THE PREPARATION AND CHARACTERIZATION OF A PHOTOREACTIVE GLUCAGON ANALOGUE AND ITS INTERACTION WITH RAT LIVER PLASMA MEMBRANES

by

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ABSTRACT

A heterobifunctional crosslinking reagent 2-nitro-4-azidophenylsulfenyl chloride (NASPCl) was synthesized and characterized. This reagent can be used to specifically attach a photoactivatable nitrophenyl azide to tryptophan containing polypeptides and proteins lacking sulfhydryl groups. The reaction of NAPSCl with glucagon, a peptide hormone containing a single tryptophan residue at position 25 and no cysteine gave one major product which could be effectively photolysed at wavelengths above 300 nm. Glucagon-NAPS could be radiolabeled by the lactoperoxidase catalyzed iodination of the peptide. The covalent labeling of protein molecules with radiolabeled glucagon-NAPS upon photolysis was demonstrated. The photosensitive glucagon was shown to activate the adenylate cyclase of hepatocyte plasma membranes with a slightly higher potency than the native hormone at equimolar concentrations. Glucagon-NAPS was, therefore, considered as an effective photoaffinity probe for labeling the glucagon receptor sites in plasma membranes of target cells.

Irradiation of hepatocyte plasma membranes in the presence of glucagon-NAPS resulted in an activated state of adenylate cyclase. The enzyme displayed a lower response to further stimulation by native glucagon, glucagon-NAPS or NaF than the response displayed by similarly treated membranes in the dark, suggestive of covalent labeling of the derivative to glucagon functional sites on the membrane.

Competitive binding studies at steady state using radiolabeled ^{125}I-glucagon and ^{125}I-glucagon-NAPS demonstrated binding of glucagon-NAPS to the same receptor sites on hepatocyte plasma membranes as native glucagon. Scatchard plot analysis of the binding isotherm curves indicated two orders of specific receptor sites: a) a high affinity-low capacity site, (glucagon, Kd = 3.52 (± 0.72) x 10^{-10}M, B_{max} = 0.34 ± 0.15 pmole/mg membrane protein; glucagon-NAPS, Kd = 1.31 (± 0.10) x 10^{-10}M, B_{max} = 0.67 ± 0.10 pmole/mg membrane protein) and b) a low affinity-high capacity site, (glucagon, Kd = 4.17(± 1.05) x 10^{-9}M, B_{max} = 3.08 ± 0.63 pmole/mg membrane protein; glucagon-NAPS, Kd = 7.28(± 1.11) x 10^{-10}M, B_{max} = 2.19(± 0.95) pmole/mg membrane protein). The binding characteristics of glucagon and glucagon-NAPS were similarly affected by guanosine 5'-triphosphate (GTP). Saturation of the binding sites at concentrations of glucagon and glucagon-NAPS producing maximal stimulation of adenylate cyclase suggested the requirement of interactions with both sites for a functional adenylate cyclase system. Hill plot analysis indicated noncooperative interactions of the peptides with the high affinity-low capacity sites and cooperative interactions with the low affinity-high capacity sites.

Covalent crosslinking of bound ^{125}I-glucagon-NAPS to hepatocyte plasma membranes upon irradiation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis identified a number of radiolabeled membrane components of which a 67,000-70,000 daltons component was significantly labeled above background and was displaced by more than 90% in the presence of excess unlabeled glucagon or glucagon-NAPS.

This membrane component was identified as the glucagon specific binding site. The apparent molecular weight of detergent solubilized glucagon-receptor complex in a GTP sensitive state was estimated by gel fractionation as 200,000-250,000. SDS-gel electrophoresis of detergent solubilized, ^{125}I-glucagon-NAPS-receptor complex, covalently crosslinked by irradiation, identified the 67,000-70,000 as well as a 50,000 and 27,500 daltons components, also observed in unsolubilized membranes. Since the latter components were not significantly labeled above background or displaced in membrane samples irradiated in the presence of unlabeled peptide, they may not be called specific receptor sites. The inhibition of covalent crosslinking of all three components in trypsinized membranes demonstrated their protein nature. Anomalous electrophoretic mobilities with different acrylamide concentrations were suggestive of the receptor peptide being a glycoprotein. Similar behaviour under reducing and nonreducing conditions was suggestive of the presence of intramolecular disulfide bonds.

In these studies we have demonstrated that the glucagon-NAPS derivative has the advantage for labeling the glucagon receptor in hepatocyte plasma membranes because it is biologically active and the binding sites for the derivative can be linked to a physiological response. The receptor site identified should represent the true glucagon receptor or a receptor subunit since upon irradiation it was covalently crosslinked by the ligand, with the receptor still being in a biologically active state.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	-	antibody
^{14}C-ATP	-	adenosine 5'-triphosphate, tetrasodium salt, [8-^{14}C]
^{3}H-cAMP	-	adenosine 3,5'-cyclic phosphate, ammonium salt, [^{3}H(G)]
App(NH)p	-	adenosine 5'-(β-γ-imido)triphosphate
BSA	-	bovine serum albumin
CD	-	circular dichroism
DIG	-	diiodoglucagon
DIT	-	diiodotyrosine
DMF	-	dimethylformamide
DNPSCl	-	2,4'-dinitrophenylsulfenyl chloride
DNDPSCl	-	2,4'-dinitro-1,5'-diphenylsulfenyl chloride
DTT	-	dithiothreitol
EDTA	-	ethylenediamine tetraacetic acid
EGTA	-	ethyleneglycol-bis-(p-amino ethyl ether) N,N'-tetra-acetic acid
GDP	-	guanosine 5'diphosphate
glucagon-NAPS	-	glucagon-2-nitro-4-azidophenylsulfenyl
glucagon-NPS	-	glucagon-2-nitrophenylsulfenyl
Gpp(NH)p	-	guanosine 5'-(β-γ-imido)triphosphate
GSH	-	glutathione
GTP	-	guanosine 5'-triphosphate
IgG	-	immunoglobulin
IR	-	infrared
KIU	-	Kallikreine Inactivator Units
LH-RH	-	luteinizing hormone releasing hormone
MIG	-	monoiodoglucagon
MIT	-	monoiodotyrosine
MS	-	mass spectra
NAPSCl	-	2-nitro-4-azidophenylsulfenyl chloride
NMR	-	nuclear magnetic resonance
NPSCl	-	2-nitrophenylsulfenyl chloride
OD	-	optical density
ORD	-	optical rotatory dispersion

SDS	-	sodium dodecyl sulfate
TCA	-	trichloroacetic acid
TES	-	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
T-RH	-	thyrotropin releasing hormone
Tris	-	tris (hydroxymethyl) aminomethane
Trp-NAPS	-	tryptophan-2-nitro-4-azidophenylsulfenyl
Trp-NPS	-	tryptophan-2-nitrophenylsulfenyl

MATERIALS

4-chloro-3-nitroaniline was purchased from Aldrich Chemical Co.; sulfuryl chloride was from Eastman; potassium phthalate from Matheson, Coleman and Bell; bovine serum albumin (fraction V), Alumina WN-3, Dowex 50W-X8 (200-400 mesh), all amino acids, 98-100% grade creatine phosphate, Rabbit Muscle Type I creatine phosphokinase, bacitracin, epinephrine, isoproterenol, iodoacetamide, iodoacetic acid, Trypsin (Type III), soybean trypsin inhibitor and aminophylline were from Sigma; [^{14}C]-ATP (40-60 mCi/mmol), [^{3}H]-cAMP (30-50 Ci/mmol) and Na^{125}I (~17 Ci/mg) from New England Nuclear; lactoperoxidase (260 units/mg, OD_{412}/OD_{280} = 0.74) and Trasylol 10,000 KIU/ml from Boehringer; Protein A-Sepharose CL-4B, Sephadex G-75 and Sephacryl S-300 from Pharmacia, Ultrogel AcA22 (1.0×10^5 - 1.2×10^6 range) from LKB; DE-52 cellulose from Whatman; N,N'-methylene-bisacrylamide and N,N,N,N'-tetramethylenediamine from Bio-Rad; ultra pure urea from Schawrtz/Mann; protein molecular weight standards from BRL; bovine-porcine glucagon was purchased from Elanco Corp. The purity of the product was evaluated by partition chromatography (Fig. 2). All other reagents used were of the highest purity available commercially.

