-HCl, pH 7.6 (8 ml) and after the addition of talc (50 mg) the contents were mixed and cenrifuged at 1 000 *g* for 20 minutes at 25°C. The pellet was washed four times with 0.1 M phosphate buffer, pH 7.0 (8 ml), discarding the washings. Following extraction of purified ^{125}I-glucagon from the talc with 50% (v/v) aqueous ethanol (6 ml) and removal of the solvent by evaporation under reduced pressure, the residue was extracted with 0.1% (w/v) ovalbumin in Tris-HCl, pH 7.6 (2 ml) and stored at -20°C in 0.2 ml aliquots. Under these storage conditions radiation destruction of the glucagon occurred so that after 3-4 weeks reprocessing through the talc procedure was necessary.

2.2.5 Binding of ^{125}I-glucagon to Liver Plasma Membranes

Rat liver plasma membranes were incubated at a final concentration of 50-100 µg protein/ml in a final reaction volume of 100 µl containing 0.1% (w/v) BSA, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5 (Buffer A), 0.1-0.2 nM ^{125}I-glucagon

and, when measuring non-specific binding, 1 μM unlabelled glucagon. Incubation was carried out for 20 minutes at 32°C. The reaction was then stopped by the addition of ice-cold Buffer A (5 ml) to the tubes and the diluted samples were immediately filtered through 0.45-μm cellulose acetate filters which had been soaked in 10% BSA overnight and washed with ice-cold Buffer A (2 ml). The tubes were rinsed once with ice-cold Buffer A (5 ml) and the rinses filtered. The filters were then washed with a final 5 ml portion of ice-cold Buffer A and the radioactivity bound to them was measured using a γ counter. Non-specific binding, measured in the presence of 1 μM unlabelled glucagon, was typically 5-10% of the total binding.

2.2.6 Direct Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes by UV Irradiation

Rat liver plasma membranes (1 mg of protein/ml) were incubated with ~1 nM ^{125}I-glucagon for 30 minutes at 32°C in a final volume of 400 μl containing 20 mM NaH_2PO_4, pH 7.5, 1 mM EDTA, 1 mg/ml BSA, a mixture of protease inhibitors [MPI : aprotinin (15 μg/ml), leupeptin (5 μg/ml), and pepstatin (7 μg/ml)] and, when used, 1 μM unlabelled glucagon. At the end of the incubation, the membranes were collected by centrifugation in an Eppendorf centrifuge and washed with 20 mM NaH_2PO_4, pH 7.5, 1 mM EDTA, 1 mg/ml BSA and MPI (500 μl). Pellets were then resuspended in the same buffer minus BSA (350 μl) and transferred to a 24-well tissue culture plate. In order to optimise the UV irradiation conditions, the plate was placed on ice either 2 cm or 10 cm from a 254 nm UV source for 5, 10 or 20 minutes. The result of this experiment (4.2.1.1) led to the adoption of 20 minutes at 10 cm from the source as the irradiation conditions for all further cross-linking experiments. After irradiation the membranes were collected by centrifugation and stored at -80°C until further use.

When investigating the effect of the presence of proteins and sugars on incorporation of bound hormone into covalently-linked product, ovalbumin, BSA and ovomucoid (each at concentrations of 0.1 mg/ml and 1 mg/ml) and GlcNAc and galactose (0.2 M) were included in the incubation buffer during the irradiation step of the procedure. The result of this experiment (4.2.1.2) led to the use of ovalbumin at 1 mg/ml in the irradiation step in all further cross-linking experiments.

2.2.7 Solubilisation of Membranes Containing Covalently Labelled Receptor

Liver membranes that had been cross-linked to ^{125}I-glucagon were solubilised in 25 mM HEPES, pH 8.0, 1% (w/v) Lubrol-PX (400 µl) at a final protein concentration of 1 mg/ml. Samples were incubated on ice for 30 minutes with periodic agitation then centrifuged at 100 000 *g* for 60 minutes. The supernatant was removed and used as a source of solubilised glucagon receptor.

2.2.8 Wheat Germ Agglutinin-Agarose Chromatography of Solubilised Covalently Labelled Receptor

A 1 ml column of WGA-agarose was packed in a 2 ml plastic syringe and washed with several column volumes of column buffer (25 mM HEPES, pH 8.0, 0.1% (w/v) Lubrol-PX, 50 mM KCl, 1 mM EDTA, 2mM $MgCl_2$) using a peristaltic pump with a flow rate set at approximately 0.3 ml/hour. A sample of solubilised covalently labelled receptor (2.2.6) was made up to 1 ml with column buffer and applied to the column, which was then washed with column buffer. Four fractions of ~1.2 ml were collected then fractions of ~0.3 ml were collected until they contained no significant radioactivity. Bound material was eluted with

column buffer containing 0.5 M GlcNAc while continuing to collect fractions of 0.3 ml, and the amount of radioactivity present in all fractions was measured using a γ counter. Fractions containing radioactive material eluted by 0.5 M GlcNAc were pooled and either precipitated for running on SDS-PAGE or concentrated using Centricon-30 microconcentrators for running on isoelectric focusing gels.

2.2.9 Chloroform/Methanol Precipitation of Proteins

Precipitation of proteins was carried out using the method of Wessel and Flügge (1984). This is a rapid and effective procedure which is unaffected by the presence of detergents in the protein sample.

Four volumes of methanol were added to one volume of the protein sample and the sample was vortexed and centrifuged at 9000 *g* for 10 seconds. One volume of chloroform was added and the vortexing and centrifugation step repeated. Three volumes of water were then added and the sample was vortexed vigorously and centrifuged for 1 minute at 9000 *g*. The upper phase was carefully removed and discarded and a further 3 volumes of methanol were added and the sample was vortexed and centrifuged for 2 minutes at 9000 *g* to pellet the protein. The supernatant was removed and the protein pellet dried under a stream of air and used immediately or stored at -20°C until further use.

2.2.10 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970).

2.2.10.1 Casting SDS-Polyacrylamide Gels

A 10% separating gel was prepared using the following components:

10 ml 1.5 M Tris-HCl pH 8.8

13.3 ml acrylamide stock

(29% w/v acrylamide, 1% w/v *NN'*-methylenebisacrylamide)

15.9 ml dH_2O

400 µl 10% (w/v) ammonium persulphate

16 µl TEMED

TEMED was added last and the gel poured immediately and overlaid with isopropanol. A 5% stacking gel was then prepared using the following components:

2.5 ml 1 M Tris-HCl pH 6.8

3.4 ml acrylamide stock

13.6 ml dH_2O

200 µl 10% (w/v) ammonium persulphate

20 µl TEMED

When the separating gel was set the isopropanol was removed and the top of the gel was washed with distilled water. The stacking gel was then poured on and the comb inserted.

2.2.10.2 Sample Preparation and Running Conditions

Samples were prepared for running on the gel by adding an equal volume of sample buffer (50 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue) or, for precipitated proteins, by taking up the proteins in 35 µl of sample buffer. Samples were incubated at 37°C for 30 minutes prior to application to the gel. Heating at higher temperatures was avoided as this has been shown for certain membrane proteins to lead to problems with aggregation and retention of the sample in the stacking gel during electrophoresis (Herberg *et al.*, 1984; Hennessey, Jr. and Scarborough, 1989). Electrophoresis was carried out using 25 mM Tris containing 0.19 M glycine and 0.1% (w/v) SDS as running buffer for 3-4 hours at 45 mA. Protein bands were visualised by staining with Coomassie blue (2.2.10.3) or silver (2.2.10.4).

2.2.10.3 Coomassie Blue Staining

After electrophoresis the gel was placed in staining solution (0.04% Coomassie brilliant blue R in 10% (v/v) methanol, 10% (v/v) acetic acid) for at least 1 hour at 40°C, then in destaining solution (10% (v/v) methanol, 10% (v/v) acetic acid) until the background was fully destained and bands clearly visible.

2.2.10.4 Silver Staining

After electrophoresis the gel was soaked in 50% (v/v) methanol for at least 2 hours. Solutions A (silver nitrate (0.8 g) dissolved in dH_2O (4 ml)) and B (14.8 M ammonia (1.4 ml) in 0.36% (w/v) NaOH (21 ml)) were prepared and solution A was added dropwise to solution B, stirring vigorously. The resulting solution was made up to 100 ml with distilled water and the gel was stained in this solution for

15 minutes with constant gentle agitation. After washing the gel in distilled water for 5 minutes with constant gentle agitation it was placed in developing solution (0.005% (w/v) citric acid in 0.019% (v/v) formaldehyde) and agitated. When bands were clearly visible the gel was washed several times with water and stored in 50% (v/v) methanol.

2.2.10.5 Autoradiography

Gels to be autoradiographed were dried and exposed to a sheet of Fuji RX medical X-ray film with an intensifying screen at -80°C for 1-20 days.

2.2.11 Isoelectric Focusing on Immobilised pH Gradients (IPGs)

2.2.11.1 Rehydration of Immobiline DryPlates

Ready-made Pharmacia-LKB pH 4-7 Immobiline DryPlates were prepared for electrofocusing by firstly cutting them into 7 mm wide strips. The strips were rehydrated overnight with a solution of 8 M urea containing 0.5-4% (w/v) Ampholine pH 3.5-10, 20% (w/v) glycerol and 2% (w/v) Lubrol-PX or NP-40 in an LKB reswelling cassette.

2.2.11.2 Sample Preparation

Samples for application onto IPGs were solubilised as described in 2.2.7. In initial experiments, designed to optimise the procedure by improving sample entry and band resolution, solubilised untreated plasma membranes (i.e. membranes which had not been subjected to the UV cross-linking procedure) were

used - in these instances, solubilised samples were prepared for electrofocusing by concentrating down to ~50 µl in Centricon-30 microconcentrators. However, when using solubilised cross-linked membranes, WGA-agarose chromatography was performed first and the GlcNAc-eluted fractions and flowthrough fractions were pooled separately and concentrated to ~50µl. Urea and Ampholine pH 3.5-10 were added to samples at final concentrations of 6 M and 2-4% respectively.

2.2.11.3 Focusing

The rehydrated gel strips were lightly blotted with water-saturated filter paper and placed on the kerosene-wetted cooling plate of a Pharmacia-LKB 2217 electrofocusing unit. The electrode strips were soaked with 10 mM glutamic acid (anode) and 10 mM sodium hydroxide (cathode). Samples (10 µl containing 10-50 µg protein) were applied either directly onto the gel surface, in silicone rubber frames placed on the gel surface or on Pharmacia-LKB sample application pieces. The effect of the sample application position was investigated by applying the sample at various points on the gel strips.

After sample application, IPGs were were initially focused at 300-500 V for 1 hour, thereafter the focusing was performed at 3000 V max and 2 mA max for 5 hours at a cooling temperature of 15°C using a Pharmacia-LKB 2297 Macrodrive 5 constant power unit. After focusing the IPG strips were fixed in an aqueous solution of 11.5% (w/v) trichloroacetic acid containing 3.5% (w/v) sulphosalicylic acid for 30 minutes then stained with either Coomassie blue or silver, or air-dried and subjected to autoradiography.

2.2.11.4 Coomassie Blue Staining

Fixed gels were placed in staining solution (0.1% (w/v) Coomassie brilliant blue G in 25% (v/v) ethanol and 8% (v/v) acetic acid) for 30 minutes at 40°C then in destaining solution (25% (v/v) ethanol and 8% (v/v) acetic acid) until the background was fully destained and the bands clearly visible. Gels were stored in destaining solution or dried.

2.2.11.5 Silver Staining

Fixed gels were silver stained using the procedure of Heukeshoven and Dernick (1985). After 2 x 10 minute washes in 10% (v/v) ethanol and 3 x 10 minute washes in water the gel was stained in 0.1% (w/v) silver nitrate for 30 minutes. After a short rinse with water development was initiated with 2.5% (w/v) sodium carbonate solution containing 0.02% (v/v) formaldehyde. Development was continued until bands were clearly visible, when the reaction was stopped with 1% (v/v) acetic acid. Three washes with water were followed by brief (10-30 seconds) treatment with 0.3% sodium (w/v) thiosulphate containing 0.15% (w/v) potassium hexacyanoferrate and 0.05% (w/v) sodium carbonate in order to clear the surface and decrease the background. The gel was rinsed for 1 minute under running tap water, washed for 3 x 10 minutes in water and stored in water or dried.

2.2.12 Isoelectric Focusing in Agarose Gels

2.2.12.1 Casting Agarose Gels

Agarose gels for isoelectric focusing were cast on 114 x 225 mm sheets of GelBond film. A few drops of water were placed on a 114 x 225 mm glass plate and a sheet of GelBond film was placed on the plate with the hydrophilic side uppermost. The film was rolled flat with a glass rod so that excess moisture and air bubbles were removed from under the gel. A second Silane-treated glass plate with a rubber spacer attached on three sides was carefully placed on top of the film and the two plates were clamped together with spring clips. The mould thus formed was warmed in a 60°C incubator before use.

Gels were formed from a solution of 2% (w/v) agarose IEF containing 12% (w/v) sorbitol, 3% (w/v) Ampholine pH 3.5-10, 1% (v/v) 2-mercaptoethanol, 2% (v/v) NP-40 and 6 M urea. The solution (18 ml per 114 x 225 mm gel) was prepared by dissolving the agarose and sorbitol in water by heating in a boiling water bath, allowing the mixture to cool to around 70°C then carefully dissolving the rest of the components. The solution was then poured into the mould and left overnight at room temperature to allow the gel to set fully.

2.2.12.2 Sample Preparation

Samples were prepared essentially as described in 2.2.11.2. The effect of addition of other detergents to the Lubrol-solubilised sample was studied by adding NP-40 or SDS to a final concentration of 2% prior to sample application. The effect of solubilisation of membranes in the presence of urea and Ampholine on band resolution was investigated by including 6 M urea and Ampholine pH 3.5-10 in the solubilisation buffer.

2.2.12.3 Focusing

The gel mould containing the set gel was opened by removing the spring clips and carefully prising the two glass plates apart with a spatula. The gel plate was removed and placed on the kerosene-wetted cooling plate of a Pharmacia-LKB 2217 electrofocusing unit. The electrode strips were soaked with 0.5 M acetic acid (anode) and 0.5 M sodium hydroxide (cathode). Samples (20 µl containing 20-100 µg protein) were applied onto Pharmacia-LKB sample application pieces which had been placed on the surface of the gel. The effect of the sample application position was investigated by applying the sample at various points between the anode and cathode.

After sample application, gels were run at 1000 V max for 90 minutes at a cooling temperature of 15°C using a Pharmacia-LKB 2297 Macrodrive 5 constant power unit. After focusing the gels were fixed and stained with Coomassie blue.

2.2.12.4 Coomassie Blue Staining

Gels were fixed in 33% (v/v) ethanol containing 10% (w/v) trichloroacetic acid for 30 minutes then washed with 33% (v/v) ethanol containing 5% (w/v) trichloroacetic acid for a further 30 minutes. After a brief rinse in destaining solution (35% (v/v) ethanol containing 10% (v/v) acetic acid) gels were placed in staining solution (0.2% (w/v) Coomassie brilliant blue R in destaining solution) for 30 minutes at 40°C then in destaining solution until the background was fully destained and bands clearly visible. Gels were stored in destaining solution or dried.

2.2.13 Solubilisation of Liver Plasma Membranes with CHAPS

Rat liver plasma membranes were suspended at a final concentration of 3-4 mg protein/ml in 25 mM HEPES, pH 7.5 containing 6 mM CHAPS*, 2 mM EDTA, 137 mM NaCl, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.007 mg/ml pepstatin and 0.1 mM PMSF. The solubilisation mixture was incubated for 30 minutes at 4°C with periodic agitation and then spun at 100 000 *g* for 1 hour at 4°C. The supernatant containing soluble active glucagon receptor was used immediately.

*Optimum CHAPS concentration was determined by carrying out the solubilisation at 0-40 mM CHAPS (section 3.2.1).

2.2.14 Assay for CHAPS-solubilised Glucagon Binding Activity

CHAPS extract (50-100 μg protein) was incubated with 0.1-0.2 nM ^{125}I-glucagon and, when measuring non-specific binding, 1 μM unlabelled glucagon at a final CHAPS concentration of 3 mM in a total volume of 120 μl. Incubations were carried out for 2 hours at 4°C then dextran-coated charcoal suspension (0.5% (w/v) dextran and 2.5% (w/v) Norit A activated charcoal) (0.4 ml) was added to each sample. After centrifugation at 2 000 *g* for 5 minutes, aliquots of the supernatants were removed and the radioactivity measured in a γ counter. Non-specific binding, measured in the presence of 1 μM unlabelled glucagon, was typically 10-20% of the total binding.

2.2.15 Sucrose Density Gradient Centrifugation

CHAPS extract (1 ml) was concentrated to ~200 µl using a Centricon-30 microconcentrator and 150µl of the concentrated extract was mixed with malate dehydrogenase (20 µl of 1 mg/ml; 2600 units/mg), catalase (20 µl of 7 mg/ml; 18600 units/mg) and β-galactosidase (10 µl of 10 mg/ml; 315 units/mg). This mixture was loaded on a 5-15% sucrose gradient containing 3 mM CHAPS, 2 mM EDTA, 137 mM NaCl, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin and 0.007 mg/ml pepstatin. The gradient was centrifuged for 12 hours at 105 000 *g* in an SW 50.1 rotor at 4°C. After centrifugation, the gradient was fractionated using an upwards displacement procedure and approximately 24 fractions of 180 µl each were collected. Two 60 µl samples from each fraction were used to assay for specific glucagon binding activity, using a half-scale version of 2.2.14. The rest of the fraction was used to carry out marker enzyme assays (2.2.16) and protein determination (2.2.3).

2.2.16 Marker Enzyme Assays

Malate Dehydrogenase - the sample (10 µl) was added to 50 mM Tris-HCl, pH 7.5, 1.5 mM oxaloacetic acid and 43 µM NADH (1 ml) and the decease in absorbance at 340 nm after 10 minutes of incubation on ice was noted.

Catalase - the sample (10 µl) was added to 50 mM Tris-HCl, pH 7.5, and 5mM hydrogen peroxide (1 ml) and the decrease in absorbance at 240 nm over a 3-minute period at room temperature was noted.

β–Galactosidase - the sample (10 µl) was added to 50 mM Tris-HCl, pH 7.5, and 2 mM *o*-nitrophenyl-β-D-galactopyranoside (1 ml). The samples were

incubated at 32°C until a clearly visible yellow colour had developed, at which point the reaction was stopped by the addition of 1 M sodium carbonate (0.5 ml). The absorbance at 410 nm was noted.

2.2.17 Gel Filtration

Gel filtration of CHAPS extract was performed on a 0.9 x 25 cm Sepharose CL-6B column which had been equilibrated with, unless otherwise stated, 25 mM HEPES, pH 7.5, 2 mM EDTA, 137 mM NaCl and 3 mM CHAPS at 4°C Unless otherwise stated, CHAPS extract (1.5 ml) was concentrated to ~350 µl using a Centricon-30 microconcentrator and the concentrated extract (300 µl) was applied to the column and run at a flow rate of ~12 ml/hour. Approximately 70 fractions of 300 µl each were collected and, for those fractions indicated, two 110 µl samples were taken from each fraction and used to assay for specific glucagon binding activity. The column was calibrated using the protein standards shown and the void volume was determined by the elution of Blue Dextran.

2.2.18 Preparation of Immobilised Glucagon (Glucagon-P150)

This procedure was designed to link glucagon covalently through the side chain of met-27 to the bromoacetyl derivative of an ethylenediamine substituted polyacrylamide gel filtration medium (Bio-Gel P-150) using the procedure of Inman (1974). Glucagon-P150 was prepared by Dr. J.G. Beeley.

Bio-Gel P-150 was substituted with ethylenediamine by reaction at 90°C for 4 hours. The product contained 600 nmoles of amino groups per mg dry weight (as determined by titration). The P150 diamine derivative was coupled with bromoacetic acid which had been activated by reaction in the dark with *N*-

hydroxysuccinimide and dicyclohexylcarbodiimide. After washing extensively with water the product, bromoacetyl-P150, was dehydrated with methanol and dried over KOH pellets.

Bromoacetyl-P150 (250 mg) was allowed to swell in water and then washed, by decantation, with 0.1 M acetic acid containing 0.1% (w/v) KI giving a final volume of 5 ml. Glucagon (3 mg) dissolved in 0.1 M acetic acid (0.5 ml) was added and the reaction mixture was stirred for six days at room temperature in the dark. The product was washed extensively with water and 0.5 M NaCl and was stored in 0.5 M NaCl containing 1% (w/v) sodium azide at 4°C. The extent of coupling was determined by estimation of glucagon remaining in the supernatant using the trinitrobenzenesulphonic acid assay for free amino groups described by Habeeb (1966).

Glucagon-P150 contained 1-3 nmoles bound glucagon/mg.

2.2.19 Determination of the Binding Capacity of Glucagon-P150

The binding capacity of the immobilised glucagon was determined by incubating washed glucagon-P150 (0-200 µl packed volume) with CHAPS extract (400 µl). In order to carry out the incubations at a final CHAPS concentration of 3 mM, the appropriate amount of glucagon-P150 was suspended in CHAPS-free solubilisation buffer in a final volume of 400 µl, then added to CHAPS extract (400 µl). Incubations were carried out at 4°C for 2 hours with constant shaking. After incubation the samples were centrifuged at 2 000 *g* for 10 minutes and the supernatants were removed. The total specific glucagon binding activity in the supernatant was determined by taking four 110 µl samples for assay and hence the percentage of activity bound to the glucagon-P150 was calculated.