INTRODUCTION

GLUCAGON

Glucagon (mobilizer of glucose) was first named by Murlin et al. in 1923 when he correctly attributed the hyperglycemic effects of extracts of pancreas on pancreatectomised animals, to the presence of a glucogenic substance. The hormonal nature and the physiological role of glucagon, however, remained relatively unnoticed for many years after, since the hyperglycemic effect was not always observed in insulin preparations. It was not until Staub et al. 1953,1955 isolated and crystallized porcine pancreatic glucagon, that the hormone became available for chemical and biological studies. Soon after isolation its molecular structure was elucidated (Bromer et al. 1956), followed by its synthesis in 1968 (Wunsch and Weignes, 1972).

Glucagon is a 29 amino acid polypeptide hormone with a molecular weight of 3485. The primary amino acid sequence of glucagon is as follows (Bromer et al. 1956, 1971):

```
 1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18
His-Ser-Gln-Gly-Thr-Phe-The-Ser-Asp-Tyr-Ser-Lys-Tyr-Leu-Asp-Ser-Arg-Arg-

 19  20  21  22  23  24  25  26  27  28  29
Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr.
```

The amino acid sequence of bovine, porcine, human, rat and rabbit glucagon have been established and found to be identical (Bromer et al. 1971; Sundby and Markussen, 1971, 1972). The amino acid composition of avian hormone was also found to be similar except for one or two amino acid substitutions. In turkey and chicken, glucagon Asp 28 residue is replaced

with Ser, and in duck glucagon Asp 28 is replaced with Ser and Ser 16 is replaced with Thr (Sundby et al. 1972; Pollock and Kimmel, 1975).

The common amino acid sequence of these hormones indicates that the structure of glucagon has been conserved through evolution, which suggests that all amino acid residues are required for its physiological functions.

Glucagon is secreted from the endocrine A-cell of the pancreatic islets of Langerhans where it is synthesized and stored, as has been shown by histochemical studies, electron microscopy and immunological techniques (Munger, 1972). The control of glucagon secretion is complex, involving changes in blood metabolites, the sympathetic and parasympathetic pathways and hormonal control by locally secreted pancreatic hormones (Alford and Chisholm, 1979).

Glucagon is transported through the portal vein to the liver which is the main target tissue possessing high affinity receptor sites. Both hepatic membrane and cytosolic enzymes degrade glucagon and a close relationship has been demonstrated between hepatic binding and hormone degradation (Ausorge et al. 1971; Rodbell, 1972; Freychet, 1976).

The fraction of glucagon that escapes the liver is distributed via the systemic circulation to other tissues and organs, the most important of which is the kidney which is responsible for its extra-hepatic clearance (Assan, 1972).

Heterogeneous molecular weight species (MW >40,000, 12,000, 9,000, 3,500 and 2,000) of glucagon-like immunologically reactive pancreatic, extra-pancreatic and gut glucagon have been reported. Extra-pancreatic and pancreatic tissue sites were shown to be responsible for the circulating

heterogeneous species of glucagon. These species arise either from post translational modifications (Tager and Markese, 1979) or are the products of specifically cleaved glicentin (gut-glucagon-like immunoreactant) precursors (Pavazola et al. 1979; Holst, 1980).

The main physiological role of the hormone is the regulation of nutrient mobilization; a reciprocal role to that of insulin which is a major regulator of nutrient storage. Through its gluconeogenic, glycogenolytic and lipolytic actions, glucagon causes the redistribution of nutrient substrates stored in the liver and adipose tissue (Lefebvre and Unger, 1972). The glucagon effects are inhibited by insulin (Exton et al. 1971; Kiss, 1978).

In vivo studies have shown that glucagon plays a secondary role to insulin in regulating basal levels of hepatic glucose output (Libjenquist and Rabin, 1979). However, glucagon plays an important role in preventing hypoglycemia together with catecholamines and cortisol (Eiger et al. 1979). The ketogenic and ureogenic properties of glucagon are defined less clearly in the in vivo studies (Alford and Chisholm, 1979). In vitro studies have shown that all urea cycle enzymes are fully stimulated in response to glucagon (Gebhardt and Mecke, 1979). The physiological and pharmacological actions of glucagon in man have been recently reviewed by Alford and Chisholm (1979).

Glucagon has been implicated in diabetes. Hyperglucagonaemia has been shown to play an important role in the development of the diabetic syndrome by accentuating hyperglycemia and ketogenesis in severe insulin deficiency. Although hyperglucagonaemia has been attributed to abnormal A cell function as a secondary effect of insulin deficiency in constrat to nondiabetics, its lack of suppression has been observed upon glucose and/or

insulin administration to diabetic subjects. Glucagon resistance in target tissues due to a decrease in the number of receptors has been suggested to explain this phenomenon (Unger and Orci, 1976). Jacobs and Cuatrecasas (1977) have presented a framework with examples for a classification of abnormalities which includes: inherited abnormalities; pathogenic-antireceptor antibodies; abnormal synthesis or degradation of the receptor; altered binding and response to ligands and regulation of receptors by heterologous ligands.

Abnormal binding of glucagon to hepatocyte receptors of hyperglucagonaemic diabetic subjects has been observed (Foucheau-Person et al. 1976; Bhathena et al. 1978; Soman and Felig, 1978). Reduced binding but without changes in adenylate cyclase stimulation was observed in liver cell membranes of chronically hyperglucagonaemic rats (Srikant et al. 1977); and there were differences in binding to fat cells between young and adult rats but not to liver plasma membranes (Lockwood and East, 1978).

From the little information available and the limited experimentation hyperglucagonaemic in diabetes can not be attributed to glucagon receptor abnormalities as yet. Not only further experimentation with diabetic subjects is required, but most important, knowledge of the glucagon receptor at the molecular level and understanding of the events that take place during hormone-receptor interactions is necessary.

PEPTIDE HORMONE RECEPTORS

The receptor concept was introduced as early as 1905 by Langley,

who suggested that combination with a "receptive substance" of the target tissue is required for drug action (Stephenson, 1956). Since then the theoretical concept of receptor has become a reality with receptors being studied as molecular entities in understanding the mechanism of hormone and drug action on target tissue and cells.

A large amount of experimental evidence has established that peptide hormones like insulin and glucagon, and neurotransmitters like epinephrine and acetylcholine, exert their primary action on target cells by binding to specific high affinity receptor sites in the plasma membranes. This evidence as well as the biochemistry and mode of action of hormones have been reviewed in a number of recent review articles (Roth, 1973; Rodbell, 1973; Cuatrecasas et al. 1975; Pohl, 1977; Greaves, 1977; Biltonen, 1977; Catt and Dufau, 1977; Pimental, 1978; Earp and Steiner, 1978; Insel, 1978; Ross et al. 1978; Sands and Richenberg, 1978; Tell et al. 1978; Levitzki and Helmreich, 1979; Rodbell, 1980).

Because of the extensive amount of information available, only the main general properties shared by peptide hormone receptors and those aspects of receptor function relevant to biological responses will be discussed, so that the relevant research with the glucagon-receptor, presented in this thesis, will be more understandable.

The protein nature of plasma membrane receptors of peptide hormones has been demonstrated with the use of digestive enzymes and the concomitant loss of hormone binding and hormone elicited action. Several receptors have been shown to contain carbohydrate moieties and in many cases the role of functional disulfide bonds has been suggested. The location of receptor proteins on the outer layer of the membrane has been

demonstrated by a variety of methods including physical, immunological and biochemical techniques as well as light and electron microscopy.

The physicochemical characterization of receptors has presented problems due to the insoluble nature of the receptor proteins, a common characteristic of intrinsic membrane components. Preformed hormone receptor complexes, however, have been isolated as soluble species by using nonionic detergents. Physical analysis of detergent solubilized receptors have demonstrated large hydrodynamic radii (60-70 Å) in relation to their sedimentation constants of 6.5-9.0 S (Catt and Dufau, 1978). Conventional fractionation techniques, affinity chromatography, radioimmunoprecipitation with specific anti-receptor-antibodies and recently photoaffinity labeling have been used to further purify or identify membrane bound or detergent solubilized hormone receptor complexes.

The biological significance of receptors has been demonstrated in most cases by one or more of the following criteria: structure and steric specificity; saturability; target cell specificity, affinity for the receptor and biological response comparable to physiological active hormone concentrations; and reversibility of the response upon removal of the hormone (Insel, 1978).

The investigation of the above parameters has distinguished three main phases in peptide hormone action. Binding of the hormone to the receptor induces an initial stimulus which is transduced through the membrane producing a second messenger, the modulation,and amplification of which results in the generation of a biological effect.

Hormone receptor studies have been facilitated by the use of tritiated and iodinated radiolabeled hormone derivatives. Their high

specific activity has made it possible to follow hormone receptor binding in vitro with whole cells and plasma membrane homogenates. Furthermore, since the observations of Sutherland and coworkers (Sutherland, 1962; Rall and Sutherland, 1962; Sutherland et al. 1968), the majority of peptide hormones have been shown to elicit membrane associated responses such as adenylate cyclase enzyme activation and production of cyclic 3'5'-adenosine monophosphate (cAMP) upon binding to plasma membrane receptors; and prolonged biological responses with target tissues. However, for certain hormones like insulin, an initial stimulus has not been identified; and for some tissues where cAMP is accepted as a second messenger of peptide hormone action, it is not known how the message is amplified.

The essential aspects of a variety of partially overlapping theoretical models which have been proposed to explain drug and hormone action is presented in a condensed form in the following sequence of reactions (Ariens et al. 1980).

$$D + R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} DR \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} DR^* \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} D + R^* \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} \underline{R} \underset{k_{-5}}{\overset{k_5}{\rightleftarrows}} R$$

where D = drug, R = receptive receptor, R^* = activated receptor, and $\underline{R}$ = nonreceptive, nonactivated receptor. K_1, K_2, K_3, K_4 and K_5 are the equilibrium constants defined as $K_i = k_{-i}/k_i$.

In the "occupation-activation" model the activated state of the receptor is in a complex form with the pharmacon; i.e. as DR^*. In the "hit-activation" model, the activated state of the receptor is predominantly in the uncomplexed form; i.e. as R^*. In both models, the stimulus is proportional to the fraction of the receptors in the activated state.

In the "two state" model, an equilibrium exists between the

receptors in the nonactivated, R and in the activated R^* state. The R^* receptor state exposes a binding site with a high affinity for agonists only, the R state exposes a site with a high affinity for only the "competing" antagonists (Ariens, 1979).

The constants which determine the fraction of the receptors involved in the drug-receptor interactions, i.e. the affinity of the drug for the receptor, are represented by K_1 and the constants for the fraction of the receptors in the activated state, i.e. the intrinsic affinity of the drug are represented by K_2.

Dose-response studies on the basis of the above equation, with whole cells or plasma membrane preparations, have provided information concerning the affinity and mechanism of interaction of the stimulatory hormone with the receptor. Dose-response curves have also been used to compare relative affinities of hormone analogues and, along with binding studies, in differentiating the amino acid residues of peptide hormones that may be involved in binding to the receptor and/or activation of the adenylate cyclase enzymes.

Comparable quantitative studies of specific binding at steady state using radiolabeled hormones in cells or membrane preparations have demonstrated high specificity for biologically active hormones and high binding affinity with dissociation constants in the order of 10^{-9} - 10^{-11} M^{-1}.

Several peptide hormones have been shown to interact with a single set of homogeneous independent receptor sites giving linear Scatchard plots (Scatchard, 1949) and Hill plots (Hill, 1910) with a slope of one (luteinizing hormone (Catt et al. 1972); growth hormone (Lesniak et al. 1973)). However, certain other hormones, like insulin (De Meyts

et al. 1976), ACTH (Lefkowitz et al. 1971), show more complex binding curves, concave Scatchard plots and Hill plots with a slope greater or less than one, indicating the presence of two or more sets of binding sites, or of site-site interactions. Negative cooperativity, observed with insulin, where increasing occupancy of receptor sites leads to a progressive reduction of binding affinity has been attributed to such interactions (De Meyts et al. 1976).

Only a few receptor molecules in the order of a few pmole/mg protein have to interact with the hormone in order to induce a massive response involving a large number of molecules.

Fairly rapid, temperature dependent rates of binding of peptide hormones to receptors have been demonstrated which agree with the fast hormone action on target cells. However, determination of rate constants and thermodynamic parameters of hormone-receptor interactions are complicated due to hormone dissociation as well as hormone and receptor degradation.

The structural arrangement of hormone receptors and adenylate cyclase catalytic and regulatory components in plasma membranes, as well as the mechanism involved in their interactions are not yet known. It is generally agreed that the receptor and cyclase molecules reside mainly in opposite halves of the cell surface membrane with the receptor on the outside. The dependence of the activation of the system upon temperature and phospholipids and the effects of agents like urea, have indicated the requirement of a lipid environment and a mobile receptor within a fluid membrane matrix as well as of hydrophobic interactions between hormone and receptor sites.

The role of hydrophobic interactions in receptor binding is supported by the presence of twofold symmetry of hydrophobic regions in insulin and glucagon (Wood et al. 1975; Pullen et al. 1976) as well as in other peptide hormones. It has been suggested that the two-fold symmetry of the hormone is reflected in a receptor structure composed of two similar or identical subunits (Rodbell et al. 1975).