As a result of this experiment (3.4.1), all further experiments were carried out using 25 µl (packed volume) of glucagon-P150 per 100 µl of CHAPS extract.

2.2.20 Affinity Chromatograpy Using Immobilised Glucagon

2.2.20.1 Binding

CHAPS extract (400-800 µl) was incubated with glucagon-P150 (100-200 µl packed volume) at a final CHAPS concentration of 3 mM for 2 hours at 4°C with constant shaking. After incubation the sample was centrifuged at 2 000 *g* for 10 minutes, the supernatant was removed and kept for later assay and the glucagon-P150 was, unless otherwise stated, washed with 3 mM CHAPS buffer (solubilisation buffer containing 3 mM CHAPS in place of 6 mM CHAPS) (1.5 ml) and eluted by one of the methods described in 2.2.20.2.

2.2.20.2 Elution Methods

Elution with Glucagon - The washed glucagon-P150 was incubated with 3 mM CHAPS buffer containing 10 µM or 100 µM unlabelled glucagon for 1 hour or overnight at 4°C with constant shaking. The sample was then centrifuged at 2 000 *g* for 10 minutes and the supernatant was removed. If assaying samples from the supernatant for specific glucagon-binding activity it was necessary to remove the unlabelled glucagon. This was attempted using two methods :

i) the supernatant was applied to an 800 µl column of dextran-coated charcoal (formed from 3 mM CHAPS buffer containing 2.5% Norit-A activated charcoal and 0.5% dextran) and the eluate was assayed for specific glucagon binding activity using four 110 µl aliquots.

ii) the supernatant was incubated with WGA-agarose (100 µl packed volume) for 2 hours at 4°C. The WGA-agarose was then packed in a 1 ml plastic syringe, washed with 3 mM CHAPS buffer (10 ml) and eluted by incubating with 3 mM CHAPS buffer (0.6 ml) containing 0.5 M GlcNAc for 2 hours. The eluate was assayed for specific glucagon binding activity using four 110 µl aliquots.

If the supernatant after elution with glucagon was to be characterised by SDS-PAGE the proteins present were precipitated using the chloroform/methanol method, run on a 10% SDS-polyacrylamide gel and visualised by silver staining.

Elution with Urea - The washed glucagon-P150 was packed in a 1 ml plastic syringe and eluted with 3 mM CHAPS buffer (0.6 ml) containing 2 M urea. The eluate was dialysed overnight against 3 mM CHAPS buffer (1 litre) and assayed for glucagon binding activity using four 110 µl aliquots.

Elution with Low pH Buffers - The washed glucagon-P150 was packed in a 1 ml plastic syringe and eluted with 3 mM CHAPS buffer adjusted to pH 4 (0.5 ml). The eluate was immediately adjusted to pH 7.5 and assayed for glucagon binding activity using four 110 µl aliquots. Elution with pH 5 buffer was carried out using the same procedure.

Elution with SDS - The washed glucagon-P150 was incubated with 3 mM CHAPS buffer containing 1% SDS (0.5 ml) for 10 minutes and centrifuged at 2 000 *g* for 10 minutes. Proteins present in the supernatant were precipitated using the chloroform/methanol method, run on a 10% SDS-polyacrylamide gel and visualised by silver staining.

2.2.21 Thermostability Experiments

2.2.21.1 Thermostability of Membrane-bound Receptor

Liver plasma membrane samples (5-10 µg protein) were incubated with 20 mM Tris pH 7.5, 5 mM EDTA, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.007 mg/ml pepstatin and 0.1 mM PMSF in a final volume of 5 µl at 50°C for the time intervals indicated. At the end of each time interval four samples were placed on ice. All samples were then assayed for glucagon-binding activity as described in 2.2.5.

2.2.21.2 Thermostability of CHAPS-solubilised Receptor

Samples of CHAPS extract (50-100 µg protein) were incubated at 35°C for the time intervals indicated. At the end of each time interval four samples were placed on ice. All samples were then assayed for glucagon binding activity as described in 2.2.14.

Chapter 3 The CHAPS-solubilised Receptor

3.1 Introduction

Treatment of rat liver plasma membranes with CHAPS has been shown to yield glucagon receptor in a soluble, active form (Herberg *et al.*, 1984; McVittie and Gurd, 1989). However, although conditions for stabilising the receptor have been optimised and its binding characteristics studied, isolation of the CHAPS-solubilised receptor has still not been achieved. The aims of the work presented in this chapter were i) to further optimise the solubilisation and stabilisation of the receptor, ii) to further characterise the receptor, using sucrose density gradient centrifugation, gel filtration and thermostability techniques, and iii) to isolate the receptor using affinity chromatography on immobilised glucagon.

Two assays for CHAPS-solubilised receptor activity have been developed. Herberg *et al.* (1984) utilised the glycoprotein nature of the receptor to develop an assay whereby glucagon-receptor complexes bind specifically to wheat germ lectin-Sepharose, whereas McVittie and Gurd (1989) used dextran-coated charcoal adsorption of free hormone, followed by recovery of glucagon-receptor complexes in the supernatant after centrifugation, as the basis of their assay. The latter method is superior to the wheat germ lectin method in that it is more rapid, cheaper and highly reproducible, and was therefore used to assay for solubilised receptor activity throughout the course of the work described in this chapter.

3.2 Solubilisation of Active Receptor with CHAPS

3.2.1 Effect of CHAPS Concentration on the Solubilisation of Active Receptor

The optimal CHAPS concentration for solubilising active glucagon receptors was determined by solubilising liver plasma membranes (4 mg protein/ml) as described in section 2.2.13 with a range of CHAPS concentrations (0-40 mM). Since adsorption of free glucagon to charcoal requires a CHAPS concentration of 3 mM or less (McVittie and Gurd, 1989), the CHAPS extracts obtained were diluted to a final CHAPS concentration of 3 mM with CHAPS-free solubilisation buffer. The diluted extracts were then assayed for specific glucagon binding activity (2.2.14) and protein (2.2.3). Figure 3.1 shows that both solubilised total protein and solubilised active receptor increase with increasing CHAPS concentration. However, while there is a large increase in receptor solubilisation between 3 and 6 mM CHAPS, further increasing the CHAPS concentration results in relatively small changes in receptor solubilisation, while protein solubilisation shows a more steady rise over the CHAPS concentrations used. For this reason, and in order to economise on CHAPS and reduce the scale of dilution required to bring the CHAPS concentration of samples to 3 mM, all further solubilisations were carried out using 6 mM CHAPS. Figure 3.2 shows that under these conditions the amount of specific glucagon binding activity is proportional to the amount of CHAPS-solubilised protein assayed.

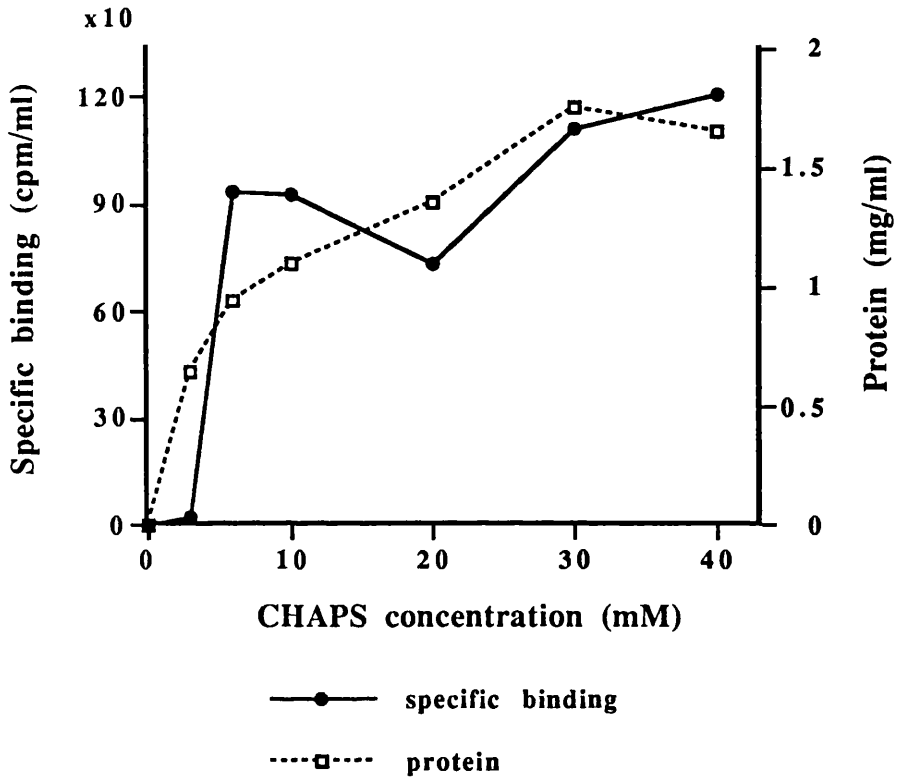


Figure 3.1 Effect of CHAPS Concentration on the Solubilisation of Active Receptor

Rat liver plasma membranes (4 mg protein/ml) were solubilised in 0-40 mM CHAPS in a final volume of 0.5 ml as described in section 2.2.13. The resulting CHAPS extracts were diluted with CHAPS-free solubilisation buffer to give a final CHAPS concentration of 3mM. Four 110 µl aliquots from each sample were then used to assay for specific glucagon-binding activity. The protein concentration of each extract was determined as described in section 2.2.3.

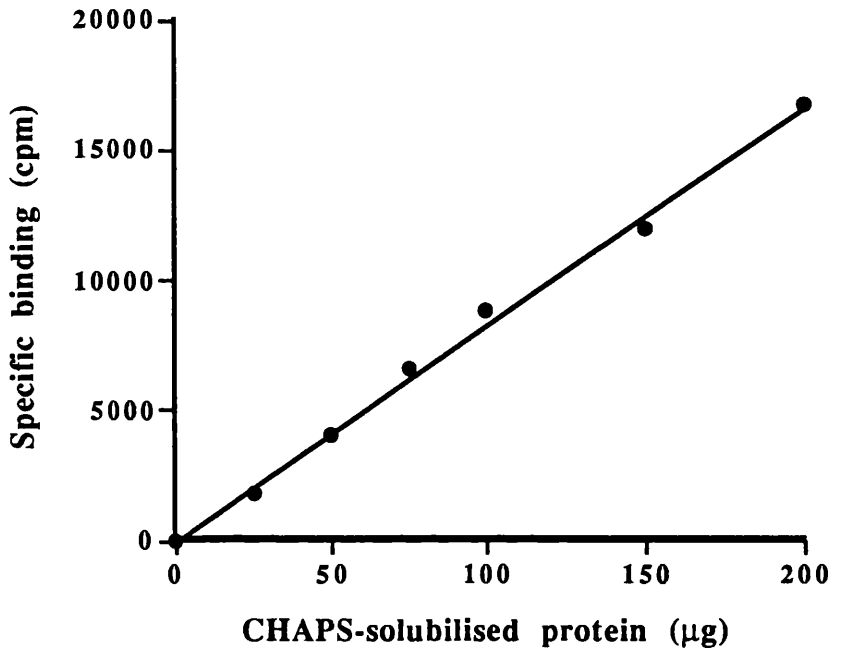


Figure 3.2 Relation of Specific Glucagon Binding to Amount of CHAPS-solubilised Protein

CHAPS extract (0-200 μg protein) was assayed for specific glucagon binding activity as described in section 2.2.14.

3.2.2 Effect of Guanine Nucleotides on Specific Glucagon Binding to the CHAPS-solubilised Receptor

The effect of guanine nucleotides on the specific binding of glucagon to the CHAPS-solubilised receptor was investigated by carrying out the solubilised receptor assay described in section 2.2.14 in the presence or absence of 100 μM GTP. It was found that the presence of 100 μM GTP had no effect on the amount of specific glucagon binding to the CHAPS-solubilised receptor. This is in contrast to the situation for the membrane-bound receptor, where the presence of 100 μM GTP resulted in a 50-60% decrease in specific glucagon binding.

3.2.3 Stability of the CHAPS-solubilised Receptor

A major problem encountered in working with the CHAPS-solubilised glucagon receptor is its instability, especially when carrying out lengthy procedures such as sucrose density gradient centrifugation or gel filtration. McVittie and Gurd (1989) showed that the stability of the receptor was greatly increased by the addition of 1 x Hanks' balanced salt solution or, to a lesser extent, 137 mM NaCl in the solubilisation buffer. The aim of the following experiments was to further increase receptor stability by looking at the effects of calcium and PMSF.

3.2.3.1 Effect of Calcium on Receptor Stability in CHAPS Extract

Initial solubilisations of liver plasma membranes with CHAPS were carried out with 1.26 mM $CaCl_2$ included in the buffer, since it has been shown that its inclusion increases the proportion of specific to total glucagon binding (McVittie and Gurd, 1989). However, the effect of calcium on receptor stability is uncertain.

Although a divalent ion chelator (EDTA) is present in the solubilisation buffer, the activity of calcium-dependent and calcium-stabilised proteases could still present a problem. The effect of calcium was investigated by solubilising liver plasma membranes with CHAPS in the presence or absence of 1.26 mM $CaCl_2$ as described in section 2.2.13. Samples of the extracts were stored for 24 hours at 4°C and -80°C and their specific glucagon binding activity was determined and compared with the activity measured immediately after solubilisation. Figure 3.3 shows that after storage at 4°C and, more surprisingly, at -80°C, the loss of receptor binding activity was greater in CHAPS extracts containing $CaCl_2$ than in those in which the salt was absent. Although the difference at 4°C is small, all further solubilisations were therefore carried out without $CaCl_2$ in the solubilisation buffer. The proportion of specific to total glucagon binding was found to be unaffected by the presence or absence of $CaCl_2$ when 137 mM NaCl was also present in the solubilisation buffer.

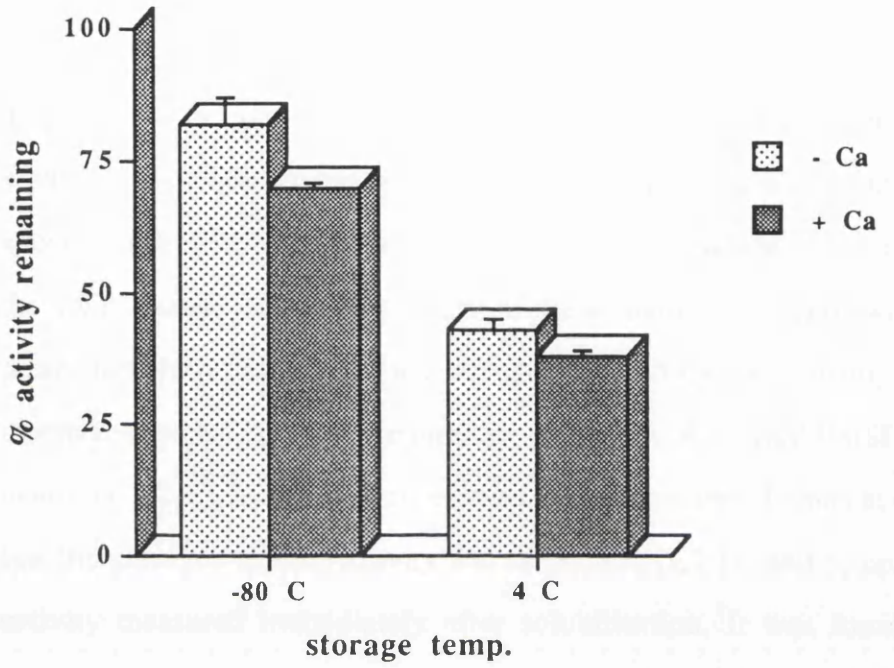


Figure 3.3 Effect of Calcium on Solubilised Receptor Stability

Liver plasma membranes (3 mg protein) were solubilised as described in section 2.2.13 in the presence or absence of 1.26 mM $CaCl_2$. Four 60 µl aliquots of the resulting extracts were taken and immediately assayed for specific glucagon binding activity as described in section 2.2.14. Aliquots (250 µl) of the extracts were stored at -80°C and 4°C for 24 hours then assayed for specific glucagon binding activity. Results are expressed as the percentage of initial activity remaining after 24 hours (n = 2).

3.2.3.2 Effect of PMSF on Receptor Stability in CHAPS Extract

Although a mixture of protease inhibitors (aprotinin, leupeptin, pepstatin and EDTA) was included in the solubilisation buffer for initial solubilisations according to the procedure of McVittie and Gurd (1989), a general protease inhibitor cocktail for animal tissue includes PMSF in addition to the inhibitors listed above (North, 1989), and it was therefore considered that the inclusion of PMSF in the solubilisation buffer might provide the solubilised receptor with additional protection from proteases. This was investigated by solubilising liver plasma membranes with CHAPS in the presence or absence of 0.1 mM PMSF as described in section 2.2.13. Samples of the extracts were stored for 24 hours at 4°C and their specific glucagon binding activity was determined (2.2.14) and compared with the activity measured immediately after solubilisation. It was found that when solubilisation was carried out in the presence of PMSF only 35% of binding activity was lost on storage for 24 hours at 4°C whereas when PMSF was absent there was a loss of 60% of activity under the same conditions. Therefore all further solubilisations were carried out in the presence of 0.1 mM PMSF.

3.3 Characterisation of the CHAPS-solubilised Receptor

3.3.1 Sucrose Density Gradient Centrifugation

Concentrated (5x) CHAPS extract was centrifuged through 5-15% sucrose gradients containing 3 mM CHAPS as described in section 2.2.15. The gradients were fractionated by upwards displacement and assayed for specific glucagon binding activity (2.2.14) and protein (2.2.3). A typical profile is shown in Figure 3.4; two peaks of binding activity were routinely seen in the positions shown. Standard curves, obtained by plotting the migration distances of marker enzymes (run on the same gradient as the CHAPS extract and assayed as described in section 2.2.16) against their sedimentation coefficients in H_2O, were found to be linear and, by interpolation, the two peaks of binding activity were found to represent species with sedimentation coefficients of 7.5 ± 0.1 S and 12.9 ± 0.6 S (n = 3). These values are somewhat higher than the single value of 4.3 S observed by Herberg *et al.* (1984) for a cross-linked ^{125}I-glucagon receptor complex solubilised with Lubrol-PX.

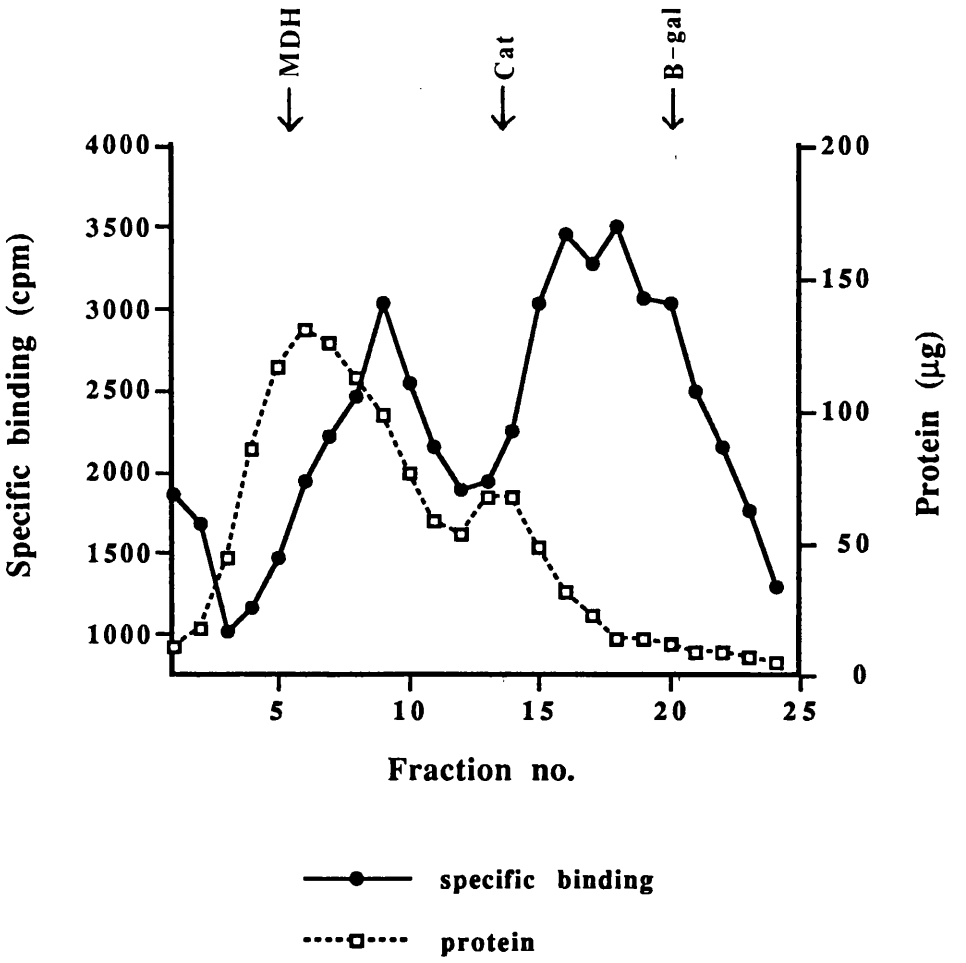


Figure 3.4 Sucrose Density Gradient Centrifugation of CHAPS Extract

Concentrated CHAPS extract (150 µl) was run on a 5-15% sucrose gradient as described in section 2.2.15. Specific glucagon binding activity was determined using two 60 µl samples from each 180 µl fraction, using a half-scale version of 2.2.14, and protein was measured as described in section 2.2.3. The positions of marker enzymes, determined using the assays described in 2.2.16, are indicated by arrows (MDH = malate dehydrogenase; Cat = catalase; β-gal = β-galactosidase).