Recent advances in the studies of hormone-receptor interactions and the involvement of guanosine-5'-triphosphate (GTP) in adenylate cyclase activation have identified three classes of components. These include the hormone receptor site (R), the catalytic site of adenylate cyclase (C), and a GTP regulatory site (N). On the basis of these findings, and the extensive studies of adenylate cyclase activation by glucagon and GTP, and the results from target size analysis studies of adenylate cyclase systems (Schlegel et al. 1979), Rodbell (1980) has postulated a model for hormone-receptor-adenylate cyclase interactions and their structural arrangement within the membrane. In this model, the role of the receptor differs from that postulated in previous studies and the GTP-regulatory protein is placed in perspective with the other membrane components.

According to this model, cell membrane receptors of peptide hormones and neurotransmitters form oligomers of RN complexes with the GTP-regulatory protein which, as a result, can not bind GTP. Binding of the hormone to R releases the inhibitory effect on N resulting in the breakdown of the RN oligomers to monomers. The RN-GTP monomer can then bind to the catalytic unit C of the adenylate cyclase, resulting in either activation or inhibition of the holoenzyme. Two types of N subunits,

stimulatory and inhibitory are responsible for RN inhibition or activation of adenylate cyclase. According to this model, therefore, the role of the receptor is to facilitate GTP ⇄ GDP exchange on the regulatory subunit N.

A number of other models have been proposed involving a precoupled receptor-cyclase complex, a mobile receptor-hormone complex and a collision coupling model (Boeynaems and Dument, 1975; Jacobs and Cuatrecasas, 1976; Tolkovsky and Levitzki, 1978).

Although all models proposed offer several possibilities to explain the hormone-receptor adenylate cyclase interactions, only the collision coupling model (Tolkovsky and Levitzki, 1978) has been tested by both binding and kinetic studies. According to these studies, the hormone-receptor cyclase complex comprises only a small fraction of the total receptor and adenylate cyclase concentrations. Its formation requires the diffusion of the hormone receptor complex in the membrane. On the basis of this model, binding studies of β-adrenergic receptors with epinephrine and an epinephrine antagonist showed a noncooperative pattern of binding and kinetic studies showed that the activations mechanism is strictly first order, both results in disagreement with the model postulated by Rodbell.

Therefore, even though some aspects of the working hypothesis concerning hormone-receptor interactions have been verified by experimentation, the question as to how adenylate cyclase is modulated by hormone-receptor interactions remains controversial.

Knowledge of the mechanism involved in the process of termination of hormone receptor interactions is also limited. Dissociation of the hormone does not appear to follow the law of mass action, and GTP, possibly through the GTP regulatory protein, has been shown to increase the rate of dissociation

of glucagon (Rodbell et al. 1974). Although degradative processes have been suggested, it has been shown that degradation reactions are independent of the hormone interactions with the receptor (Freychet et al. 1972). It would appear, therefore, that other mechanisms may be involved, whose nature remains still unknown.

PHOTOAFFINITY LABELING BY BIFUNCTIONAL REAGENTS

There are a large number of reagents used for chemical modification of proteins. One category, the bifunctional reagents, has the advantage that they can be used for both inter- and intramolecular crosslinking of specific residues of peptides and proteins (Wold, 1972; Henkin, 1977).

Intramolecular crosslinking of specific amino acid residues, provided that the native structure of the protein is maintained, can provide information about inter-residue distances and identify proximal amino acid residues (Hartman and Wold, 1967).

Intermolecular crosslinking may join molecules of the same or different kinds. The products can be used as models for studying protein-protein interactions, as high molecular weight analogues or as substances combining the desirable attributes of both components into one as well as for use as an affinity label (Cuatrecasas and Anfinsen, 1971).

Despite the variety of conventional bifunctional reagents, their use, however, is limited. In the case of homobifunctional reagents, X-R-X, there are limitations in terms of reactivity and specificity. In the case of heterobifunctional reagents, X-R-Y, their use can also be limited by the specificities of the two reactive groups as well as by the uncertainty in the sequence of their reaction. Some of the limitations may be overcome if the second functional group is sufficiently different that well-controlled sequential reactions of the reactive groups are possible. For both homo- and heterobifunctional reagents it is necessary to detect whether cross-

linking is intra- or intermolecular (Wold, 1972).

Heterobifunctional reagents have been especially useful in the study of a large number of biological processes that take place in living systems, where an organic molecule, "the ligand", interacts with a biological macromolecule, "the receptor", as for example in the case of enzyme-substrate (Erecinska, 1977), antibody-antigen (Fleet et al. 1969) or hormone-receptor interactions (Tae, 1977).

The labeling of the ligand with the reagent provides the receptor with an analogue of the natural ligand so that natural interactions can take place, resulting in a noncovalent complex. By the subsequent reaction of the second reactive group, the receptor can be covalently labeled and the crosslinked product can be isolated and characterized.

For maintaining the specificity of the binding interactions, there are certain requirements that should determine the selection of the reagent. These requirements for the labeling process of the ligand have been defined (Knowles, 1972):

a) specificity in the ligand modification,

b) modification should not alter the conformation of the ligand,

c) the precursor species of the second reactive group should be chemically inert and small,

d) the modification should not alter the ligand-receptor binding interactions or the activity of the ligand, and

e) the reaction conditions of either reactive group should not alter the stability of the reagent or the ligand.

In contrast to the necessary specificity required in the modification of the ligand, the covalent labeling of the receptor should be nonselective, with a wide range of reactivities. The requirements for

the covalent labeling of the ligand to the receptor should be:

a) a chemically inert precursor species,

b) a nonselective reactive species which, when generated, does not rearrange intramolecularly to a less reactive entity

c) a reactive species with a short half life, which will react before dissociation of the ligand occurs,

d) the generation of the reactive species should not result in the dissociation of the bound ligand from the receptor.

Most common reagents used react with nucleophiles, and this can be limiting since not all of the amino acid residues have nucleophilic side chains. Furthermore, in the studies of membrane receptors, lipids and glycoproteins with oligosaccharide chains are present and they may be part of the receptors themselves. Also, in aqueous solutions, water may react much faster than any of the functional groups of the amino acid side chains. Therefore, in order to satisfy the above requirements for the covalent labeling of the receptor, a reactive species cannot be added externally to aqueous solutions of proteins, instead it must be generated in situ from a stable precursor species.

Two such types of chemical intermediates with a wide range of reactivity are carbenes and nitrenes, which comprise the photoaffinity labels (Knowles, 1972). These reactive species can be produced thermolytically as well as photolytically. Since the integrity of biological systems is limited to a certain temperature range, the photolytic generation is preferred for it can be carried out at long enough wavelengths to avoid any photo-oxidation and radiation-induced damage. The chemistry and photoactivation of nitrenes is discussed in Chapter I.

In the last few years, covalently linking biological macromolecules with bifunctional reagents containing an azide moiety which can be converted to a highly reactive nitrene upon irradiation, has been widely used. Chemical modification with these reagents has provided radiolabeled hormone photoprobes and other protein molecules resulting in covalently linked ligand-receptor complexes after irradiation which are much easier to isolate and to study than a purely reversible ligand-receptor complex (Bayley and Knowles, 1977; Das and Fox, 1979; Ji, 1979; Chowdry and Westheimer, 1979; Hanstein, 1979).