3.3.2 Gel Filtration on Sepharose CL-6B

Sepharose CL-6B was used in all gel filtration experiments due to its wide fractionation range of 10^4-4 x 10^6 Da. This fractionation range was desirable because, although Herberg *et al.* (1984) reported a molecular weight of 119 kDa for the glucagon receptor in Lubrol-PX, other groups have reported much higher molecular weights for the receptor in Lubrol-PX (Giorgio *et al.*, 1974) and digitonin (Mason and Tager, 1985).

Initial experiments were carried out on a 46 ml column. However, using a column of this size meant that in order to obtain a manageable number of fractions (this being limited by the need to economise on ^{125}I-glucagon), the fraction volume had to be large (~1.2 ml) and, keeping the scale of the receptor assay the same as described in section 2.2.14, only a small proportion (110 µl) of each fraction could be used. As a result of this and the high degree of dilution of sample on the column, amounts of activity measured were so small as to be unreliable, especially as specific binding represented such a small proportion (<10 %) of total binding. Therefore, a smaller (16 ml) column was used. While the resolution attainable on this column was not as high as on the larger one, smaller, more concentrated fractions could be collected and hence the receptor assay was more reliable with much higher amounts of specific binding detectable. For these reasons the smaller column was used for all the experiments described in this section.

As the glucagon receptor is known to be a glycoprotein, its molecular weight may not correlate well to the molecular weight calibration curve constructed from globular proteins. Therefore a calibration curve relating elution volume to Stokes' radius (R_S) was established, the aim being to obtain an R_S value(s) for the glucagon receptor and use this, together with a sedimentation coefficient value(s) obtained from sucrose density gradient centrifugation, to calculate the molecular weight as shown in section 3.3.3, a procedure used previously to determine the molecular weight of the receptor in Lubrol-PX (Herberg *et al.*, 1984).

3.3.2.1 Gel Filtration of Concentrated CHAPS Extract

Concentrated (5x) CHAPS extract was run on Sepharose CL-6B as described in section 2.2.17 and fractions 19-42 were assayed for specific glucagon binding activity (2.2.14) and protein (2.2.3). Figure 3.5 shows that the majority (~70%) of glucagon binding activity eluted in the void volume, indicating that under these conditions much of the solubilised receptor exists in a highly aggregated form with a molecular weight of over 4 x 10^6 Da. However, the fact that lower levels of activity are detected over the rest of the range of fractions assayed suggests that although the majority of receptor is highly aggregated, a small proportion of the total receptor population exists in a series of less aggregated forms. The protein profile shows a peak at the void volume but the majority of protein eluted after the void volume. This suggests that the aggregation of the receptor is specific to a certain degree; i.e. aggregation to the extent seen does not occur simply as a result of non-specific aggregation of all or most of the solubilised proteins.

3.3.2.2 Gel Filtration of Unconcentrated CHAPS Extract

Although considered unlikely, it was thought possible that the high degree of aggregation of the receptor seen in section 3.3.2.1 could be brought about by concentration of CHAPS extract prior to gel filtration. This hypothesis was tested by running unconcentrated CHAPS extract on Sepharose CL-6B as described in section 2.2.17 and assaying fractions 19-42 for specific glucagon binding activity (2.2.14) and protein (2.2.3). Figure 3.6 shows that, as with concentrated extract, the majority of activity is eluted in the void volume, although the proportion of the total activity in this peak is slightly lower (~60%) than it is with concentrated extract (~70%). Thus concentration of CHAPS extract appears to have little effect on receptor aggregation, and hence in all the following gel filtration experiments concentrated (5x) extract was used due to the higher levels of activity detectable.

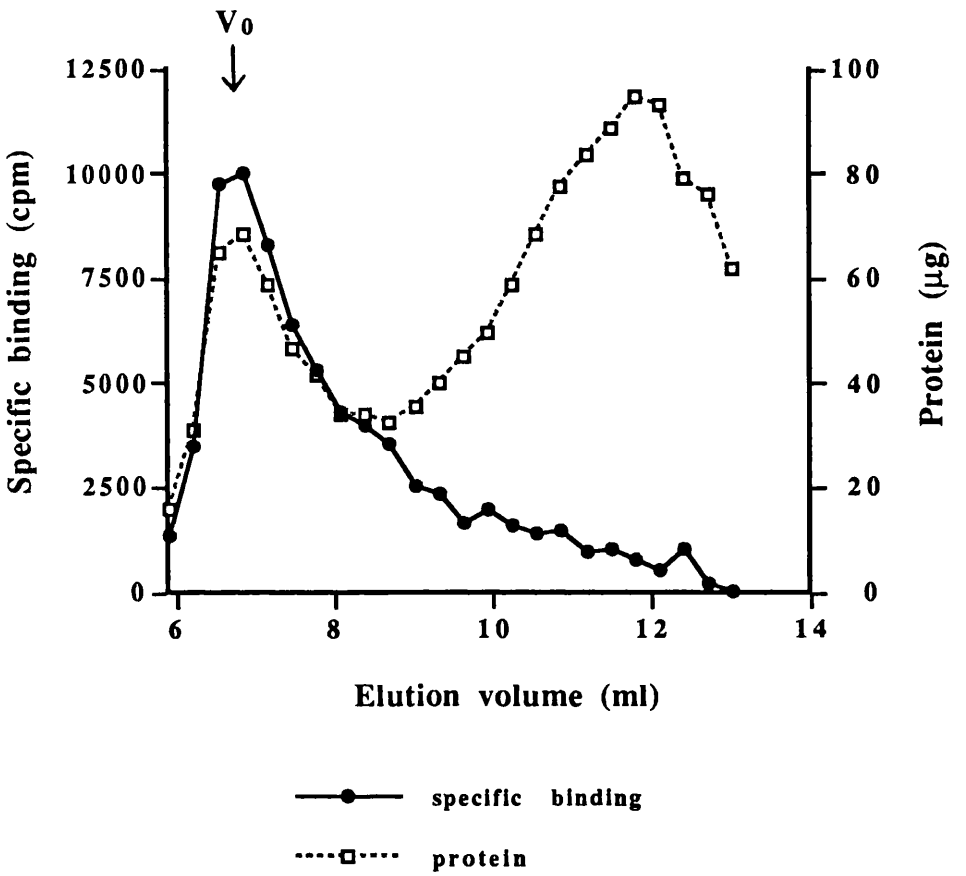


Figure 3.5 Gel Filtration on Sepharose CL-6B of CHAPS Extract

Concentrated CHAPS extract (300 µl) was run on a 0.9 x 25 cm Sepharose CL-6B column as described in section 2.2.17. Fractions (310 µl) were analysed for specific glucagon binding activity using two 110 µl samples (section 2.2.14) and for protein (2.2.3). The void volume (V_O) was determined by the elution of Blue Dextran.

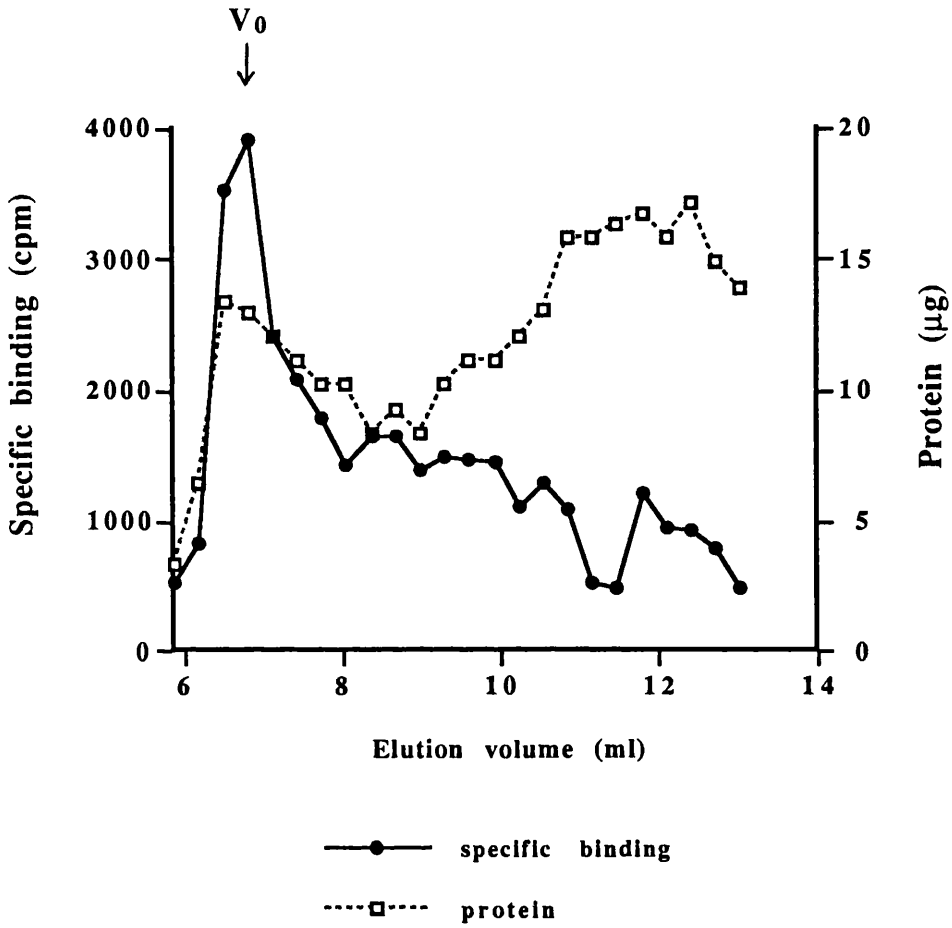


Figure 3.6 Gel Filtration on Sepharose CL-6B of Unconcentrated CHAPS Extract

CHAPS extract (300 µl) was run on a 0.9 x 25 cm Sepharose CL-6B column as described in section 2.2.17, except that the extract was not concentrated prior to application onto the column. Fractions (310 µl) were analysed for specific glucagon binding activity using two 110 µl samples (section 2.2.14) and for protein (2.2.3).

3.3.2.3 Effect of Increased NaCl Concentration on Receptor Aggregation

Receptor aggregation after solubilisation is most likely to occur as a result of hydrophobic interactions. However, in order to eliminate the possibility of aggregation through electrostatic interactions, gel filtration was carried out at a higher NaCl concentration (637 mM as opposed to 137 mM), although it should be added that if aggregation is indeed brought about mainly by hydrophobic interactions, an increase in salt concentration could lead to further aggregation. CHAPS extract was prepared as described in section 2.2.13 in the presence of 637 mM NaCl then run on Sepharose CL-6B as described in section 2.2.17 except that the column buffer contained 637 mM NaCl. Fractions 19-40 were assayed for specific glucagon binding activity as described in section 2.2.14. It was found that the specific activity of CHAPS extract in 637 mM NaCl was approximately one third of that in 137 mM NaCl. Figure 3.7 shows that increasing the NaCl concentration to 637 mM has no effect on the gel filtration profile, with ~70 % of activity again eluted in the void volume, and therefore has no effect on receptor aggregation. All further experiments were carried out in 137 mM NaCl.

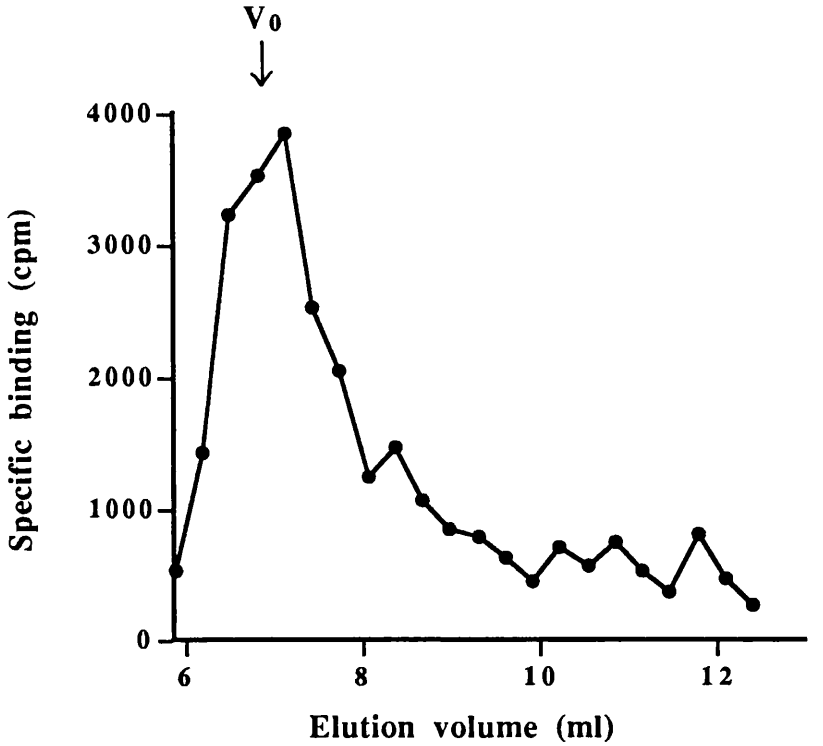


Figure 3.7 Gel Filtration on Sepharose CL-6B of CHAPS Extract in High Salt

CHAPS extract (1.5 ml) was prepared as described in section 2.2.13, except that the concentration of NaCl was 637 mM. After concentration to 300 µl the extract was run on a 0.9 x 25 cm Sepharose CL-6B column as described in section 2.2.17, except that the column buffer contained 637 mM NaCl. Fractions were assayed for specific glucagon binding activity using two 110 µl samples (section 2.2.14).

3.3.2.4 Effect of Sucrose on Receptor Aggregation

The results of experiments 3.3.2.1-3.3.2.3, suggesting a high degree of receptor aggregation in CHAPS extract, contradict the result obtained from sucrose density gradient centrifugation (3.3.1) which identifies much smaller glucagon-binding species, as evidenced by their sedimentation coefficients. It was therefore considered that sucrose might prevent receptor aggregation or, because CHAPS extracts applied to sucrose gradients were prepared in the absence of sucrose, that it might promote disaggregation of the receptor. This was tested by preparing CHAPS extract as described in section 2.2.13 and making it 10% (w/v) with respect to sucrose prior to gel filtration using column buffer containing 10% (w/v) sucrose. Fractions 19-40 were assayed for specific glucagon binding activity. The proportion of activity in the void volume peak was found to be lower (~50% of the total activity) in the presence of sucrose (Figure 3.8) than in its absence (3.3.2.1), suggesting at least a small degree of disaggregation in the presence of sucrose.

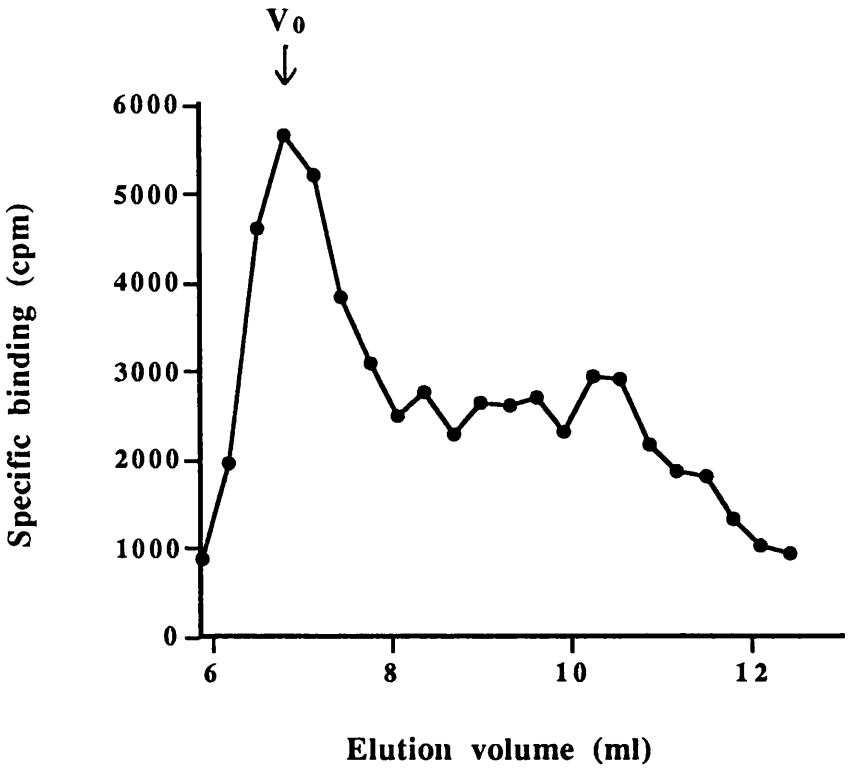


Figure 3.8 Gel Filtration on Sepharose CL-6B of CHAPS Extract in 10% Sucrose

Concentrated CHAPS extract was made 10% (w/v) with respect to sucrose and 300 µl was run on a 0.9 x 25 cm Sepharose CL-6B column as described in section 2.2.17, except that the column buffer also contained 10% (w/v) sucrose. Fractions were assayed for specific glucagon binding activity using two 110 µl samples (section 2.2.14).

3.3.2.5 Effect of Increased Spin Time in Preparation of CHAPS Extract on Gel Filtration

Experiment 3.3.2.4, although showing that sucrose has a small effect on the aggregation state of the receptor, does not explain why still 50% of active receptor appears to be highly aggregated while sucrose density gradient experiments do not give any evidence of the receptor in such a high state of aggregation. It was considered that the spin conditions in the sucrose density gradient experiments (105 000 *g* for 12 hours) could result in highly aggregated material of the size suggested by gel filtration experiments being pelleted and so undetected after fractionation of the gradient. Therefore, CHAPS extract was prepared as described in section 2.2.13 except that the solubilisation mixture was spun at 105 000 *g* for 12 hours instead of at 100 000 *g* for 1 hour. The concentrated extract was then run on Sepharose CL-6B as described in section 2.2.17 and fractions 20-47 were assayed for specific glucagon binding activity as described in section 2.2.14, using a higher concentration (0.5 nM) of ^{125}I-glucagon than normal due to the expected low amounts of activity present. Figure 3.9 shows that no activity eluted in the void volume, indicating that the high molecular weight material seen in the previous experiments was indeed pelleted using the longer centrifugation. The implications of this for the solubilisation of the glucagon receptor with CHAPS are discussed in section 5.1.1. Figure 3.9 also shows a peak of binding activity at an elution volume of ~10.4 ml; this was routinely observed as the peak of lowest molecular weight but no other peaks could be resolved. A calibration curve was obtained by plotting $\sqrt{}$ - log (K_{av}) against R_s where

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume for the protein

V_o = column void volume

V_t = total bed volume

. The calibration curve obtained was linear and gave an R_S value of 5.8 nm for the species giving the peak of activity shown in Figure 3.9. This compares with a value of 6.3 nm obtained by Herberg *et al.* (1984) for a cross-linked ^{125}I-glucagon-receptor complex solubilised in Lubrol-PX.

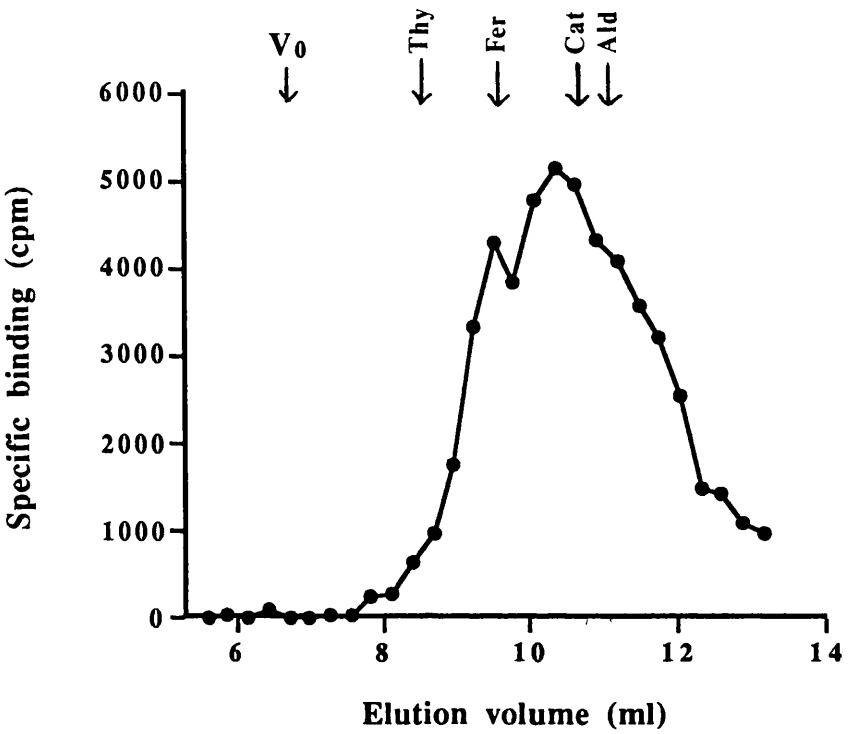


Figure 3.9 Gel Filtration on Sepharose CL-6B of CHAPS Extract Spun at 105 000 *g* for 12 Hours

CHAPS extract (1.5 ml) was prepared as described in section 2.2.13, except that the solubilised membranes were spun at 105 000 *g* for 12 hours. After concentration to 250 µl the extract was made 10% (w/v) with respect to sucrose and 300 µl of concentrated extract was run on a 0.9 x 25 cm Sepharose CL-6B column as described in section 2.2.17, except that the column buffer also contained 10% (w/v) sucrose. Fractions were assayed for specific glucagon binding activity using two 110 µl samples (section 2.2.14). The elution positions of calibration proteins are indicated by arrows (Ald = aldolase; Cat = catalase; Fer = ferritin; Thy = thyroglobulin).

3.3.3 Estimation of the Minimum Molecular Weight of the CHAPS-solubilised Receptor

The gel filtration experiments described in section 3.2 show that, under the solubilisation conditions used, the majority of the glucagon receptor population exists in a highly aggregated form which can be pelleted by increasing the time and rate of centrifugation during preparation, while a smaller proportion appears to be present as a series of less aggregated species. By using the sedimentation coefficient ($s_{20,\omega}$) and Stokes radius (R_s) values of the smallest species given by sucrose density gradient and gel filtration experiments, and assuming that these correspond to the same species, it was possible to estimate a minimum molecular weight for the receptor under these conditions using the following equation,

$$M_r = \frac{6\pi N\eta_{20,\omega} \cdot R_s \cdot s_{20,\omega}}{(1 - \nu\rho_{20,\omega})}$$

where ν (partial specific volume (ml/g)) was assumed to be 0.738, the average for all the marker proteins and thus an additional assumption is made that, as has been observed for the receptor in Lubrol-PX (Herberg *et al.*, 1984), there is no significant binding of detergent to the receptor. As the partial specific volume of CHAPS (0.75) is very similar to this value, running of CHAPS extract on sucrose gradients containing D_2O was not carried out since no change in the observed sedimentation coefficient of the receptor due to detergent binding would have been observed and hence this would not have been a suitable method for determining the true partial specific volume (for further discussion see section 5.1.2). The minimum molecular weight was calculated to be approximately 190 kDa and it is that the smallest form of the receptor in 6 mM CHAPS is a trimer of the 60 kDa species seen in cross-linking studies (see section 5.1.2 for discussion).

3.3.4 Effect of CHAPS Concentration on Receptor Aggregation

It was shown in section 3.2.1 that increasing the CHAPS concentration above 6 mM has little effect on the amount of active receptor solubilised from liver plasma membranes. However, it is possible that an increase in CHAPS concentration,whilst having no effect on the quantity of receptor solubilised, may have an effect on the aggregation state of the receptor that is solubilised and thus the proportion of solubilised activity that exists in the highly aggregated form seen in gel filtration experiments (3.3.2). To test this, liver plasma membranes were solubilised as described in section 2.2.13 using CHAPS concentrations of 6 mM and 20 mM. The resulting extracts were then centrifuged at 105 000 *g* for 12 hours in order to pellet highly aggregated material as shown in section 3.3.2.5. The supernatants were diluted to 3 mM CHAPS and assayed for specific glucagon binding activity as described in section 2.2.14. It was found that the percentage of activity remaining in the supernatant was unaffected by the CHAPS concentration (19.7% and 17.2% in 6 mM and 20 mM CHAPS respectively), indicating that the aggregation state of the solubilised receptor is unaffected by changes in CHAPS concentration.

3.3.5 Thermostability of the CHAPS-solubilised Receptor

The effect of heat on the binding activity of the CHAPS-solubilised receptor was investigated for possible further evidence of receptor heterogeneity. Preliminary experiments, carried out to determine a temperature at which <10% of the initial specific glucagon binding activity in CHAPS extract was present after a suitable time interval, led to the adoption of 35°C as the temperature used in all thermostability experiments involving the CHAPS-solubilised receptor.

A timecourse for the thermostability of the receptor in CHAPS extract was carried out as described in section 2.2.21.2. Figure 3.10 shows that biphasic decay of receptor binding activity was observed : a rapid loss of binding activity was seen over the first 30 minutes (22% of activity remaining), followed by a much slower decrease so that 18% of activity still remained after 60 minutes. This suggests that there are two receptor populations with differing thermostability, one accounting for ~75% of the total population, with a half-life of ~9 minutes at 35°C, and the other accounting for ~25%, with a half-life of ~128 minutes at 35°C. The inclusion of 100 μM GTP in the incubation mixture had no effect on the observed thermostability. Similar experiments using liver plasma membranes, carried out as described in section 2.2.21.1, showed monophasic decay (Figure 3.11), suggesting the presence of a homogeneous receptor population with a half-life of ~7 minutes at 50°C (it should be noted that thermostability experiments involving membranes were performed at 50°C, reflecting the greater stability of the receptor in membranes compared with the CHAPS-solubilised receptor). It is therefore possible that the results observed with CHAPS extract were not due to the thermostability of the glucagon receptor but to changes in some other constituent(s) of CHAPS extract (discussed in section 5.1.2). Since the relative proportions of the two receptor populations in CHAPS extract with differing thermostability correlate approximately with the proportions of receptor of high or low aggregation state, it was proposed that the observed biphasic decay of the receptor in CHAPS extract

might be a reflection of the aggregation state of the receptor, with the highly aggregated form(s) having a lower thermostability than the less highly aggregated forms. This hypothesis was tested by carrying out a timecourse for the thermostability of the receptor in CHAPS extract which had been spun at 105 000 *g* for 12 hours (see section 3.3.2.5); i.e. using only 'less aggregated' receptor. However, reliable assessment of the specific glucagon binding activity of this material was not possible due to a high proportion (>80%) of non-specific binding in a small amount of total binding, especially after any heat treatment.

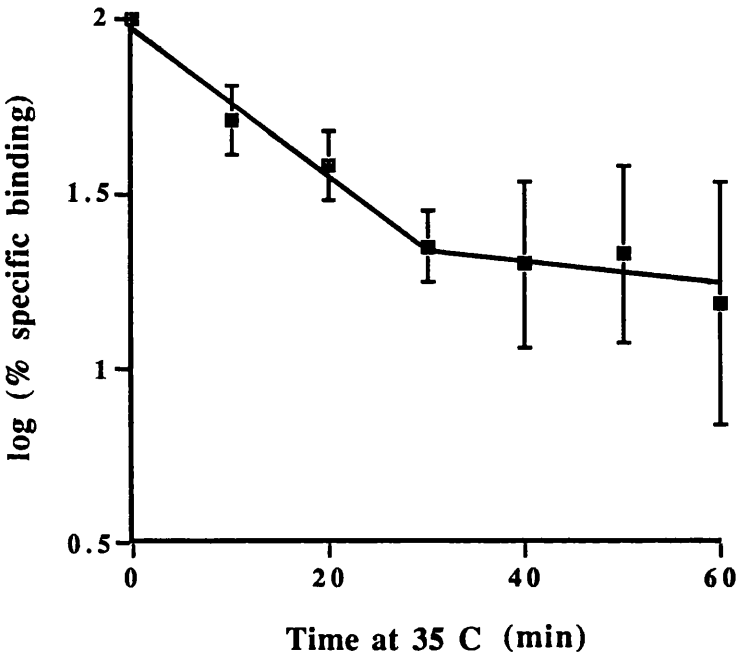


Figure 3.10 Thermostability of CHAPS-solubilised Receptor

Samples of CHAPS extract (50-100 μg protein) were incubated at 35°C for the time intervals shown and assayed for specific glucagon binding activity as described in section 2.2.14. Results are expressed as the log of the percentage of initial binding activity remaining after each time interval (n=4).

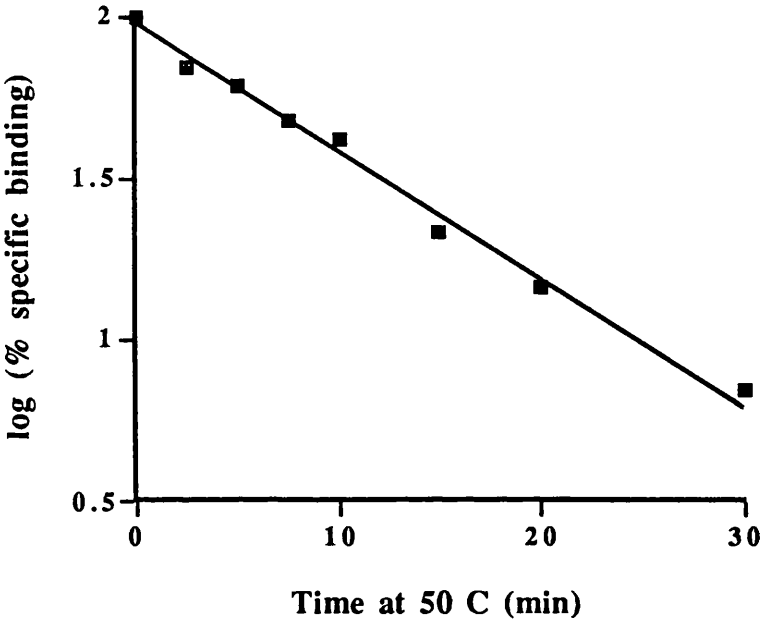


Figure 3.11 Thermostability of Membrane-bound Receptor

Liver plasma membranes were incubated at 50°C for the time intervals shown as described in section 2.2.21.1 and assayed for specific glucagon binding (2.2.14). Results are expressed as the log of the percentage of initial binding activity remaining after each time interval (n=2).

3.4 Affinity Chromatography Using Immobilised Glucagon

Immobilised glucagon (glucagon-P150) was prepared as described in section 2.2.18. Specific binding of CHAPS-solubilised receptor to glucagon-P150 was demonstrated by incubating CHAPS extract with either glucagon-P150 or bromoacetyl-P150 (the activated P150 derivative to which glucagon had not been coupled) for two hours at 4°C with constant shaking at a final CHAPS concentration of 3 mM. After centrifugation, the supernatants were assayed for specific glucagon binding activity as described in section 2.2.14. Table 3.1 shows that binding of receptor activity to glucagon-P150 is specific as 83% of activity was shown to bind to it whereas only 2% of activity was bound to the bromoacetylated derivative.

3.4.1 Determination of the Binding Capacity of Glucagon-P150

The binding capacity of the immobilised glucagon was determined as described in section 2.2.19. Figure 3.12 shows that, using 400 µl of CHAPS extract, the amount of activity bound is proportional to the packed volume of glucagon-P150 up to a volume of approximately 100 µl. At volumes above this value 100 % of activity was found to be bound. Therefore in all further experiments 25 µl (packed volume) of glucagon-P150 per 100 µl of CHAPS extract was used.

INCUBATION	SPECIFIC BINDING ACTIVITY IN SUPERNATANT (cpm)	ACTIVITY BOUND TO P150 DERIVATIVE (cpm)
CHAPS extract	13 400	---
CHAPS extract + glucagon-P150	2 300 (17%)	11 100 (83%)
CHAPS extract + bromoacetyl-P150	13 100 (98%)	300 (2%)

Table 3.1 Specific Binding of CHAPS-solubilised Receptor Activity to Glucagon-P150

CHAPS extract (500 µl) was incubated with glucagon-P150 or bromoacetyl-P150 (100 µl packed volume), or with no additions, at a final CHAPS concentration of 3 mM at 4°C for 2 hours. After centrifugation , 4 x 110 µl aliquots of the supernatants were assayed for specific glucagon binding activity as described in section 2.2.14. The figures in brackets represent the percentage of the total binding activity with which the P150 derivatives were incubated.

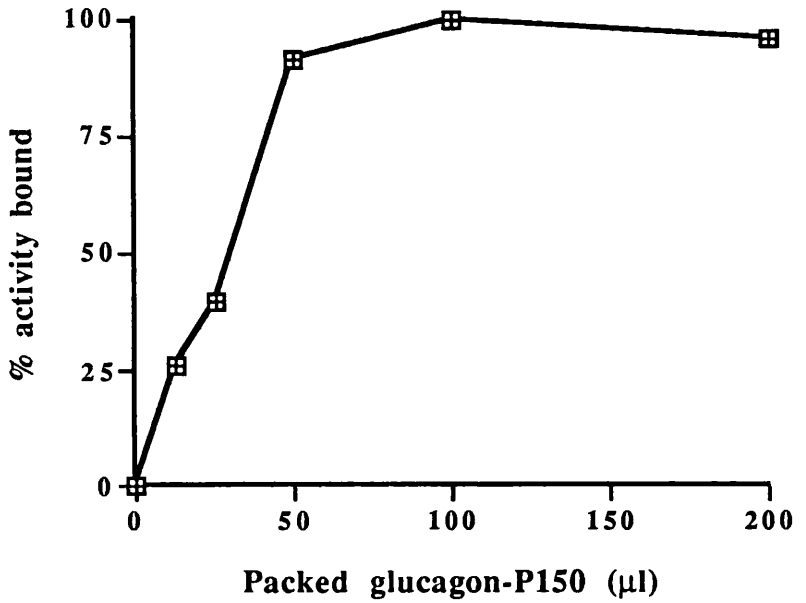


Figure 3.12 Determination of the Binding Capacity of Glucagon-P150

The binding capacity of glucagon-P150 was determined as described in section 2.2.19. Results are expressed as the percentage of activity present in 400 µl of CHAPS extract bound to glucagon-P150.

3.4.2 Elution of Active Receptor from Glucagon-P150

Sections 3.4 and 3.4.1 show that the glucagon-P150 we prepared bound the CHAPS-solubilised receptor specifically and in a manner proportional to the amount of immobilised ligand present. The experiments presented in this section describe the results of various methods which were used to elute the receptor from glucagon P-150 in an active form. In all these experiments, unless otherwise stated, the receptor was bound to glucagon-P150 and the resulting glucagon-P150-receptor complex was washed as described in section 2.2.20.1.

3.4.2.1 Elution with Glucagon

The method of choice for elution of the bound receptor from glucagon-P150 is elution with glucagon itself because of the potentially high degree of specificity afforded by this method. However, in order to be able to assay for specific glucagon binding after elution, the unlabelled glucagon in the eluate must be removed, or at least its concentration must be reduced to such a level that it does not interfere with the assay. By using a competition binding assay curve for CHAPS extract (McVittie and Gurd, 1989) it could be seen that an unlabelled glucagon concentration of 1 nM or lower would be required in order to achieve this. However, if elution was carried out using 10 μM glucagon, simple dilution of the eluate to 1 nM glucagon was obviously not feasible due to the very large scale of dilution required. Therefore it was necessary to use other methods, described below. In each case the washed glucagon-P150-receptor complex was incubated with 10 μM glucagon for one hour at 4°C with constant shaking then centrifuged at 2 000 *g* for ten minutes, after which the supernatant was removed and treated for removal of glucagon before assaying for specific glucagon binding activity.

ii) Removal of Glucagon using Wheat Germ Agglutinin - Agarose

It has been shown previously that the CHAPS-solubilised receptor can be bound reversibly to wheat germ agglutinin (Herberg et al., 1984). Therefore it was considered that incubation of glucagon-eluted material with excess WGA-agarose followed by extensive washing could be an effective way of removing glucagon from the eluate. The WGA-agarose-receptor complex could then be eluted with GlcNAc and assayed.

In order to determine the volume of wash buffer required to reduce the concentration of glucagon to below 1 nM a preliminary experiment was carried out whereby 0.5 ml (i.e. the same volume as used to elute glucagon-P150) of 10 μM unlabelled glucagon containing a small amount (~250 000 cpm) of ^{125}I-glucagon as a tracer was incubated with 100 μl of packed WGA-agarose which was then packed in a 1 ml plastic syringe and washed with varying amounts of 3 mM CHAPS buffer before elution with 0.5 M GlcNAc. Assuming that the proportion of unlabelled glucagon in the eluate was equivalent to the proportion of ^{125}I-glucagon present, it was found that a wash of at least 10 ml was required to bring the concentration of unlabelled glucagon to below 1 nM in the GlcNAc-eluted fraction and therefore this volume was routinely used in the washing step of the procedure.

Supernatants after glucagon-elution were treated with WGA-agarose as described in section 2.2.20.2. However, no specific glucagon binding activity was recovered using this method. In order to investigate possible reasons for this an experiment was carried out whereby CHAPS extract was incubated with WGA-agarose in the presence or absence of 10 μM glucagon for two hours at 4°C. The WGA-agarose was then packed in a 1 ml plastic syringe and washed and eluted as described in section 2.2.20.2. It was found that no activity was present in the eluate, regardless of whether the CHAPS extract initially contained 10 μM glucagon or not, suggesting that the receptor was either being lost during the washing step before elution, or that it was remaining bound to the WGA-agarose during the

elution step. This was investigated further by varying (i) the wash volume and (ii) the elution time. Samples of CHAPS extract were incubated with 100 µl (packed volume) of WGA-agarose at 4°C for two hours, then washing and elution were carried out as shown in Table 3.2. This table shows that

i) when a 10 ml wash was used, no receptor activity was eluted with GlcNAc whereas the use of a 1 ml wash resulted in the elution of 56% and 52% of bound activity after two and twenty hours respectively, and

ii) increasing the elution time from two to twenty hours had no effect on the amount of activity eluted by GlcNAc.

These results suggest that activity bound to WGA-agarose is lost during a 10 ml washing step. This problem could be overcome by reducing the volume of buffer used to wash the WGA-agarose; however, this was not possible since a wash of at least 10 ml was required to give a sufficiently low glucagon concentration. Therefore this method of removing glucagon after elution from glucagon was considered unfeasible.

TREATMENT	ACTIVITY RECOVERED (%)
1 ml wash, 2 hr elution	56
10 ml wash, 2 hr elution	0
1 ml wash, 20 hr elution	52
10 ml wash, 20 hr elution	0

Table 3.2 Effect of Washing Volume and Elution Time on Recovery of Active Receptor from WGA-agarose

CHAPS extract was incubated with 100 µl (packed volume) of WGA-agarose at 4°C for 2 hours. The WGA-agarose was then washed with the volume of 3 mM CHAPS buffer shown and incubated with 0.5 M GlcNAc (0.5 ml) for the time interval shown. Eluates were then assayed for specific glucagon binding activity as described in section 2.2.14. Results are expressed as the percentage of activity eluted compared with untreated CHAPS extract assayed at the appropriate time intervals.

ii) Removal of Glucagon by Dextran-coated Charcoal Treatment

This method was based on the principle of the solubilised receptor assay; i.e. utilising dextran-coated charcoal to adsorb free glucagon. Supernatants after glucagon-elution were run through 800 µl charcoal-dextran columns and assayed for specific glucagon binding activity as described in section 2.2.20.2 (i). In order to ensure that this method would reduce the concentration of glucagon to the necessary level a preliminary experiment was carried out whereby 0.5 ml (i.e. the same volume as used to elute glucagon-P150) of 10 µM unlabelled glucagon containing a small amount (400 000 cpm) of ^{125}I-glucagon as a tracer was run through a column of dextran-coated charcoal of either 200 µl or 800 µl which had previously been washed with 3 mM CHAPS buffer. The eluate was then counted for ^{125}I. Assuming that the proportion of unlabelled glucagon removed by the column was equivalent to the proportion of ^{125}I-glucagon tracer removed, it was found that a 200 µl charcoal column was effective in reducing the glucagon concentration in the eluate from 10 µM to 30 nM whereas using an 800 µl column it was possible to reduce the concentration to ~0.8 nM which was thus deemed sufficient to enable the glucagon binding assay to be performed. However, treatment of supernatants after glucagon-elution by such a method resulted in the recovery of no specific glucagon binding. At this stage it was impossible to say whether the failure to recover activity was due to the receptor not being eluted by glucagon under the conditions used or to the receptor losing activity during the elution process.

The experiments described above have shown that while it is possible that failure to detect receptor activity after elution with glucagon may have been due to loss of activity during the treatments to remove glucagon from the eluate, it is equally possible that it may have been due to failure to elute the receptor from glucagon-P150 using glucagon. This hypothesis was tested by carrying out elutions

as described in section 2.2.20.2 using 100 μM glucagon (i.e. 10x the concentration used in previous experiments) and using incubation times of one and twenty hours. Proteins in the supernatants after elution were precipitated by the chloroform/ methanol method described in section 2.2.9 and characterised by running on 10% SDS-PAGE gels (2.2.10). Using the figure quoted by McVittie and Gurd (1989) for the amount of glucagon receptor in CHAPS extract (at least 200 fmol/mg CHAPS extract protein) it was calculated that if 800 μl of CHAPS extract was used at the start of an affinity chromatography experiment, and assuming that 100% of receptor activity was bound to the glucagon-P150, there would be enough receptor present to be visualised by silver staining, even if a relatively small proportion, e.g. 20%, was eluted from the glucagon-P150 (see section 5.1.3). However, no bands were seen on silver stained gels after carrying out the procedure described above, indicating that elution of the receptor with glucagon was not achieved, even after using 100 μM glucagon and incubating for 20 hours.

3.4.2.2 Elution with Urea

The effect of urea on the specific glucagon binding activity of the CHAPS-solubilised receptor was investigated by carrying out the assay described in section 2.2.14 in the presence of 0-1 M urea. Figure 3.14 shows that specific binding decreases with increasing urea concentration so that in the presence of 1 M urea only 7% of activity remains. In order to see if this loss of activity was reversible, CHAPS extract was incubated with 2 M urea then dialysed overnight against 3 mM CHAPS buffer at 4°C. The specific glucagon binding activity was then determined. It was found that 45% (taking the activity of untreated CHAPS extract left at 4°C overnight as 100%) of activity was recovered after dialysis. Therefore an elution strategy was devised whereby the glucagon-P150-receptor complex was eluted with 2 M urea and dialysed as described in section 2.2.20.2. However, it was found that no activity was recovered in the dialysate after this procedure. Possible reasons for this are discussed in section 5.1.3.