More important, this approach offers an advantage in membrane receptor characterization, over conventional affinity labeling because the ligand-receptor interactions can be characterized prior to covalent incorporation of the ligand to the receptor, by experiments carried out in the dark.

Although the efficacy of nitrenes to covalently label lipids and hydrophobic amino acid side chains of membrane proteins has been questioned recently (Bayley and Knowles, 1978a,b), because of the longevity and electrophilic nature of nitrenes, successful photolabeling of receptor molecules with nitrenes has been reported. For brevity, only examples of photolabeled peptide hormone receptors are shown in Table I, since they are relevant to the photoaffinity labeling of glucagon receptor.

OBJECTIVES

The biological significance of glucagon has been discussed and its central role in the studies of hormone receptor interactions has

TABLE 1

Photoaffinity labeling of peptide hormone receptors

Reagent	Peptide	Activity of photolabeled receptor	Species photolabeled	Reference
4-fluoro-3-nitrophenyl azide	N-glucagon	N.D.	23-25K membrane receptor components	Bregman et al. 1977; 1978
2-nitro-5-azidobenzoic acid	cholecystokinin	irreversible stimulation	N.D.	Galardy and Jamieson, 1977
N-(2-nitro-4-azidophenyl) ethylenediamine	enkephalin	irreversible inactivation	N.D.	Lee et al. 1979
4-azidophenyl amino acid analogues	angiotensin	irreversible inactivation	N.D.	Hazum et al. 1979
p-azidophenylalaninamide	neurophysin	irreversible inhibition of ligand binding	N.D.	Escher and Guillemete, 1979; Kwok and Moore, 1970
4-azidophenyldiazonium	bradykinin	partial inactivation	N.D.	Klausner et al. 1980
2-nitro-5-azidobenzoyl glycine	oxitocin	inhibition of oxitosin stimulated urea release	N.D.	Escher et al. 1979
2-nitro-5-azidophenyl sulphenyl chloride	corticotropin (ACTH)	inactivation of receptor mediating steroidgenesis	100 K	Stadel et al. 1978
2,4-dinitro-5-azido fluorobenzene	"	N.D.	N.D.	Ramachandran et al. 1980
2-nitro-4-azidophenyl sulfenyl chloride	"	N.D.	N.D.	Canova Davis and Ramachandran, 1980; Muramoto and Ramachandra, 1980

TABLE 1 (continued)

Reagent	Peptide	Activity of photolabeled receptor	Species photolabeled	Reference
(4-azidobenzoyl)-N-hydroxysuccinimide	$N^{\epsilon B29}$ and $N^{\alpha A1}$, $N^{\epsilon B29}$ insulin	N.D.	90K, 130K membrane component of rat adipocytes	Yip et al. 1978, 1980
4-fluoro-3-nitrophenyl azide	insulin	N.D.	310K oligomer with 137K and 45K subunits rat adipocytes membrane components	Jacobs et al. 1979
4-azido-benzoic acid	A1, B1 or B29 insulin	N.D.	90K component of rat liver plasma membranes	Wisher et al. 1979
p-azido-benzoyl N-hydroxy succinimide ester	$N^{\alpha A1}$ and $N^{\epsilon B29}$ insulin	N.D.	130K component of rat liver plasma membranes	Yeung et al. 1980
N^{α}-4-azido-2-nitrophenyl glycine N-hydroxysuccinimido ester; 4'-nitrophenyl-(4-azido-2-nitrophenyl)-acetate	B2-B1 insulin B1 insulin	permanent activation of lipogenesis in adipocyte cells	N.D.	Diaconescue et al. 1980
"	B2-B1 insulin	N.D.	300K and 600K subunits of nonionic detergent solubilized adipocyte plasma membranes	Kuehn et al. 1980

N.D. = not determined

been mentioned. However, the knowledge available concerning the nature of the glucagon receptor is limited as well as the understanding of glucagon membrane interactions. Therefore, the task undertaken in this work aimed in further characterization of the receptor by the use of the photoaffinity labeling technique. Photoaffinity labeling of the glucagon receptor by the same technique has been attempted (see Table I, Bergman et al. 1977). However, the photoprobe used was inactive even though it competed with native glucagon at equimolar concentration, in binding to liver plasma membranes.

It was considered, therefore, important in the covalent labeling of glucagon that the selected photoaffinity reagent did not alter either the activity or the binding characteristics of glucagon for the receptor. The importance of the biological significance of the amino acid residues of glucagon (discussed in Chapter III), had to be considered. Further, it was important that the amino acid residue modified was proximal or in the region involved in binding to the receptor, without, however, altering the binding characteristics of the hormone.

The chemical modification of Trp-25 residue of glucagon with nitrosulfenyl reagents (Epand and Cote, 1976) was shown to have equal or even enhanced potency to stimulate adenylate cyclase and to bind to receptor. Also, the carboxyl terminus of the hormone has been shown either by chemical modifications or from partial sequences of glucagon, to be important in binding of the hormone to plasma membrane (Epand, 1980). Therefore Trp-25 was selected as a candidate for covalent labeling with the photoaffinity reagent, 2-nitro-4-azidophenylsulfenyl chloride. The

synthesis of the reagent, modification of glucagon and characterization of the derivative, are described in Chapter I. The biological potency of the derivative in terms of adenylate cyclase activation and binding to liver plasma membranes, as well as the covalent photolabeling of the receptor are described in Chapter II. The molecular weight characterization of the glucagon receptor, as well as the characterization of other photolabeled products are described in Chapter III.

CHAPTER I

Synthesis and Characterization of 2-nitro-4-azidosulfenyl chloride (NAPSCl) and its Application to the Preparation of the Photoreactive Glucagon Derivative: Glucagon-NAPS

CONTENTS OF CHAPTER I

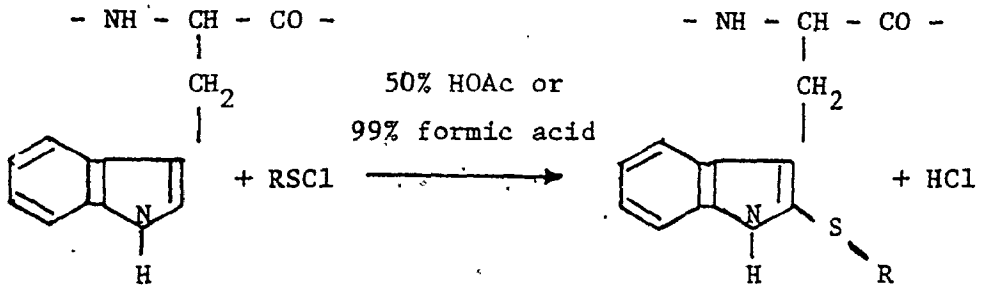
RESULTS

INTRODUCTION

The rationale which led to the selection of tryptophan-25 residue as the candidate for the modification of glucagon has been presented in the Introduction. The reagent required for such a modification had to be first of all specific and selective for tryptophan. Secondly, since the technique of photoaffinity labeling was to be applied it was required that the reagent carried a photoreactive species, in this case a phenyl azide.