3.4.2.3 Elution with Low pH Buffers

A final attempt to elute the receptor from glucagon-P150 in an active form was made using low pH buffers, using the procedure described in section 2.2.20.2. Care was taken to ensure that the eluate was at low pH for as little time as possible by eluting into buffer containing the appropriate volume of 1 M Tris base required to bring the pH back to 7.5. However, specific glucagon binding activity was not recovered using either pH 4 or pH 5 buffers. Possible reasons for this are discussed in section 5.1.3.

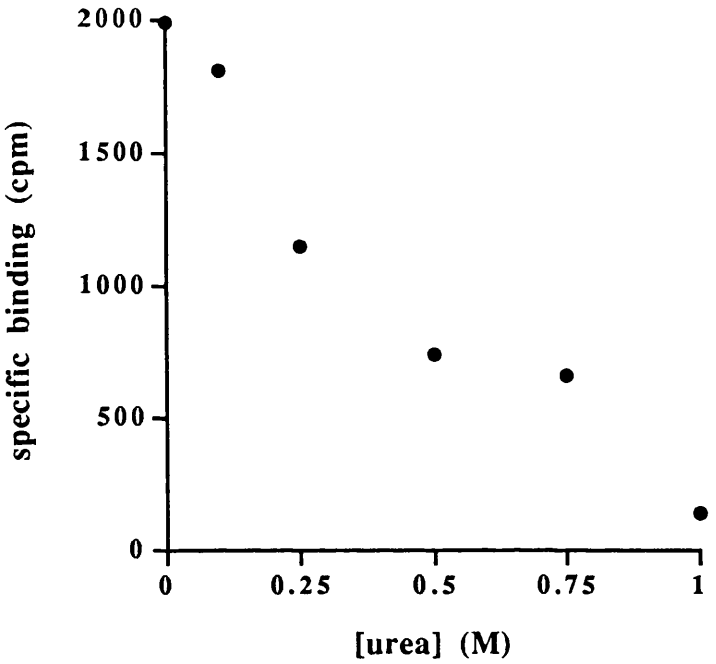


Figure 3.13 Effect of Urea on Solubilised Receptor Binding Activity

Specific glucagon binding activity in CHAPS extract was measured in the presence of the indicated concentrations of urea, using the assay described in section 2.2.14.

3.4 Elution of Receptor from Glucagon-P150 with SDS

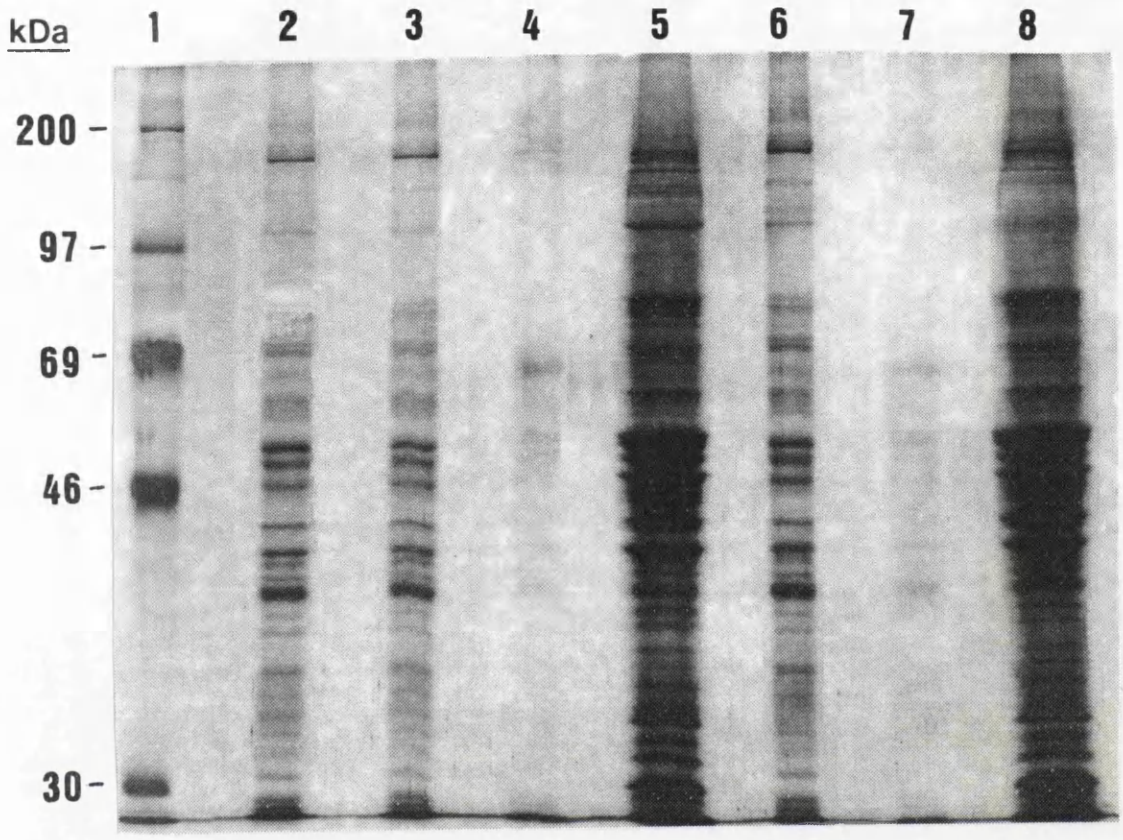
As elution of the active receptor had not been achieved using the methods described in sections 3.4.2.1-3.4.2.3 elution with SDS was carried out; obviously this would not result in the elution of active receptor but would give some indication of the specificity of binding of the receptor to glucagon-P150 and therefore an indication of the potential degree of purification afforded by this method.

CHAPS extract was incubated with glucagon-P150 and washed as described in section 2.2.20.1. As a control, CHAPS extract was incubated with bromoacetyl-P150 using a similar procedure. The P150 derivatives were then eluted with SDS as described in section 2.2.20.2. Figure 3.14 (lanes 5 and 8) shows that the SDS-eluted material from both glucagon-P150 and bromoacetyl-P150 contained the majority of proteins originally present in the CHAPS extract (these lanes are somewhat overloaded due to the unexpectedly high amount of protein present in the eluates). The inclusion of two additional washing steps, one of them with an increased NaCl concentration (1 M), before elution, had no effect on the band pattern seen on silver stained SDS-PAGE gels (Figure 3.15), and the high salt wash (lane 2) contained very little protein.

These results showed that elution with SDS was not suitable as a method for elution of the receptor from glucagon-P150 due to the simultaneous elution of non-specifically bound proteins which could not be removed by washing in high salt. Therefore, due to the unsuitability of this elution method, and of those elution methods described previously, this line of research was discontinued.

Figure 3.14 SDS-PAGE of SDS-eluted Material from Glucagon-P150 and Bromoacetyl-P150 After Incubation With CHAPS Extract

CHAPS extract (800 µl) was incubated with glucagon-P150 (200 µl) or bromoacetyl-P150 (200 µl) and centrifuged as described in section 2.2.20.1. The affinity matrix was then washed (2.2.20.1) and eluted with SDS (2.2.20.2). The samples listed opposite were run on a 10% SDS polyacrylamide gel and silver stained (2.2.10).



Lane 1: molecular weight markers

2: CHAPS extract

3: supernatant after incubation of glucagon-P150 with CHAPS extract

4: wash of glucagon-P150 after incubation with CHAPS extract

5: SDS-eluted material from glucagon-P150 after incubation with CHAPS extract and washing

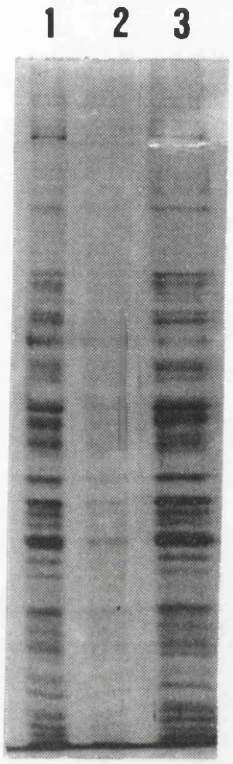
6: supernatant after incubation of bromoacetyl-P150 with CHAPS extract

7: wash of bromoacetyl-P150 after incubation with CHAPS extract

8: SDS-eluted material from bromoacetyl-P150 after incubation with CHAPS extract and washing

Figure 3.15 SDS-PAGE of SDS-eluted Material from Glucagon-P150

CHAPS extract (800 µl) was incubated with glucagon-P150 (200 µl) and centrifuged as described in section 2.2.20.1. The affinity matrix was then washed as described in section 2.2.20.1 but with the inclusion of two additional washing steps, one of them with high salt (1 M NaCl), and eluted with SDS (2.2.20.2). The samples listed opposite were run on a 10% SDS polyacrylamide gel and silver stained (2.2.10).



Lane 1: supernatant after incubation of CHAPS extract with glucagon-P150

2: high salt wash of glucagon-P150 after incubation with CHAPS extract

3: SDS-eluted material from glucagon-P150 after incubation with CHAPS extract and washing

3.5 Summary

Solubilisation of rat liver plasma membranes with CHAPS yielded glucagon receptor in an active form. Increases in CHAPS concentration above 6 mM resulted in no further increase in solubilised activity as measured by a charcoal/dextran binding assay, suggesting the possibility of conformational denaturation of the receptor at higher detergent concentrations. This aspect of receptor solubilisation, and the subsequent stability of the solubilised receptor, are discussed in section 5.1.1.

Size characterisation of the CHAPS-solubilised receptor was carried out by the combined use of sucrose density gradient centrifugation and gel filtration. These methods showed the receptor to exist predominantly in a highly aggregated form in CHAPS. This aggregation appeared to be specific and was unaffected by changes in ionic strength or CHAPS concentration. Removal of highly aggregated material allowed the identification of a 190 kDa species as the smallest species present in CHAPS and this is proposed to be a trimer of receptor subunits. This and aspects of receptor aggregation are discussed in section 5.1.2.

Thermostability studies on the CHAPS-solubilised receptor showed biphasic decay of binding activity at 35°C. This is discussed in section 5.1.2 in terms of the possibility of the existence of heterogeneous receptor populations.

Binding of CHAPS-solubilised receptor to immobilised glucagon was shown. However, elution with glucagon, urea or low pH buffers did not result in elution of active receptor, while SDS-PAGE analysis of glucagon-eluted material showed no elution of receptor even at higher glucagon concentrations and increased time of elution. Elution with SDS resulted in the elution of proteins which were presumably bound non-specifically to the immobilised glucagon. These results are discussed in section 5.1.3.

Chapter 4 Receptor Desensitisation and Phosphorylation

4.1 Introduction

As described in section 1.7, treatment of intact hepatocytes with glucagon, or other ligands which stimulate inositol phospholipid metabolism, leads to a cAMP-independent desensitisation of glucagon-stimulated adenylate cyclase activity. As this process is mimicked by the phorbol ester TPA it has been proposed that protein kinase C plays a crucial role in the mechanism of desensitisation, bringing about the phosphorylation of either the glucagon receptor or the stimulatory guanine nucleotide regulatory protein G_s, or both of these components (Murphy *et al.*, 1987). The aim of the experiments described in this chapter was to ascertain whether phosphorylation of the glucagon receptor takes place during the desensitisation process, as has been shown for other receptors.

The β-adrenergic receptor was shown to be phosphorylated during desensitisation by incubating cells in the presence of ^{32}P-labelled ATP, both before and during the desensitisation procedure, purifying the receptor and identifying it as a ^{32}P-labelled protein on SDS-PAGE (Nambi *et al.*, 1985). However, the use of a similar procedure to identify phosphorylation of the glucagon receptor during desensitisation was not possible due to the unavailability of a procedure to purify the receptor to homogeneity. Therefore the following methods were used: i) isoelectric focusing of partially purified covalently labelled ^{125}I-glucagon-receptor complex on immobilised pH gradients and agarose IEF gels, and ii) comparison of the thermostability of the receptor in membrane fractions prepared from control and desensitised hepatocytes.

4.2 Isoelectric Focusing of Glucagon Receptor

Phosphorylation of the receptor would bring about a change in its isoelectric point and hence its focused band position on isoelectric focusing gels. Therefore, if the receptor was labelled by covalent cross-linking to ^{125}I-glucagon, it would be possible to distinguish between phosphorylated and unphosphorylated receptor by running solubilised cross-linked membrane preparations on isoelectric focusing gels without the need for purification of the receptor to homogeneity. However, before this could be carried out using membrane preparations from control and desensitised cells, it was necessary to optimise both the cross-linking procedure and the isoelectric focusing of solubilised membranes.

4.2.1 Direct Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes by UV Irradiation

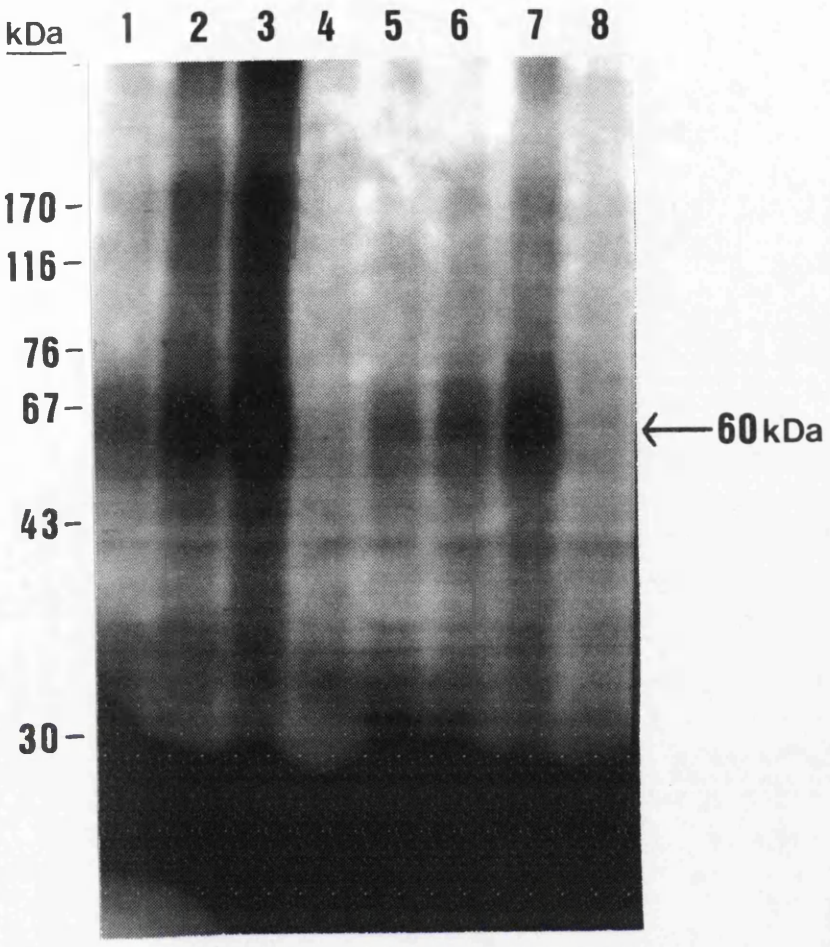
Iwanij and Hur (1985) showed that cross-linking of ^{125}I-glucagon to its receptor in plasma membranes could be achieved directly by UV irradiation, without the need for photoaffinity reagents. Using this procedure they identified a labelled glucagon-receptor complex of M_r 62 kDa. However, the amount of membrane-bound ^{125}I-glucagon incorporated into the cross-linked complex was low (0.5-1.2%). The following experiments were carried out to try to improve the incorporation by varying the UV irradiation conditions used and investigating the effect of protein and sugar additives on cross-linking.

4.2.1.1 Effect of UV Irradiation Conditions on Cross-linking

Liver plasma membranes were incubated with ^{125}I-glucagon and UV irradiated as described in section 2.2.6, carrying out the irradiation for 5, 10 and 20 minutes with the sample either 2 cm or 10 cm from the 254 nm UV source. The samples were then analysed by SDS-PAGE and autoradiography (2.2.10). Figure 4.1 shows the incorporation of label into a band of M_r ~60'000 ., correlating well with the results obtained by Iwanij and Hur (1985) using a similar procedure. The specificity of labelling of this band is shown by its abolition when binding and cross-linking were carried out in the presence of 1 μM unlabelled glucagon (lanes 4 and 8). The incorporation of label into this band was quantitated by excising the corresponding areas of the dried gel and counting them for ^{125}I. Figure 4.2 shows that at both UV source distances incorporation of label into the 60 kDa receptor band increased with increasing time of UV irradiation and that incorporation at each time point was greater with the UV source at 2 cm than at 10 cm. Figure 4.1 shows that, with the UV source at 2 cm from the sample, increasing time of irradiation led to an increase in the appearance of labelled material of higher molecular weight, including a band at ~170 kDa which may represent a trimeric aggregate of the receptor. Under these conditions, labelled material was also retained by the stacking gel (not shown). These aggregates were seen to a lesser extent with the UV source at a distance of 10 cm from the sample, even with an irradiation time of 20 minutes. Therefore, irradiation conditions adopted for use in future experiments were 20 minutes at a distance of 10 cm from the UV source; although the amount of label incorporated into the 60 kDa band was lower using this regime than with a similar time of irradiation at 2 cm from the UV source, the amount of aggregated material was less, a potentially important advantage when addressing problems associated with sample entry in isoelectric focusing gels. Using these irradiation conditions, approximately 1% of membrane-bound ^{125}I-glucagon was incorporated into the 60 kDa cross-linked receptor band.

Figure 4.1 Autoradiograph Showing The Effect of UV Irradiation Conditions on Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes

Rat liver plasma membranes (400 μg) were incubated with ^{125}I-glucagon and UV irradiated as described in section 2.2.6 under the irradiation conditions shown. The samples were then analysed by SDS-PAGE and autoradiography (2.2.10).



Irradiation Conditions (distance from UV source, time of irradiation):

Lane 1: 2 cm, 5 min.
2: 2 cm, 10 min.
3: 2 cm, 20 min.
4: 2 cm, 20 min. (+ 1 μM unlabelled glucagon)
5: 10 cm, 5 min.
6: 10 cm, 10 min.
7: 10 cm, 20 min.
8: 10 cm, 20 min. (+1 μM unlabelled glucagon)

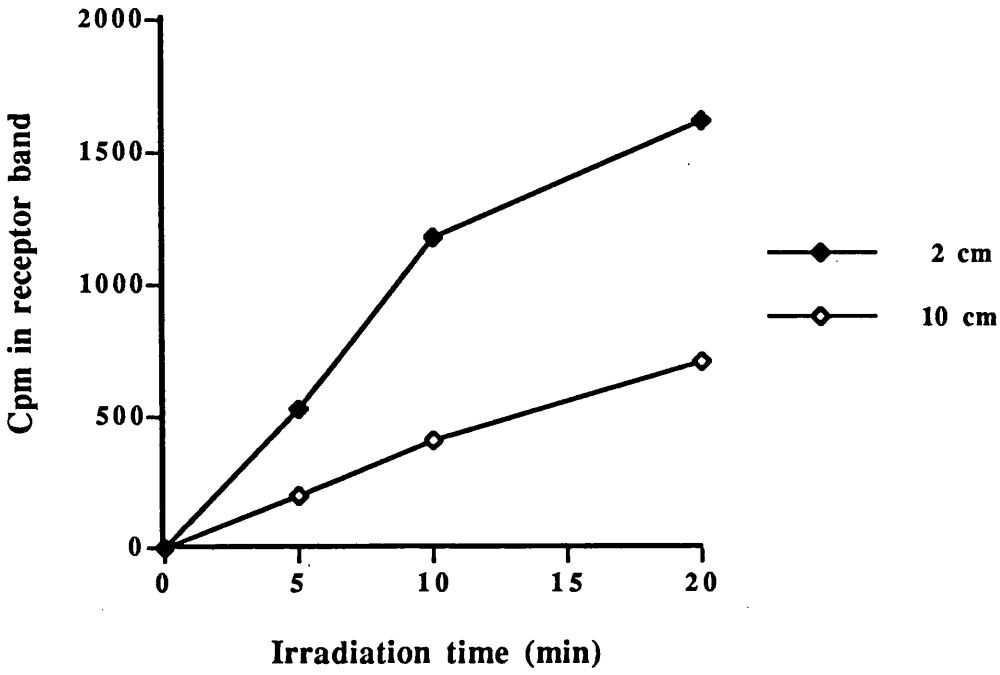


Figure 4.2 Effect of UV Irradiation Conditions on Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes

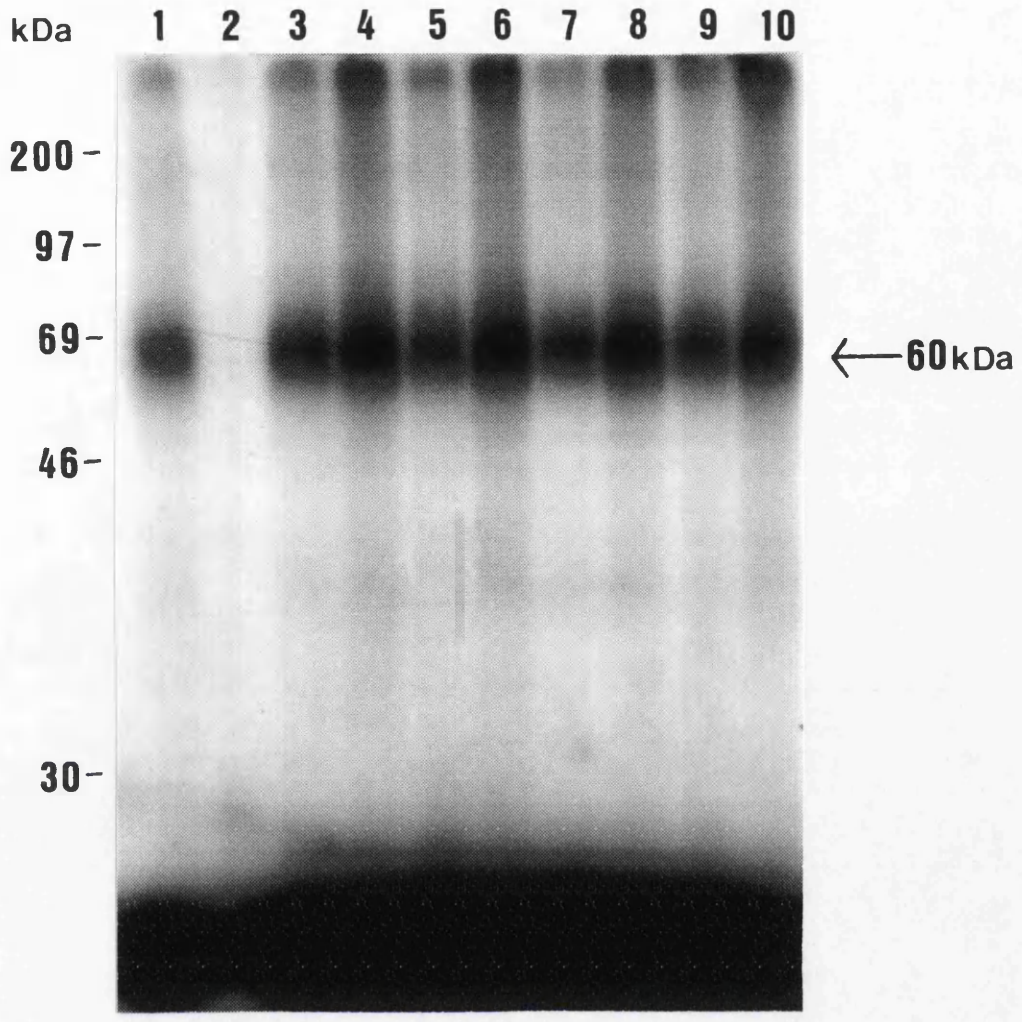
Rat liver plasma membranes (400 μg) were incubated with ^{125}I-glucagon and UV irradiated as described in section 2.2.6 under the irradiation conditions shown. The samples were then analysed by SDS-PAGE and autoradiography. The areas of the gel corresponding to the labelled 60 kDa receptor bands shown by autoradiography (Figure 4.1) were excised and counted for ^{125}I.