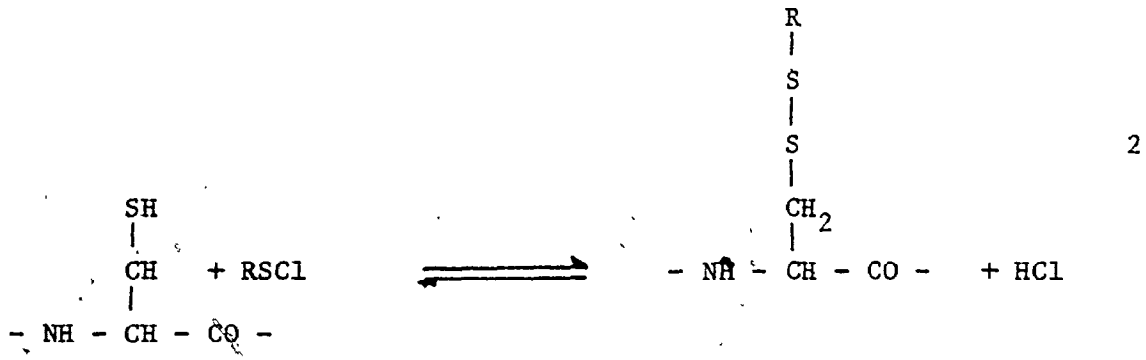
It has been previously reported that sulfenyl halides are highly specific reagents for tryptophan and cysteine residues of polypeptides and proteins (Scoffone et al. 1968; Fontana et al. 1968a,b; Veronese et al. 1968, 1970; Epand and Cote, 1976; Ramachandran et al. 1979) and since the reaction conditions are very mild, selective modification is readily achieved.

The reactions of 2-nitrophenylsulfenyl chloride (NPSCl), 2,4-dinitrophenylsulfenyl chloride (DNPSCl), (Scoffone et al. 1968), and of 2,4-dinitro-1,5-diphenylsulfenyl chloride (DNDPSCl) (Veronese et al. 1970) with tryptophan and tryptophan containing peptides in 30-50% acetic acid or 99% formic acid resulted in a modified tryptophan with a thioether function in position 2 of the indole ring [Eq. 1].



1

Cysteine is converted into an asymmetric disulfide and can be reversibly recovered by treatment with reducing agents (β-mercaptoethanol, thioglycolic acid or sodium borohydride) [Eq. 2] (Fontana et al. 1968a).



2

The selectivity of the reaction with tryptophan has been explained on the basis that sulfenyl halides react as electrophiles towards aromatic groups in the presence of Lewis acids, producing aromatic monosulfides (Buess and Khorash, 1950). With more reactive aromatic species, e.g. resorcinol or N,N'-dimethyl-aniline, no catalyst is needed and the reaction occurs readily. The indole ring of tryptophan behaves as a very reactive aromatic species towards these reagents. The reactivity of indole and substituted indoles towards sulfenyl halides has been reported (Wieland and Sarges, 1962; Fontana et al. 1966a,b). The lack of reactivity of other aromatic amino acid side chains such as those of phenylalanine and tyrosine has been explained in terms of less nucleophilicity towards sulfenyl halides than the indole ring (Scoffone et al. 1968).

Efficiency and selectivity of the reaction can be further ensured by the presence of a nitro group in the ortho-position of the aromatic ring which stabilizes the sulfenyl function against hydrolysis (Di Nunno et al. 1966). Also, the acidic reaction conditions inhibit sulfenamide formation and thus minimize nonspecific labeling of the peptide (Scoffone et al. 1968). In addition to that, the presence of the nitro group confers the advantage of light absorption in the visible region at wavelengths above 300 nm where the proteins are nonabsorbant and, therefore, the reaction of the reagent can be quantitated spectrophotometrically (Scoffone et al. 1968). Furthermore, it makes it possible to carry the irradiation studies in the visible region thus avoiding photolytic damage when biological systems are studied. Another advantage is that the presence of the nitro group decreases the half-life of the nitrene (i.e. it increases its reactivity) (Fleet et al. 1972).

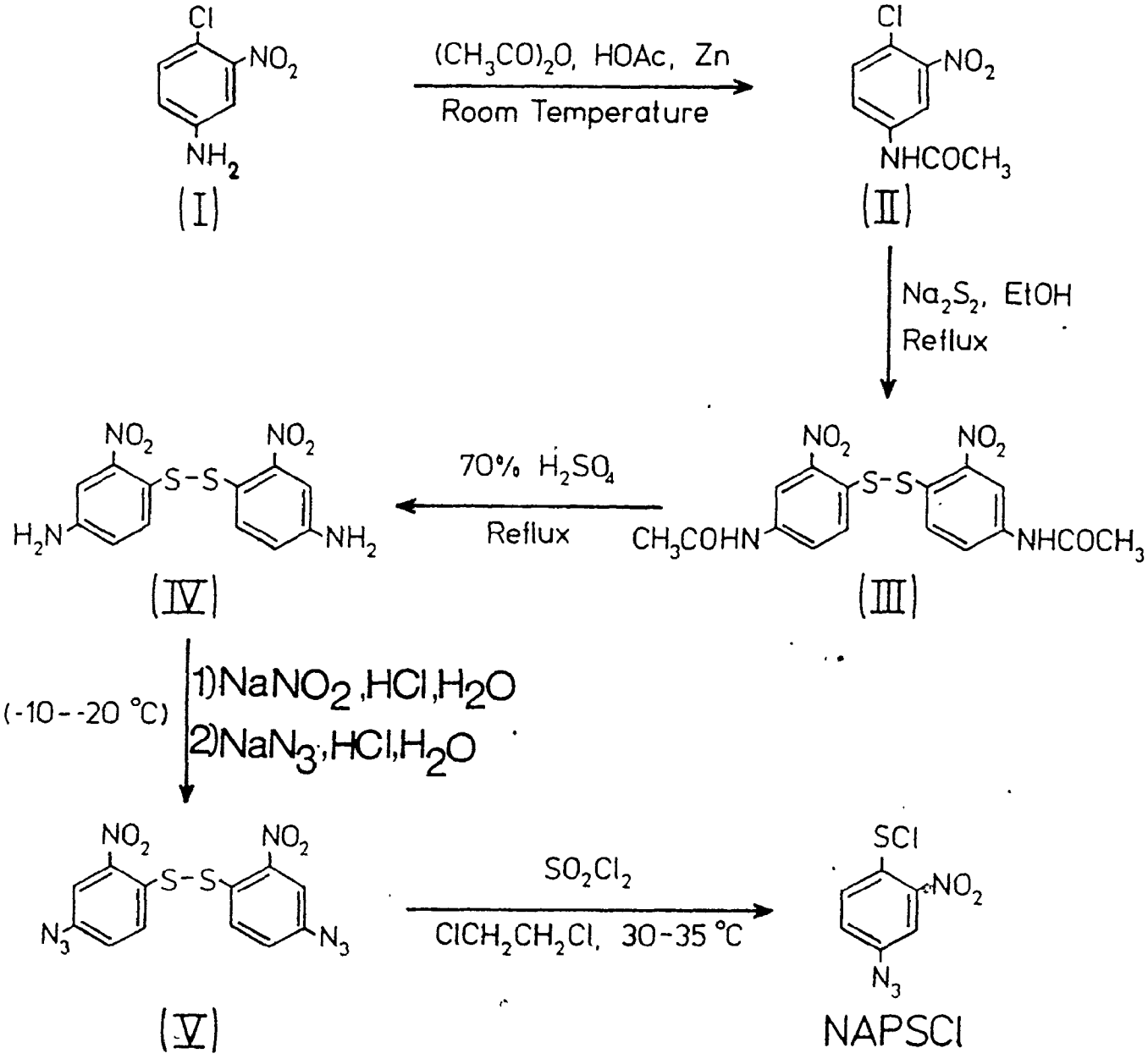
Taking into account the advantages of sulfenyl halides as tryptophan modifying reagents, as discussed previously, it was concluded that a nitrosulfenyl reagent with an azide molecule at the p-position would satisfy the requirements for the bifunctional photoaffinity reagents sought. Since such a reagent was not commercially available, its synthesis was undertaken.