4.2.1.2 Effect of Proteins and Sugars on Cross-linking

Previous work carried out in this laboratory (J.G. Beeley, unpublished work) had suggested that the presence of ovalbumin or BSA during the irradiation step of the cross-linking procedure resulted in an increase in the amount of membrane-bound ^{125}I-glucagon incorporated into the cross-linked receptor complex. The effect of these two proteins plus another protein, ovomucoid, and two sugars, GlcNAc and galactose, was investigated by incubating membranes with ^{125}I-glucagon and UV irradiating essentially as described in section 2.2.6, except that samples were irradiated in the presence of ovalbumin, BSA, ovomucoid, GlcNAc and galactose or with no additions. The samples were then analysed by SDS-PAGE and autoradiography (Figure 4.3). The areas of the dried gel corresponding to the labelled 60 kDa bands were excised and counted for ^{125}I. Figure 4.4 shows that in the presence of either ovalbumin, BSA or ovomucoid at a concentration of 1 mg/ml, the incorporation of membrane-bound ^{125}I-glucagon into the cross-linked 60 kDa receptor band was almost doubled while the same proteins at a concentration of 0.1 mg/ml resulted in increases in incorporation of approximately 20%. The presence of 0.2 M galactose or 0.2 M GlcNAc resulted in increases in incorporation of approximately 80% and 35% respectively. Determination of glucagon-binding prior to irradiation showed comparable levels of membrane-bound glucagon in all samples, therefore ensuring that the increases in incorporation seen were not due to differences in the amount of glucagon bound to the membranes before irradiation. Possible reasons for these results are discussed in section 5.2.1. However, since the principal aim of this experiment was to increase the amount of ^{125}I-glucagon incorporated into the cross-linked 60 kDa receptor band, this line of investigation was not carried any further. All future cross-linking experiments were carried out using 1 mg/ml ovalbumin in the irradiation step. Under these conditions approximately 2% of membrane-bound ^{125}I-glucagon was incorporated into the 60 kDa cross-linked receptor band.

Figure 4.3 Autoradiograph Showing the Effect of Proteins and Sugars on the Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes

Liver plasma membranes (400 μg) were incubated with ^{125}I-glucagon and UV irradiated as described in section 2.2.6 in the presence or absence of the proteins and sugars shown. The samples were then analysed by SDS-PAGE and autoradiography (2.2.10).



Addition during UV irradiation:

Lane 1: no addition
2: " " (1 μM unlabelled glucagon in binding step)
3: BSA (0.1 mg/ml)
4: BSA (1 mg/ml)
5: ovalbumin (0.1 mg/ml)
6: ovalbumin (1 mg/ml)
7: ovomucoid (0.1 mg/ml)
8: ovomucoid (1 mg/ml)
9: GlcNAc (0.2 M)
10: Gal (0.2 M)

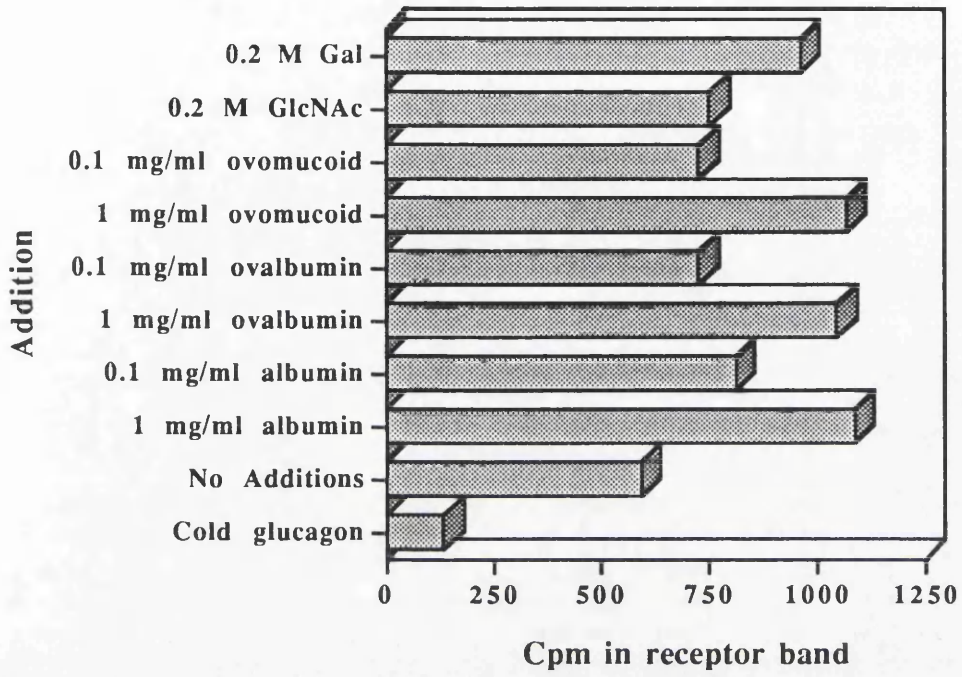


Figure 4.4 Effect of Proteins and Sugars on Cross-linking of ^{125}I-glucagon to Liver Plasma Membranes

Liver plasma membranes (400 µg) were incubated with ^{125}I-glucagon and UV irradiated as described in section 2.2.6 in the presence or absence of the proteins and sugars shown. The samples were then analysed by SDS-PAGE and autoradiography (2.2.10) and the areas of the gel corresponding to the 60 kDa receptor bands shown by autoradiography (Figure 4.3) were excised and counted for ^{125}I.

4.2.2 Partial Purification of Cross-linked Receptor By WGA-agarose Chromatography

Before application onto isoelectric focusing gels, cross-linked membrane samples were chromatographed on WGA-agarose. This was done not only to partially purify the covalently-labelled receptor but also to minimise interference from free ^{125}I-glucagon on isoelectric focusing gels by removing non-covalently bound hormone from the sample (further removal was achieved in subsequent concentration or precipitation steps).

Cross-linked membrane samples were solubilised in 1% Lubrol-PX as described in section 2.2.7. Solubilised samples were then run on 1 ml WGA-agarose columns as described in section 2.2.8. A typical elution profile (Figure 4.5) shows that most of the counts, representing free ^{125}I-glucagon, were eluted in the flowthrough while cross-linked glucagon-receptor complexes eluted as a small peak after the introduction of 0.5 M GlcNAc to the elution buffer. These peak fractions were pooled and the proteins present were precipitated as described in section 2.2.9 and analysed by SDS-PAGE and autoradiography (2.2.10). Figure 4.6 shows the presence of a labelled band of approximately 60 kDa, confirming that the labelled GlcNAc-eluted material represented the covalently labelled glucagon receptor. The absence of free ^{125}I-glucagon in this lane demonstrates the effectivity of WGA-agarose chromatography followed by chloroform/methanol precipitation as a method for the removal of non-covalently bound labelled glucagon from the sample. When the flowthrough fractions shown in Figure 4.6 were pooled, precipitated and analysed by SDS-PAGE and autoradiography, no covalently labelled material was seen, indicating only one receptor population.

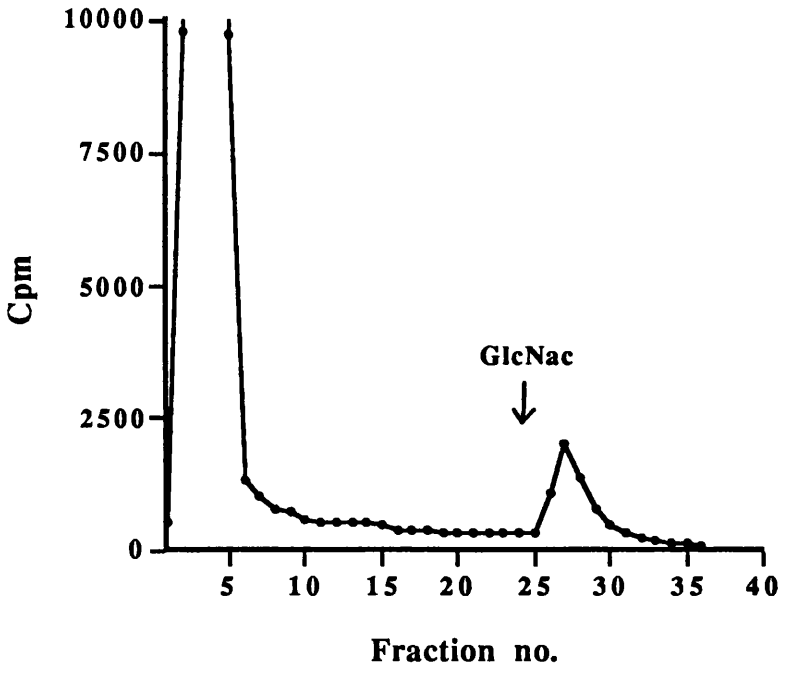


Figure 4.5 WGA-agarose Chromatography of Covalently Labelled Receptor

Covalently labelled liver plasma membranes (400 μg) were solubilised in 1% (w/v) Lubrol-PX (2.2.7) and run on a 1 ml WGA-agarose column as described in section 2.2.8. A typical elution profile is shown. The vertical arrow denotes the point at which the column was eluted with 0.5 M GlcNAc.

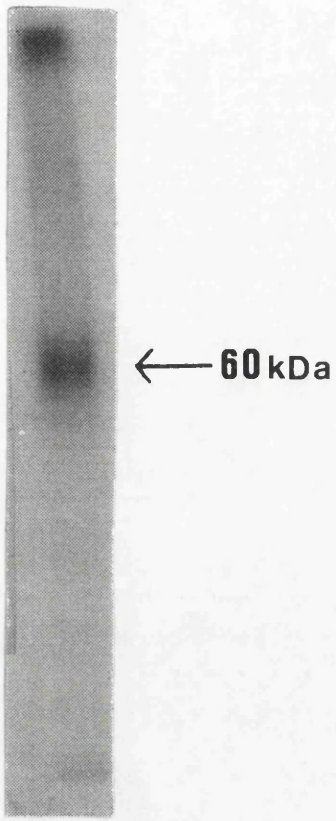


Figure 4.6 SDS-PAGE of Covalently Labelled Receptor Following WGA-agarose Chromatography

Covalently labelled liver plasma membranes (400 μg) were solubilised in 1% (w/v) Lubrol-PX (2.2.7) and run on a 1 ml WGA-agarose column as described in section 2.2.8. The GlcNAc-eluted fractions containing labelled material (Figure 4.5) were pooled and precipitated as described in section 2.2.9 then analysed by SDS-PAGE and autoradiography (2.2.10).

4.2.3 Isoelectric Focusing on Immobilised pH Gradients

Immobilised pH gradients (IPGs) offer considerable advantages over traditional carrier ampholyte (CA) gels for isoelectric focusing in that the pH gradient is stable with time, due to the covalent anchoring of the pH gradient to a polymer matrix, resulting in greatly improved reproducibility of IEF patterns. For isoelectric focusing of covalently cross-linked glucagon receptor, pH 4-7 IPGs were used. These were obtained in ready-made dry form from Pharmacia-LKB and allow entry of molecules with molecular weights up to 500 kDa. Although work described earlier in this thesis (3.3) has shown that the glucagon receptor exists in CHAPS predominantly in a highly aggregated form with a molecular weight of greater than 2 x 10^6 Da, Herberg *et al.* (1984) reported a molecular weight of 119 kDa for the Lubrol-solubilised covalently-labelled receptor, well below the cut-off point for entry into the IPGs used.

4.2.3.1 Optimisation of Isoelectric Focusing of Solubilised Plasma Membranes on pH 4-7 IPGs

Initial experiments were carried out to optimise the procedure by improving sample entry and band resolution. These experiments were done using untreated plasma membranes which were solubilised with Lubrol-PX in the same way as described for cross-linked membranes (2.2.7). Solubilised samples were then concentrated to ~50 µl and urea and Ampholine pH 3.5-10 were added to final concentrations of 6 M and 2% (w/v) respectively. Samples (10 µl) were applied to pH 4-7 IPG strips which had been rehydrated as described in section 2.2.11.1, using 6 M urea, 20% (w/v) glycerol, 0.5% (w/v) Ampholine and 2% (w/v) Lubrol-PX. Focusing was initially carried out at low voltage (300-500 V for 1 hour) as this has been shown to improve sample entry (Görg *et al.*, 1988). Thereafter the voltage was increased to 3000 V and focusing was continued as described in section

2.2.11.3. After focusing, the gel strips were silver stained (2.2.11.5). Stained gels routinely showed some unfocused material at and around the point of sample application, with resolution of some bands away from this point over the rest of the length of the gel strip (Figure 4.7). This indicated that while some of the material in the sample was able to enter the gel and focus, much of the material was either unable to enter the gel or, once entered, precipitated at or near the point of entry.

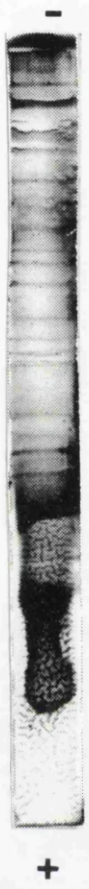


Figure 4.7 Isoelectric Focusing of Liver Plasma Membranes on a pH 4-7 IPG

A sample of Lubrol-solubilised liver plasma membranes (2.2.11.2) containing 6 M urea and 2% (w/v) Ampholine pH 3.5-10 (10 µl containing ~10 µg protein) was run (2.2.11.3) on a pH 4-7 IPG which had been rehydrated as described in section 2.2.11.1 using 8 M urea containing 0.5% (w/v) Ampholine pH 3.5-10, 20% (w/v) glycerol and 2% (w/v) Lubrol-PX. After focusing, the gel was silver stained using the procedure described in section 2.2.11.5.

Extensive studies were undertaken to try to increase the proportion of sample entering the gel and focusing into clearly resolved bands. However, it was found that no improvement was made by increasing the concentration of Ampholine to 4% (w/v) in either the rehydration solution or the sample, contrary to the findings of Rimpilainen and Righetti (1985), or by replacing Lubrol-PX with NP-40. Similarly, it was found that neither the position nor the method of sample application had an effect on focusing. Hence no significant improvement in sample entry and band resolution could be made on that shown in Figure 4.7. Nevertheless, samples of partially purified covalently labelled glucagon receptor were run on IPGs as described below.

4.2.3.2 Isoelectric Focusing of Covalently Labelled Glucagon Receptor on pH 4-7 IPGs

IPGs were rehydrated as described in section 2.2.11.1 using 6 M urea, 20% (w/v) glycerol, 4 % (w/v) Ampholine pH 3.5-10 and 2% (w/v) Lubrol-PX. Samples of partially purified covalently labelled receptor (4.2.2) were prepared for focusing as described in section 2.2.11.2 with urea and Ampholine at final concentrations of 6 M and 4% (w/v). ^{125}I-glucagon was prepared for focusing by taking up in 25 mM HEPES, pH 8.0, containing 2% (w/v) Lubrol-PX, 6 M urea and 4% (w/v) Ampholine pH 3.5-10. Samples (10 µl) were applied on sample application pieces which had been placed on the IPG strips approximately 2 cm from the anode. Focusing was then carried out as described in section 2.2.11.3. After focusing, the strips were air-dried and subjected to autoradiography. Figure 4.8 shows that the sample containing ^{125}I-glucagon focused into three distinct bands well away from the sample application point whereas the sample containing covalently labelled receptor resulted in all labelled material remaining at the point of sample application. The difference in the behaviour of these two samples indicates that the failure of covalently labelled receptor to enter the gel and focus may have

been due to the presence of receptor aggregates and these aggregates hindering the entry of less aggregated species into the gel, as discussed in section 5.2.1. Therefore the use of IPGs was discontinued and focusing was attempted using agarose gels.

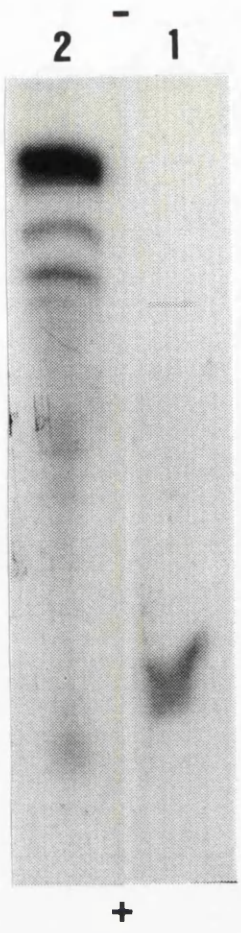


Figure 4.8 Isoelectric Focusing of Covalently Labelled Receptor on a pH 4-7 IPG

IPGs were rehydrated as described in section 2.2.11.1 using 6 M urea, 20% (w/v) glycerol, 4 % (w/v) Ampholine pH 3.5-10 and 2% (w/v) Lubrol-PX. The following samples were then applied to the strips:

Lane 1 - Partially purified covalently labelled glucagon receptor, i.e. GlcNAc-eluted fractions from WGA-agarose chromatography (section 4.2.2) prepared as described in section 2.2.11.2.

Lane 2 - ^{125}I-glucagon in 25 mM HEPES, pH 8.0, containing 1% (w/v) Lubrol-PX

Both samples contained 6 M urea and 4% (w/v) Ampholine pH 3.5-10. Focusing was carried out as described in section 2.2.11.3. After focusing, the strips were air-dried and subjected to autoradiography.

4.2.4 Isoelectric Focusing on Agarose IEF Gels

It was shown in section 4.2.3.2 that while focusing of some components of samples of Lubrol-solubilised plasma membranes on pH 4-7 IPGs was achieved, focusing of covalently labelled glucagon receptor was unsuccessful, probably due to problems with sample entry. Therefore, focusing was carried out on agarose gels. These gels have a larger pore size than the IPGs used and so will facilitate the entry of larger molecules.

4.2.4.1 Optimisation of Isoelectric Focusing of Solubilised Plasma Membranes on Agarose IEF Gels

Initial experiments were carried out using 1% (w/v) agarose gels containing 3% (w/v) Ampholine pH 3.5-10 and 2% (v/v) Lubrol-PX. However, when samples of Lubrol-solubilised untreated membranes were applied to these gels and focusing was carried out, it was found that all stained material remained at the site of sample application, regardless of the position on the gel. Therefore, gels containing urea were prepared using a modification of the method of Olsson and Låås (1981) as described in section 2.2.12.1. Due to the greatly reduced mechanical stability of agarose gels prepared in the presence of urea, it was necessary to increase the agarose concentration to 2% (w/v). The addition of sorbitol to the gel solution also provided the gel with greater stability. However, although Olsson and Låås (1981) reported the use of 2% agarose gels containing 8 M urea, it was found that using urea concentrations above 6 M led to the gel breaking up upon opening the casting mould. Therefore, all gels contained 6 M urea.

Samples of Lubrol-solubilised plasma membranes were prepared as described in section 2.2.12.2, using final concentrations of urea and Ampholine pH 3.5-10 of 6 M and 2% (w/v) respectively, and run on a 2% (w/v) agarose IEF gel

which had been prepared as described in section 2.2.12.1. Samples were applied to the gel on sample application pieces which had been placed on the gel at different positions. Focusing was carried out as described in section 2.2.12.3 and the gel was Coomassie stained (2.2.12.4). Figure 4.9 shows that focusing of components into distinct bands was only achieved when the sample was applied near the anode. Application at other positions resulted in most stained material remaining at or near the anodic side of the application point while material which did enter the gel failed to focus, with considerable streaking of material toward the anode. However, even when the sample was applied near the anode there was some stained material remaining at the anodic side of the application point and band resolution in the area immediately adjacent to the cathodic side was poor. It was considered that possible contamination of membranes with ribosomes (Evans, 1978) might lead to the presence of RNA-protein aggregates after solubilisation which could migrate to the anodic side of the sample application piece but fail to enter the gel, thus giving rise to the patterns shown in Figure 4.9. However, treatment of membranes with RNAase at concentrations of 10 μg/ml and 50 μg/ml had no effect on the electrofocusing patterns observed.

Attempts were made to reduce the amount of material remaining at the sample application point and to increase the resolution of the focused bands. The addition of SDS to the sample, as described by Schmidt-Ullrich and Wallach (1977), or the solubilisation of membranes with NP-40 or CHAPS instead of Lubrol-PX afforded no improvement. The effects of increasing the concentration of Ampholine pH 3.5-10 in the sample to 6% (w/v), and the effects of solubilising membranes in the presence of 6% (w/v) Ampholine or 6 M urea and 6% (w/v) Ampholine are shown in Figure 4.10. This shows that the addition of 6% (w/v) Ampholine to the sample after solubilisation or the inclusion of 6% (w/v) Ampholine in the solubilisation buffer resulted in a decrease in the amount of material remaining at the application point and better band resolution with less streaking. However, surprisingly, the inclusion of 6 M urea in the solubilisation

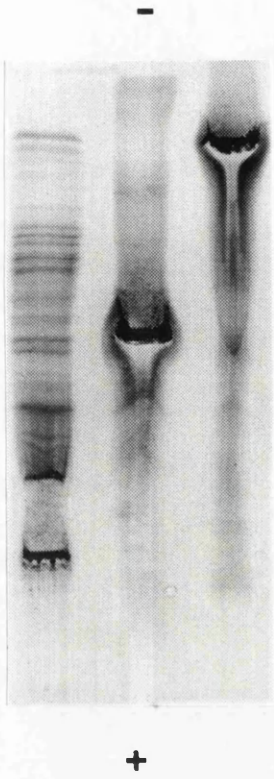
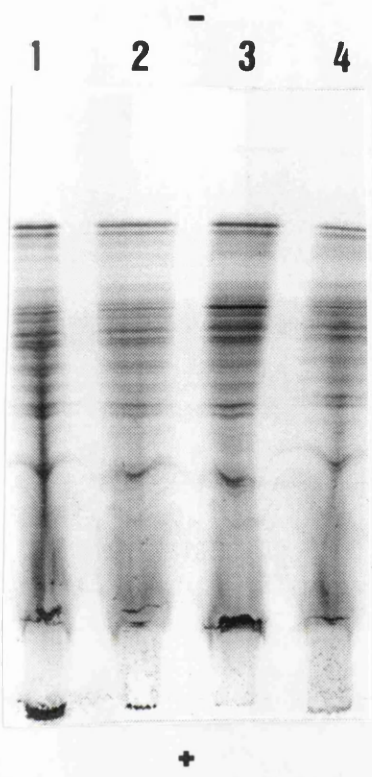


Figure 4.9 Effect of Sample Application Position on Isoelectric Focusing of Liver Plasma Membranes on Agarose IEF Gels

Lubrol-solubilised (2.2.7) liver plasma membranes containing 6 M urea and 2% (w/v) Ampholine pH 3.5-10 (20 µl containing ~50 µg protein) were applied at three different positions on a 2% (w/v) agarose IEF gel which had been prepared as described in section 2.2.12.1. Focusing was carried out as described in section 2.2.12.3 and the focused gels were Coomassie stained (2.2.12.4).

Figure 4.10 Effect of Ampholine and Urea on Isoelectric Focusing of Liver Plasma Membranes on Agarose IEF Gels

Liver plasma membranes were solubilised as described in section 2.2.7 in the presence or absence of 6% (w/v) Ampholine pH 3.5-10 and 6 M urea as indicated. All samples contained urea at a final concentration of 6 M and Ampholine at a final concentration of 2% (w/v) or 6% (w/v) as indicated. Samples (20 µl containing ~50 µg protein) were applied ~1 cm from the anode of a 2% (w/v) agarose IEF gel which had been prepared as described in section 2.2.12.1 and focusing was carried out as described in section 2.2.12.3. After focusing, the gel was Coomassie stained (2.2.12.4).



Lane 1: membranes solubilised in 1% (w/v) Lubrol-PX, Ampholine added to 2% (w/v)

2: membranes solubilised in 1% (w/v) Lubrol-PX, Ampholine added to 6% (w/v)

3: membranes solubilised in 1% (w/v) Lubrol-PX , 6% (w/v) Ampholine

4: membranes solubilised in 1% (w/v) Lubrol-PX, 6% (w/v) Ampholine, 6 M urea

buffer as well as 6% (w/v) Ampholine led to no further improvement in band resolution. As a result of this experiment, all further samples for electrofocusing were prepared with 6% (w/v) Ampholine included in the solubilisation buffer.

In order to ensure that UV treatment of membranes did not result in an increase in aggregation of the membrane proteins and hence affect their behaviour on isoelectric focusing gels, a sample of membranes was irradiated using a 254 nm UV source at a distance of 10 cm from the sample for 20 minutes (i.e. the conditions used in the procedure for cross-linking ^{125}I-glucagon to membranes) then the sample was solubilised as described in section 2.2.12.2 in the presence of 6% (w/v) Ampholine pH 3.5-10 and run on a 2% (w/v) agarose IEF gel as described in section 2.2.12.3. Figure 4.11 shows that UV treatment had no effect on the isoelectric focusing of the membrane components.

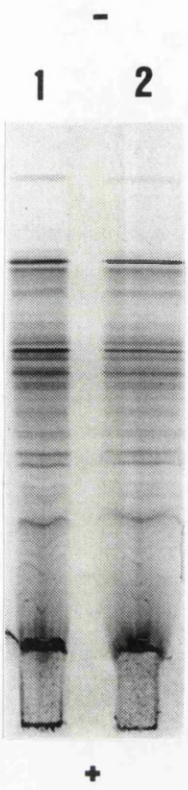


Figure 4.11 Effect of UV Irradiation of Liver Plasma Membranes on Their Isoelectric Focusing on Agarose IEF Gels

Liver plasma membranes (400 µg) were irradiated using a 254 nm UV source at a distance of 10 cm from the sample for 20 minutes then solubilised as described in section 2.2.12.2 in the presence of 6% (w/v) Ampholine pH 3.5-10 and run on a 2% (w/v) agarose IEF gel as described in section 2.2.12.3, applying samples ~1 cm from the anode. After focusing, the gel was Coomassie stained (2.2.12.4).

Lane 1: untreated membranes

2: UV irradiated membranes

4.2.4.2 Isoelectric Focusing of Covalently Labelled Glucagon Receptor on Agarose IEF Gels

Following optimisation of the procedure as described in section 4.2.4.1, samples of covalently labelled glucagon receptor were run on agarose IEF gels. 2% (w/v) agarose gels were prepared as described in section 2.2.12.1. Samples of partially purified covalently labelled receptor (4.2.2) were prepared for focusing as described in section 2.2.12.2, solubilisation being carried out in the presence of 6% (w/v) Ampholine pH 3.5-10. Focusing was then carried out as described in section 2.2.12.3, applying samples approximately 1 cm from the anode. After focusing, the gels were air-dried and subjected to autoradiography. However, as with IPGs (4.2.3.2), it was found that all labelled material in samples of covalently labelled receptor remained at the sample application point. This was again probably due to the presence of receptor aggregates failing to enter the gel and these aggregates hindering the entry of less aggregated species into the gel. Aggregation of the receptor had been observed previously in this study in different detergents (sections 3.3.2, 4.2.1.1, 4.2.3) and is discussed further in section 5.2.1).

Due to the inability to obtain focused bands representing covalently labelled receptor on either IPGs or agarose IEF gels, the use of this method as a means of detection of phosphorylated receptor was discontinued.

4.3 Thermostability of Receptor in Control and Desensitised Membranes

A second approach used to investigate possible phosphorylation of the glucagon receptor during desensitisation of glucagon stimulated adenylate cyclase activity was to look at the thermostability of the receptor in control and desensitised hepatocyte membranes. The hypothesis to be tested was that phosphorylation would lead to a change in the thermostability of the receptor and therefore if a difference in the thermostability was observed, it would then be possible to indirectly demonstrate phosphorylation of the receptor by treating desensitised membranes with phosphatases and seeing a change back to the thermostability observed in control membranes. Similarly, control membranes could be treated with protein kinase C and the receptor thermostability compared with that in desensitised membranes.

Isolated hepatocytes from fed male Sprague-Dawley rats were prepared by Dr. A. Savage according to the method of Berry and Friend (1969). Desensitisation of glucagon-stimulated adenylate cyclase activity in intact hepatocytes was carried out with 10 nM vasopressin using the method of Murphy *et al.* (1987), again by Dr. A. Savage. Initially, washed crude membrane fractions were prepared from both control (untreated) and desensitised hepatocytes using the method of Houslay and Elliott (1979). However, reliable assessment of the thermostability of the glucagon receptor in these membrane preparations, using the procedure described in section 2.2.21.2, was not possible due to a high degree of non-specific binding which resulted in specific binding forming only ~20% of total binding. Therefore, purification of plasma membranes from both control (untreated) and desensitised hepatocytes was carried out as described in section 2.2.2. Due to the time involved, these preparations were carried out in the presence of a "phosphatase inhibitor cocktail" of 0.1 μM okadaic acid, 30 mM sodium pyrophosphate, 10 mM EDTA and 1 mM phosphoserine. Adenylate cyclase assays (Houslay *et al.*, 1976), using

the cyclic AMP-binding subunit of protein kinase prepared from bovine adrenal gland (Brown *et al.*, 1972) to assay the cyclic AMP produced, on plasma membrane preparations which had been stored at -80°C demonstrated that the membranes from vasopressin-treated cells showed 70% of the glucagon-stimulated adenylate cyclase activity of membranes from control cells, this being shown by statistical analysis to be a significant difference. Therefore it was demonstrated that, after preparation and storage, membranes from vasopressin-treated cells still showed desensitisation of glucagon-stimulated adenylate cyclase activity compared with membranes from control cells.

Preliminary experiments were carried out using plasma membrane preparations from control cells to determine a temperature at which ~10% of the initial specific glucagon binding activity of the membranes was present after a suitable time interval. This led to the adoption of 50°C as the temperature used in subsequent experiments. The thermostability of the receptor in these preparations was similar to that observed in plasma membrane preparations prepared from whole liver (3.3.5). Timecourses for the thermostability of the receptor in control membranes were carried out and desensitised membranes were carried out as described in section 2.2.21.2. Figure 4.12 shows that there was found to be no difference in the thermostability of the receptor from the two types of membrane, each showing a half-life of ~8 minutes at 50°C. This indicates that either i) the receptor is not phosphorylated during desensitisation or ii) the receptor is phosphorylated but that this does not bring about a change in the thermostability of the receptor. This is discussed in section 5.2.2. As a result, further experiments using phosphatases and kinases were not carried out. Therefore confirmation of receptor phosphorylation awaits purification of the receptor (see section 5.3).

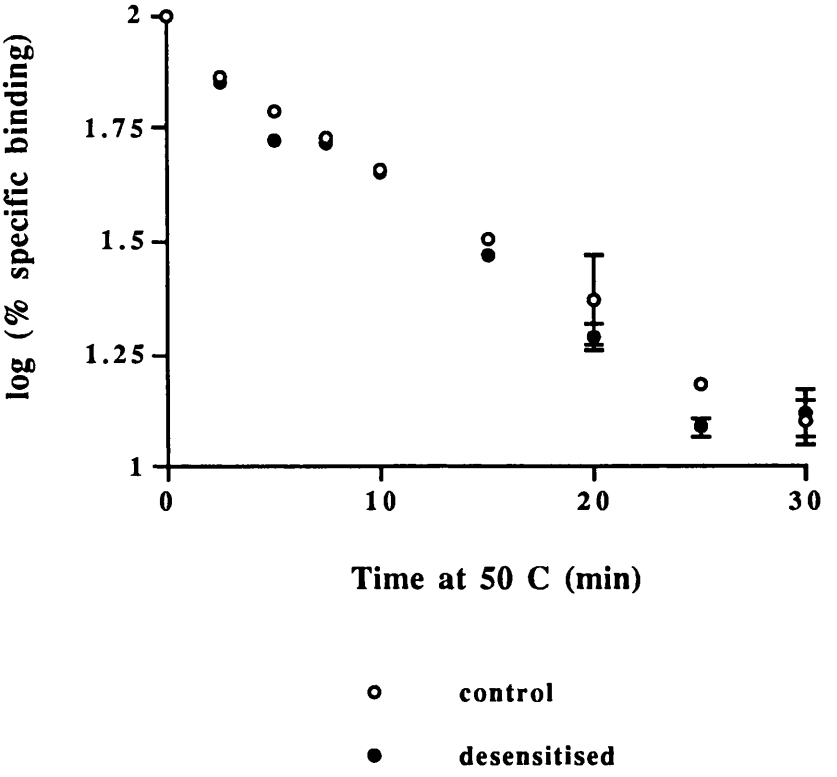


Figure 4.12 Thermostability of Receptor in Control and Desensitised Membranes

Plasma membranes were prepared as described in section 2.2.2. Samples of membranes (10 μg protein) were incubated at 50°C for the time intervals shown as described in section 2.2.21.1 and assayed for specific glucagon binding (2.2.14). Results are expressed as the log of the percentage of initial binding activity remaining after each time interval (n=3).

4.4 Summary

Direct cross-linking by UV irradiation of ^{125}I-glucagon to rat liver plasma membranes resulted in the identification of a specifically labelled peptide of M_r ~60 kDa. Incorporation of bound label into cross-linked material was shown to be increased in the presence of proteins and sugars and is discussed in section 5.2.1.

Isoelectric focusing of partially purified samples of cross-linked receptor on both immobilised pH gradients and IEF agarose gels resulted in labelled material remaining at the site of sample application, despite optimisation of both procedures to give good focused band patterns for solubilised membranes. The problems encountered in isoelectric focusing of covalently labelled receptor are discussed in section 5.2.1 in terms of receptor aggregation.

The thermostability of the receptor was shown to be the same in plasma membrane preparations from both control and desensitised cells. This result indicates that either (i) the receptor is not phosphorylated during desensitisation or (ii) the receptor is phosphorylated but that this does not result in a change in the thermostability of the receptor. This is discussed in section 5.2.2.

Chapter 5 Discussion

In this chapter an overall analysis of the results presented in Chapters three and four will be given and discussed in the light of previous and current work in the relevant areas.

5.1 The CHAPS-solubilised Receptor

5.1.1 Receptor Solubilisation and Stability

Solubilisation of integral membrane proteins by detergents does not always simply follow the disruption of the lipid bilayer (for review see Silvius, 1992). Differential solubilisation of membrane proteins can arise as a result of different overall free energies of formation for the micelles into which they become incorporated, differing ease with which detergent molecules can compete with endogenous lipid molecules for solvation of the protein-lipid interface, and changes in protein-protein interactions which may influence the overall free energy of solubilisation.

Previous attempts to solubilise the glucagon receptor with detergents have usually resulted in a loss of activity, the exception being when the zwitterionic detergent CHAPS was used (Herberg *et al.*, 1984). Solubilisation of active receptor increased greatly between 3 and 6 mM CHAPS, probably reflecting the fact that over this range of concentrations the critical micelle concentration of CHAPS was reached. However, further increasing the CHAPS concentration above 6 mM resulted in relatively small changes in the solubilisation of active receptor even though the total protein solubilised increased steadily over this range. Since the recovery of specific binding activity after solubilisation with 6 mM CHAPS was only approximately 30 % of that in membranes, the failure to see increased activity with CHAPS concentrations above 6 mM could not be due to complete solubilisation of active receptor at these concentrations. It is possible that at CHAPS concentrations above 6 mM there was no further solubilisation of receptor.

However, it is also possible that further solubilisation of the receptor did occur at higher CHAPS concentrations but that the receptor solubilised at these concentrations lost activity, a proportion of which was not recovered on dilution of the CHAPS to 3 mM for assay. Indeed, assays of liver plasma membranes solubilised at CHAPS concentrations of 6 mM and above, without dilution to 3 mM CHAPS prior to assay, showed total abolition of specific glucagon binding activity. This suggests that at CHAPS concentrations at or above 6 mM conformational denaturation of the receptor occurs which is partially reversible upon dilution of CHAPS to 3 mM, although this result could also be due to the failure of the solubilised receptor assay at CHAPS concentrations above 3 mM. The ratio of detergent to lipid in the protein-containing micelle is often critical (Lund *et al.*, 1989) and in the case of the glucagon receptor it is likely that increasing CHAPS concentration leads to progressive removal of residual lipid from the protein and subsequent inactivation. That reversible changes in receptor conformation occur upon the addition of CHAPS is supported by the observation of McVittie and Gurd (1989) that previously solubilised inactive receptors can be activated by the addition of Hanks' balanced salt solution. The observation of reduced thermostability of the CHAPS-solubilised receptor compared with that of the membrane-bound receptor (see section 5.1.2) provides further evidence of conformational changes in the receptor upon solubilisation. Another possibility is that at CHAPS concentrations of 6 mM and above, dissociation of G_s from the receptor occurs, thus reducing the receptor binding affinity, and that upon dilution of CHAPS to 3 mM, "reconstitution" of G_s with the receptor takes place, thus restoring higher affinity binding. However, if this was the case, a reduction in binding similar to that given by the GTP-induced dissociation of G_s in membranes (50-60%) might be expected, whereas total abolition of binding was in fact observed. Also, solubilised receptor preparations assayed at 3mM CHAPS showed no sensitivity to guanine nucleotides, indicating the absence of functional interaction of the receptor with G_s, and thus precluding the possibility of "reconstitution" of dissociated G protein upon

dilution of CHAPS. The question of interaction of G_s with the CHAPS-solubilised receptor is addressed in greater detail in section 5.1.2. The failure to observe specific glucagon binding upon assay at higher concentrations of CHAPS could also reflect the loss of required conformational features of glucagon itself at these concentrations.

The instability of the CHAPS-solubilised receptor during storage provides further evidence that receptor-micelle interactions provided by CHAPS do not replace the receptor-membrane interactions required for stable binding activity (McVittie and Gurd, 1989). Perturbations of the conformational stability of membrane proteins by detergents can arise as a result of changes in the environmental fluidity upon incorporation of the protein into micelles (Milder *et al.*, 1991), binding of detergent to the protein at sites not normally occupied by lipid (Simmonds *et al.*, 1982) or displacement from the protein surface of lipids needed to stabilise the protein structure (Robinson *et al.*, 1980). Changes in protein-protein interactions may also lead to instability after solubilisation. However, it was shown (section 3.2.2) that the observed instability of the receptor in CHAPS extract is due in part to the action of endogenous proteases. The small increase in stability brought about by the removal of calcium from the solubilisation buffer suggests that the solubilised receptor may be a target for calcium-dependent and/or calcium-stabilised proteases. The addition of PMSF to a mixture of protease inhibitors used by McVittie and Gurd (1989) resulted in almost a halving of the activity lost after storage for 24 hours at 4°C, suggesting significant action of serine proteases on the receptor in CHAPS extract. It is possible that solubilisation of the receptor by CHAPS increases the likelihood of proteolysis by exposing proteolytically sensitive regions of its structure.

5.1.2 Characterisation of the CHAPS-solubilised Receptor

Characterisation of the CHAPS-solubilised receptor by sucrose density gradient centrifugation and gel filtration on Sepharose CL-6B (section 3.3) showed that the receptor exists in CHAPS extract predominantly in a highly aggregated state, with a small proportion of the total receptor population existing in a series of less aggregated forms. The relative proportions of receptor activity (~70%) and total solubilised protein (~25%) eluted in the void volume of gel filtration experiments suggest that the aggregation is to some degree specific; i.e. that the high molecular weight aggregates are a result of self-aggregation of the glucagon receptor or aggregation between the receptor and other specific proteins, rather than general non-specific aggregation or "incomplete" solubilisation of the membranes. The occurrence of the receptor in the membrane as part of a multimeric complex of proteins has been suggested previously (Schlegel *et al.*, 1979), although Houslay *et al.* (1977), using the same technique of target size analysis, suggested that the receptor is an independent mobile species in the membrane. The identification of other possible proteins which may specifically aggregate with the glucagon receptor on solubilisation is outside the scope of this study.