As a starting material 4-chloro-3-nitroaniline was chosen as the closest precursor species for the azide synthesis. The chlorine molecule could be substituted by sulfur thus providing the precursor species for the sulfenyl halide. Since, on the one hand, the reaction conditions for an amine to an azide conversion were not favourable for the integrity of the sulfenyl chloride moiety, and on the other hand those for the chlorine substitution by sulfur were not favourable for the stability of the azide species, it was decided to proceed through a bis(phenyl)

intermediate. After the azide synthesis was completed, the disulfide bond could be cleaved under milder conditions selected to give the reagent required.

A. Synthesis of the photoaffinity reagent: 2-nitro-4-azido-phenylsulfenyl chloride (NAPSCl)

The synthesis of 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl) reagent was accomplished by a series of 5 reactions as shown in Scheme I. Initially the reaction of 4-chloro-3-nitroaniline with Na_2S_2 was attempted but it failed to give the required disulfide. This is due to the presence of the electron donating group $-NH_2$ on the aromatic ring which deactivates the ring towards nucleophilic substitution of the chlorine molecule by the disulfide anion. To overcome this effect the aniline was converted into an acetanilide by applying the procedure of Vogel (1965a) for acetylation of aromatic amines (Step 1). Although the acetamido group is also deactivating towards nucleophilic substitution like $-NH_2$, it is much less powerful because the electron withdrawing capacity of the carbonyl group tends to neutralize the electron-donating power of the nitrogen. Nucleophilic substitution of the chlorine by disulfide was therefore now possible, following a method analogous to that used for the preparation of bis(o-nitrophenyl)disulfide (Bogert and Stull, 1941) (Step 2).


Cl
NO2
NH2
(I)
(CH3CO)2O, HOAc, Zn
Room Temperature
Cl
NO2
NHCOCH3
(II)
Na2S2, EtOH
Reflux
NO2
NO2
S-S
CH3COHN
NHCOCH3
(III)
70% H2SO4
Reflux
NO2
NO2
S-S
H2N
NH2
(IV)
(-10--20 °C)
1)NaNO2,HCl,H2O
2)NaN3,HCl,H2O
NO2
NO2
S-S
N3
N3
(V)
SO2Cl2
ClCH2CH2Cl, 30-35 °C
SCl
NO2
N3
NAPSCl

Scheme I

Thiol groups react with oxygen or other oxidizing agents such as I_2 (at alkaline pH) to give short lived free radicals which rapidly dimerize to the disulfide. While sulfur compounds are good nucleophiles, most of them are poor leaving groups because of the low polarity of the C-S bond (Parker and Kharash, 1960)

After the synthesis of the disulfide, it was necessary to recover the amino group at the p-position because it is required as a precursor for the azide synthesis. This was accomplished by hydrolyzing the acetanilide using the method of Vogel (1956b) for deacetylation of derivatives of aromatic amines (Step 3).

The azide derivative was subsequently obtained by reacting the diazonium salt of the aniline with hydrazoic acid according to the procedure for phenyl azide synthesis (Fleet et al. 1972) (Step 4). During azide formation the disulfide bond remains stable. It has been previously shown (Parker and Kharash, 1960) that in the presence of the nucleophiles SCN^-, I^-, N_3^-, $C_6H_5NH_2$, the phenyldisulfide molecules tested were recovered unchanged, indicating that even reactive disulfides are not cleaved by these relatively powerful nucleophiles. The order of nucleophilicity for the displacement of mercaptides from disulfides has been shown to be as follows (Parker and Kharash, 1960): $C_2H_5S^- > C_6H_5S^- > CN^- > OH^- > DNPS^- > N_3^- > SCN^-$. The final reaction included the chlorolysis of the disulfide (Kuhle, 1973) by using the mild chlorinating agent sulfuryl chloride and thus avoiding any attack of the halogen on the aromatic ring (Step 5).

The starting reagent as well as the product of all the reactions were characterized by proton NMR, IR and Mass Spectra. In all cases,

the results agreed with those expected for the reported compound.

B. Reactivity and specificity of NAPSCl

Even though the reactivity and specificity of nitrosulfenyl halide molecules for Trp and Trp-containing polypeptides have been studied (Scoffone et al. 1968; Veronese et al. 1970), it was necessary to show that the sulfenyl chloride moiety of NAPSCl shared these same properties. This was considered essential before the modification of glucagon was attempted. It should be noted again that in this case glucagon is an ideal polypeptide because it contains only one Trp residue and has no cysteine (Bromer et al. 1957). The isoelectric point of glucagon lies near pH 7.0, and its solubility in the physiological pH range is quite low (<50 µg/ml). However, its solubility is greater than 10 mg/ml at pH 2-3 and pH 9-11. The absorptivity of glucagon in dilute aqueous acid (pH 2.0, at λmax 278 mm) is 8310 $M^{-1}cm^{-1}$, with E = 2.38·$Lg^{-1}cm^{-1}$ (Gratzer et al. 1967). The solubility properties of both the reagent NAPSCl and glucagon under the acidic reaction conditions required for the Trp-modification as well as the reactivity of NAPSCl contributed to a high yield of modified hormone.

The successful modification of Trp-25 residue of glucagon by sulfenyl halides has been reported previously (Veronese et al. 1970; Epand and Cote, 1976). However, since the glucagon-NAPS derivative would be used to specifically label the glucagon-receptor, it was important to characterize the derivative and to show that this chemical modification fulfilled the conditions for the modification of ligands (see Introduction).

C. Photochemical properties

Aromatic azides were first successfully applied as photoaffinity labels by Fleet et al. (1972), Knowles (1972) and by Richards and his groups (Yoshioha et al. (1973); Hew et al. (1973)) in studies of haptene-antibody interactions. The photochemical intermediates produced are nitrenes which are chemically stable with a relatively long life time (10^{-4} sec) (Knowles, 1972). Aryl nitrenes half lives, however, can vary with their substituents and a range of reagents of different reactivities have been reported.

The reactions open to nitrenes have been studied (Knowles, 1972; Hanstein, 1979) (Scheme II).

Nitrenes generated from aliphatic azides tend to stabilize themselves through intramolecular rearrangement, rather than intermolecular insertion reactions (Hanstein, 1979) [Eq. 3].

$$R - CH_2 - N_3 \xrightarrow{N_2} R - CH_2 - \ddot{N} \longrightarrow R - CH = NH \qquad 3$$

Aromatic azides are stable in the dark and at moderate temperatures, but the resultant nitrene can undergo direct insertion, abstraction coupling, or addition reactions which can result in the covalent attachment of the ligand to the site of interactions. Aryl nitrenes are not very susceptible to rearrangement and if that occurs, their major products are azepines whose ring expansion usually results in the attachment of the label to neighbouring nucleophiles (Knowles, 1972).