Aggregation of proteins following removal of detergent from solubilised membranes has been observed (Valpuesta *et al.*, 1986). However, the possibility of aggregation occurring only upon dilution of CHAPS extracts to a final concentration of 3 mM (the concentration at which sucrose density gradients and gel filtration were carried out) was discounted by the observation that centrifugation of 6 mM CHAPS for 12 hours at 105 000 *g* results in the sedimentation of ~80% of binding activity and the absence of any highly aggregated activity in the void volume of gel filtration experiments, indicating that aggregated material exists in the 6 mM CHAPS extract. Similar results with 20 mM CHAPS extract suggest that aggregation is not decreased by increasing CHAPS concentration. Indeed, although many membrane proteins exhibit progressive deoligomerisation at increasing

detergent concentrations or detergent:lipid ratios, some show increased aggregation under these circumstances, without necessarily being denatured (Silvius, 1992). CHAPS concentration appears to have little or no effect, however, on the aggregation state of the glucagon receptor over the concentration range used.

Aggregation was unaffected by increasing NaCl concentration up to a concentration of 1 M, suggesting that electrostatic interactions do not play an important role in the aggregation of the CHAPS-solubilised receptor. Over this range of NaCl concentrations hydrophobic interactions due to decreased solvation of the protein would not be expected to be promoted. Therefore it is unlikely that increased hydrophobic interactions counteracting any reduction in electrostatic interactions were responsible for the failure to see a change in the aggregation state of the receptor upon increasing the NaCl concentration. Therefore, it is likely that hydrophobic rather than electrostatic interactions are involved in the mechanism of aggregation. However, sucrose appeared to have a small disaggregating effect by an as yet unknown mechanism.

Removal of highly aggregated material by centrifugation enabled the determination of the Stokes radius of the smallest active species present to be made. The use of this value together with the S value of the smallest species observed in sucrose density gradient centrifugation (confirmation that the smallest species from each method were identical by pooling of material from the density gradient and running on the gel filtration column, or vice versa, was not possible due to the loss of activity; therefore this had to be assumed) led to the calculation of a minimum molecular weight of ~190 kDa for the CHAPS-solubilised receptor. An assumption made in this calculation is that any binding of CHAPS to the receptor is insufficient to significantly alter the S value or Stokes radius. Although many membrane proteins, after solubilisation, have been shown to bind significant amounts of non-ionic detergents, some have been shown to bind very little, e.g. glycoprotein IIb and III of platelets (Jennings and Phillips, 1982), and indeed the glucagon receptor has been shown to bind no significant amount of Lubrol-PX (Herberg *et al.*, 1984).

Furthermore, because of the low micelle number and low molecular mass of CHAPS, detergent binding may be anticipated to have a relatively small effect on hydrodynamic measurements compared with non-ionic detergents. It was not possible to assess the binding of CHAPS to the receptor by the determination of the S value in both H_2O and D_2O since the partial specific volume of CHAPS is so similar to that of the globular marker proteins used that the S value would be the same in both solvents, using the globular marker proteins for calibration, whether significant amounts of CHAPS were bound to the receptor or not (Clarke, 1975).

The recent isolation of a cDNA clone for the glucagon receptor (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993) has shown that the receptor is a 485-amino acid protein with a predicted molecular size of 54 962 Da (see section 5.3). Treatment of covalently labelled receptor with Endo F has suggested that the receptor contains at least four N-linked glycans accounting for 18 kDa (Iyengar and Herberg, 1984) and, indeed, the predicted amino acid sequence of the receptor shows the presence of four potential N-glycosylation sites on the extracellular N-terminal domain. Therefore the true molecular size of the native receptor might be expected to be somewhat higher than the 55 kDa peptide backbone. Indeed, SDS-PAGE analysis of covalently labelled receptor in this study and in previous work (Iyengar and Herberg, 1984; Iwanij and Hur, 1985) has led to the identification of an apparent molecular weight of 60-63 kDa, although if N-glycans do indeed account for 18 kDa then even this could be an underestimate of the true molecular size. Therefore, based on a monomer molecular weight of 60-70 kDa, it is proposed that the 190 kDa species identified in hydrodynamic experiments represents a trimer of receptor subunits. The identification of a species of similar molecular weight in membranes by SDS-PAGE analysis (see section 5.2.1) suggests that the observed size of the CHAPS-solubilised receptor is not an artifact of solubilisation. Furthermore, the size reported here is similar to that obtained for the membrane-bound receptor using target size analysis (Houslay *et al.*, 1977). However, the possibility that it represents a complex between the receptor and other different proteins cannot be

ruled out. In particular, association with the stimulatory G protein remains a possibility. However, in agreement with the findings of Herberg *et al.* (1984), the CHAPS-solubilised receptor was shown to bind ^{125}I-glucagon in a guanine nucleotide insensitive manner, compared with membranes which showed a 50-60% reduction in ^{125}I-glucagon binding in the presence of GTP. Furthermore, the hydrodynamic experiments described here were carried out on the unoccupied receptor which is proposed to be "uncoupled " from the stimulatory G protein. Indeed, the extent of coupling of the β_2-adrenergic receptor to G_s after solubilisation with sodium cholate has been shown to depend on the exposure of membranes to ligands prior to and during solubilisation, exposure to β-agonist prior to solubilisation resulting in increased coupling. Herberg *et al.* (1984) showed that the lack of guanine nucleotide effect in solution was not due to the functional absence of G_s in the CHAPS solution since it could reconstitute guanine nucleotide and hormonal sensitivity to *cyc*$^-$ S49 cell membrane adenylate cyclase. Hence it was suggested that reconstitution into a lipid environment is necessary for receptor-G_s interactions to occur, as shown for the β_2-adrenergic receptor.

Determination of the molecular size of the receptor in other detergents has yielded conflicting results. Giorgio *et al.* (1974) showed the presence of glucagon-binding proteins of over 1.5 x 10^6 Da after treatment of labelled membranes with Lubrol-PX. However, Herberg *et al.* (1984) proposed the Lubrol-solubilised cross-linked receptor to be a dimer of 119 kDa which was shown not to be associated with the stimulatory G protein.

Thermostability studies on the CHAPS-solubilised receptor (section 3.3.5) suggest the presence of two receptor populations, one accounting for ~75% of the total population and having a half-life of ~9 minutes at 35°C and the other accounting for ~25% with a half-life of ~128 minutes at 35°C. However, corresponding experiments on liver plasma membranes show monophasic decay, indicating the presence of a homogeneous receptor population with a half-life of ~7 minutes at 50°C. It is possible, therefore, that the biphasic effect observed with

CHAPS extract could be brought about by the combined effects of heat denaturation and increased activity of proteases, the latter not being a contributing factor in experiments with membranes due to the decreased susceptibility to proteolysis of the membrane-bound receptor. Alternatively, the relative proportions of the two populations suggest that they could represent the highly aggregated and less aggregated species observed in the molecular size determination experiments; i.e. that the thermolabile population represents the highly aggregated receptor while the thermostable population represents the less aggregated species. However, reliable assessment of the specific glucagon binding activity of the less aggregated material was not possible due to a high proportion (>80%) of non-specific binding in a small amount of total binding, especially after any heat treatment.

A further possibility is that the two populations of different thermostability represent populations of receptor which are associated with and dissociated from G_s, since conformational changes in the receptor brought about by interaction with G_s might be expected to bring about a change in the thermostability of the receptor. However, this is unlikely since experiments carried out in the presence of guanine nucleotides resulted in no change in the thermostability of the CHAPS-solubilised receptor, and, as described above, the receptor did not exhibit functional interaction with the stimulatory G protein after solubilisation.

5.1.3 Affinity Chromatography Using Immobilised Glucagon

Biospecific affinity chromatography is by far the most useful and successfully applied method for the purification of integral membrane proteins and has played an essential role in the purification of hormone receptors, e.g. the insulin receptor (Cuatrecasas, 1972). Isolation of the CHAPS-solubilised receptor was attempted using biospecific affinity chromatography on immobilised glucagon (section 3.4). The high degree of specificity afforded by this method gives it the

potential for a substantial degree of purification in one step. This is particularly important for the glucagon receptor in view of its instability after solubilisation. Immobilised glucagon was prepared by the attachment of methionine-27 in glucagon to bromoacetamidoethyl-polyacrylamide. The methionine residue in glucagon can be modified without altering its receptor binding affinity (Coolican *et al.* 1982). The immobilised glucagon binds CHAPS-solubilised receptor specifically and in a manner proportional to the amount of immobilised ligand present. However, selective elution of receptor, either in active or inactive form, is not possible using the methods described here, as discussed below.

The method of choice for elution is to elute with glucagon itself as this is potentially a highly specific method. However, the removal of glucagon to a concentration at which assay for the soluble receptor can be carried out would be required. This proved to be highly problematical. Removal of glucagon by binding the glucagon-eluted receptor to WGA-agarose then washing with an adequate volume of buffer was unsuccessful. It was shown that solubilised receptor bound to WGA-agarose is lost during the course of washing with the required volume of buffer. This indicates that binding of the receptor to WGA-agarose in the presence of CHAPS is less stable than the binding of cross-linked receptor to the agglutinin in Lubrol-PX (see section 4.2.2). Therefore it is possible that loss of receptor bound to WGA-agarose after elution was the reason for the inability to detect receptor using this procedure; alternatively, it may have been due to failure to elute the receptor from glucagon-P150. Similarly, attempts to remove glucagon by passing glucagon-eluted material through dextran-coated charcoal columns failed to yield active receptor, due either to loss of activity through this procedure or to failure to elute receptor from glucagon-P150. This was tested by elution of glucagon-P150-receptor complexes with glucagon and analysis of the eluate by SDS-PAGE and showed, as far as the sensitivity of the silver staining procedure allows, that no receptor is eluted even after incubation with 100 μM glucagon for 20 hours. With CHAPS extract containing at least 200 fmol receptor/mg soluble

protein (McVittie and Gurd, 1989) it is calculated that the starting material in a typical affinity chromatography experiment described here contains approximately 10 ng receptor. Since the silver staining procedure used is capable of detecting as little as 0.1-1.0 ng of polypeptide in a single band (Sambrook *et al.*, 1989), elution of less than 10% of bound activity (assuming 100% of applied activity binds) should still be readily detectable. Hence it appears that the receptor binds to the immobilised ligand with very high affinity. If the affinity of the complex is very high, the time required for complete dissociation may be quite appreciable, even with high concentrations of competing ligand in the elution buffer. If this is indeed a problem, it could possibly be overcome by modification of the immobilised ligand to reduce its affinity, as has been shown in the use of immobilised glucagon in the purification of anti-glucagon antibodies (Murphy, 1974). An alternative approach to reducing the affinity to the immobilised ligand would be to increase or decrease (depending on the relative importance of ionic and hydrophobic interactions) the salt concentration in the binding buffer but this could also decrease the subsequent interaction with free ligand in affinity elution (Dean *et al.*, 1985).

Elution of the receptor with urea was considered a feasible procedure from the finding that inactivation of CHAPS-solubilised receptor binding activity after treatment with 2 M urea is partially reversible after overnight dialysis. Therefore the failure to recover active receptor after elution of glucagon-P150-receptor complexes with 2 M urea followed by overnight dialysis was surprising. Whilst inactivation by urea during elution and subsequent failure to recover activity after dialysis is possible, the preliminary experiments described above showed this not to be the case when treating CHAPS extract with 2 M urea, and it seems more likely that the receptor was not eluted by 2 M urea. Similarly, when elution was carried out with pH 4 or pH 5 buffers there was no recovery of activity. Since great care was taken to ensure that the eluates were adjusted back to pH 7.5 as quickly as possible, it again seems more likely that failure to recover activity was due to the absence of elution of the receptor rather than any inactivation during elution.

Elution of glucagon-P150-receptor complex with SDS followed by analysis on SDS-PAGE shows that a small amount of the majority of the proteins originally present in CHAPS extract bind to glucagon-P150. These proteins were also seen after SDS elution of bromoacetyl-P150 which has been incubated with CHAPS extract and therefore probably represent proteins which bound non-specifically to the acrylamide derivative support material. The absence of significant amounts of protein in a high salt wash of glucagon-P150-receptor complexes indicates that this non-specific binding arises as a result of hydrophobic interactions.

5.2 Receptor Desensitisation and Phosphorylation

5.2.1 Photoaffinity Labelling and Isoelectric Focusing of Glucagon Receptor

Direct cross-linking of ^{125}I-glucagon to its receptor by UV irradiation leads to the identification of a specifically labelled glucagon-receptor complex of M_r ~60 000, correlating well with the results obtained by Iwanij and Hur (1985) using a similar procedure. The presence of labelled material of higher molecular weight, including material which is too large to enter the stacking gel, again demonstrates aggregation of the receptor, here occurring even in the presence of SDS. The observation of a labelled band of molecular weight ~170 kDa indicates a trimeric aggregate of the receptor, as seen in the CHAPS-solubilised state (see section 5.1.2), although there is no evidence of the putative receptor dimer observed by Herberg *et al.* (1984) and Iwanij and Hur (1985).

The increase in the incorporation of membrane-bound ^{125}I-glucagon into the cross-linked receptor band in the presence of added proteins is surprising. The most likely explanation of this effect is that the presence of additional protein during the irradiation step protects the receptor from UV-promoted degradation. However, it is

questionable whether this explanation could also account for the observation of a smaller increase in cross-linking in the presence of sugars. A reduction in non-specific binding of ^{125}I-glucagon to the tube surface can be discounted since the proteins and sugars were added only during the cross-linking step, and the amount of specific binding of ^{125}I-glucagon to the receptor was shown to be the same for each sample prior to irradiation. The nature of this effect merits further investigation; however, since the main purpose here was to increase the amount of incorporation of bound label into cross-linked label, no further investigation was carried out.

Partial purification of cross-linked receptor was carried out by WGA-agarose chromatography. The absence of any labelled material in the flowthrough indicates that, in terms of binding to wheat germ agglutinin, there is only one receptor population. This is in agreement with Herberg *et al.* (1984), who found no labelled receptor remaining in solution after exposure to wheat germ agglutinin-Sepharose, but in contrast to the findings of Iwanij and Vincent (1990) who found separate populations of covalently labelled receptor that bound or did not bind to the agglutinin. However, Mason and Tager (1985), using digitonin-solubilised non-cross-linked glucagon-receptor complexes, showed that high affinity receptors bind to wheat germ agglutinin whereas low affinity receptors do not bind. This might indicate that differential glycosylation could alter receptor affinity. Hence it is possible that using the cross-linking procedure described here only high affinity glucagon-receptor complexes underwent covalent cross-linking and this is why all covalently labelled material was seen to bind to wheat germ agglutinin.

Isoelectric focusing of covalently labelled glucagon receptor gives further demonstration of the tendency of the receptor to form large aggregates. Early problems encountered in the isoelectric focusing of membrane proteins in immobilised pH gradients (poor sample entry, smearing and lack of sharply focused bands) were overcome by the addition of carrier ampholytes to both the gel and the solubilised protein sample (Rimpilainen and Righetti, 1988). The

subsequent improvement in focusing given by this procedure was proposed to arise as a result of the formation of detergent-carrier ampholyte mixed micelles which greatly improve the solubility of the membrane proteins under study. However, here it is shown that even after such treatment of samples of partially purified covalently labelled glucagon receptor and the inclusion of carrier ampholyte in the gel rehydration solution, all labelled material remains at the point of sample application. Since ^{125}I-glucagon focuses into distinct bands under the same conditions it appears that the inability of the glucagon receptor to focus is due to the presence of receptor aggregates. These aggregates may be too large to enter the gel matrix and exacerbate the problem by clogging the gel surface and hindering the entry of less aggregated forms of the receptor into the gel (Görg *et al.*, 1988). This problem is perhaps not surprising in view of the observations that some labelled material does not enter the stacking gel on SDS-PAGE under conditions where aggregation may be expected to present less of a problem, and that in the CHAPS-solubilised state a large proportion of the receptor activity is in a form over 2 x 10^6 Da, well over the exclusion limit for IPGs. Similarly, using larger pore size agarose IEF gels, even after extensive optimisation of the procedure so that good resolution of the components of solubilised membrane preparations was achieved, all labelled material in samples of covalently labelled receptor remain at the sample application point, presumably due to the same reasons as described for IPGs. In view of the reported molecular weight of 119 kDa for the Lubrol-solubilised cross-linked receptor by Herberg *et al.* (1984), the problems described above may seem surprising. However, it should be pointed out that their gel filtration profile did not show whether aggregated labelled material was eluted at or near the void volume; in view of the results of SDS-PAGE it is likely that this material did exist. Indeed earlier work showed the presence of glucagon-binding proteins of over 1.5 x 10^6 Da after treatment of labelled membranes with Lubrol-PX (Giorgio *et al.*, 1974).

5.2.2 Thermostability of Receptor in Control and Desensitised Membranes

For many proteins, the biologically active conformation is only marginally more stable than unfolded, inactive conformations. The effect of temperature on the stability of proteins is a result of changes in the free energy of the protein. This in turn is determined by the enthalpy change for unfolding and the difference in heat capacity between the unfolded and folded conformations (Pace, 1990). Hence the stability of a protein at a certain temperature gives an indication of the degree of unfolding shown by that protein at that temperature. Here it was proposed that phosphorylation of the glucagon receptor might result in a change in its conformation which in turn could alter the tendency of the receptor to unfold at higher temperature. Hence the thermostability of the phosphorylated receptor, as determined by glucagon binding, might be expected to differ from that of the unphosphorylated receptor.

The thermostability of the glucagon receptor was found to be the same in plasma membranes preparations from both control and vasopressin-desensitised cells. The presence of a "phosphatase inhibitor cocktail" during membrane preparation and the subsequent demonstration that membranes prepared from desensitised cells still showed desensitisation of glucagon-stimulated adenylate cyclase activity compared with membranes prepared from control cells, allows the possibility of dephosphorylation of receptor during membrane preparation to be discounted. Therefore the failure to observe any change in thermostability indicates that either (i) the receptor is not phosphorylated during desensitisation or (ii) the receptor is phosphorylated but that this does not result in a change in the thermostability of the receptor. Of these two possibilities the second seems to be the more likely. Phosphorylation of the β_2-adrenergic receptor has been shown to occur at sites in the third cytoplasmic loop or the C-terminal domain, regions which provide linear recognition sequences for coupling to G_s (Kobilka, 1992). Therefore

it is possible that phosphorylation might affect G protein coupling without having a major effect on the overall conformation of the receptor which is stabilised by the transmembrane regions. Therefore phosphorylation, whilst altering G protein coupling, might not affect the thermostability of the receptor.

Recent isolation of a cDNA clone for the glucagon receptor by an expression cloning strategy (Jelinek *et al.*, 1993) has shown that a sequence motif important for G protein coupling is situated directly next to a putative serine phosphorylation site, and in addition there are several other potential sites (serine and threonine) for phosphorylation in the carboxyl terminal domain. This suggests that, as expected, phosphorylation of the receptor and subsequent uncoupling from the stimulatory G protein may form the molecular mechanism of desensitisation of glucagon-stimulated adenylate cyclase. The ability of the phorbol ester TPA (Murphy *et al.*, 1987) and diacylglycerol (Newlands and Houslay, 1991) to mimic desensitisation indicates that this phosphorylation occurs as a result of stimulation of protein kinase C. Indeed, the predicted amino acid sequence of the glucagon receptor contains a potential phosphorylation site for protein kinase C (Svoboda *et al.*, 1993). It remains to be seen whether, in a situation analogous to desensitisation of the β_2-adrenergic receptor (Kobilka, 1992), there are also rôles for protein kinase A and/or a glucagon receptor specific protein kinase.

5.3 Future Prospects

The recent isolation of a cDNA clone for the glucagon receptor (Jelinek *et al.*, 1993; Svoboda *et al.*, 1993) has shown that this receptor is a 485-amino acid protein with a predicted molecular size of 54 962 Da and a predicted structure similar to that of other G protein coupled receptors, with seven transmembrane domains, three extracellular and three cytoplasmic loops. The extracellular domain contains four potential N-linked glycosylation sites while the cytoplasmic domains

contain, as described above, a sequence motif important for G protein coupling and several potential phosphorylation sites. The expression cloning strategy used detected only one glucagon receptor cDNA, and the expressed receptor was shown to increase the intracellular concentration of both cAMP and calcium, indicating that only one receptor is responsible for the two types of signalling observed in hepatocytes, as shown for the calcitonin receptor (Chabre *et al.*, 1992). It is possible, therefore, that the stimulation of the two signalling pathways may be brought about by the coupling of two different G proteins to a single receptor population. However, this does not preclude the possibility of other forms of receptor heterogeneity, e.g. differing affinities for glucagon, arising as a result of differential glycosylation.

The isolation of a cDNA clone represents a major advance in the area of glucagon receptor research. However, the studies described in this thesis could be furthered by using the predicted amino acid sequence of the cloned receptor to generate specific peptides for the raising of monoclonal antibodies which could then be used for purification of the receptor. Purified receptor would enable confirmation to be made of phosphorylation during desensitisation of glucagon-stimulated adenylate cyclase, while reconstitution of the receptor in appropriate membrane systems with guanine nucleotide binding proteins and adenylate cyclase and/or phospholipase C would enable full characterisation of the active receptor and its mechanisms of signal transduction to be made. The availability of a cDNA clone for the glucagon receptor would also enable gene products resulting from site-directed mutagenesis to be reconstituted into such systems in order to assess the functionality of specific domains of the receptor.

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