The aryl nitrenes generated upon photolysis can occur in two electronic states i.e. singlet and triplet, containing paired or unpaired electrons, respectively. The two electronic states differ

SCHEME II

Reactions of nitrenes:

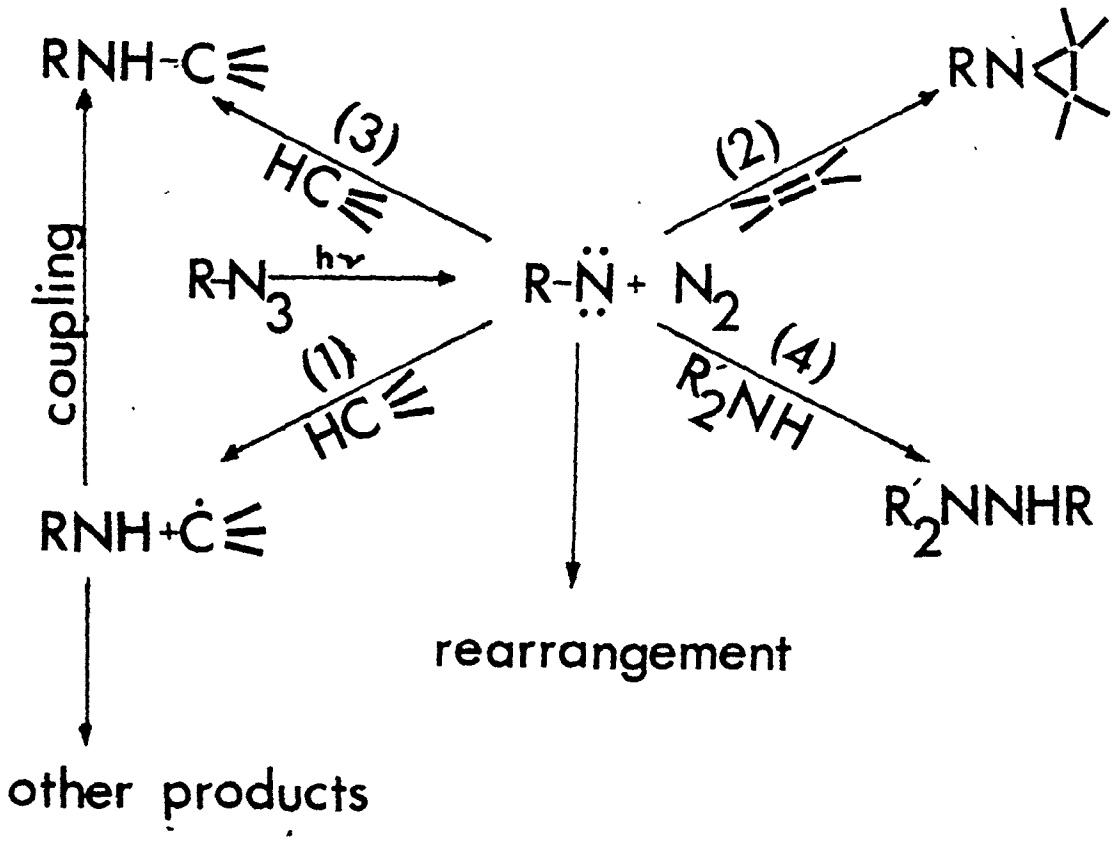
1) abstraction (normally of hydrogen from carbon)

2) cycloaddition

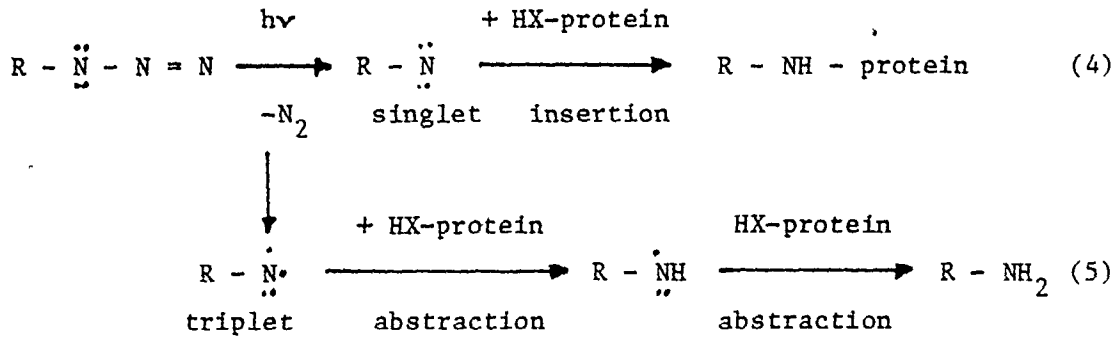
3) direct insertion (usually into C-H bonds)

4) attack by nucleophiles

5) rearrangement



drastically in their reactivities [Eq.4,5] and only singlet nitrenes are useful for photoaffinity labeling (Hanstein, 1979).



Triplet nitrenes usually abstract hydrogen, and are converted into amines and only rarely have insertion reactions been observed (Hanstein, 1979). Recent analytical studies of the photolysis products of phenyl and nitrophenyl azides have shown that only a small percent (5-10) of the total nitrenes generated are triplets which are converted into amines. Azo compounds and anthranil were the other products detected. This led to the postulation of a multiplicity of nitrene species as a result of intersystem crossing of singlet to triplet azides and although nitro substituents may increase the electrophilicity of the singlet nitrene, it was suggested that they may promote such intercrossing thus increasing the fraction of triplet nitrenes. Even though no intermolecular insertion products were isolated, the intramolecular insertion product observed suggested that the nitrophenyl nitrenes may react "intramolecularly" with neighbouring nucleophiles of proteins upon generation in situ at a binding site of a biological macromolecule (Nakayama et al. 1979).

Since the life time of the nitrene intermediate species (10^{-4} sec) is relatively long, a question is raised concerning the possibility of movement away from the ligand-receptor complex and of labeling loci other

than the active site. Such a situation may be of importance where small molecules, rapidly exchanging with proteins, are concerned. This is less significant, however, in protein-protein interactions, because the larger molecules should have slower exchange rates and generation of the nitrene would not be expected to modify these interactions sufficiently to cause dissociation. Nitrophenyl azides have an advantage in this case since the nitro substituent increases the nitrene reactivity, as has been discussed and, therefore, they have been successfully used to photolabel protein molecules (see Introduction).

The choice of proper wavelength is important in photoaffinity labeling and requires the knowledge of the absorption characteristics of the photoaffinity ligand. High efficiency in photolabeling can be achieved if the light emission peak coincides with the absorption maxima of the ligand. The light sensitivity of the receptor molecule photolabeled has to be taken into consideration as well, to prevent photolytic damage.

With aromatic azides, in addition to the spectrum of the parent hydrocarbon, they also absorb between 300-400 nm due to the azido group. Nitrophenyl azides with absorption maxima between 250-300 nm and 350-400 nm can be expected to be photolysed at both ranges. Since the minimum photolytic damage to protein is to be preferred, the less-energy rich radiation above 350 nm should be chosen. This not only ensures the integrity of the membrane or protein photolabeled, but it also results in a more photostable product (Hanstein, 1979).

The energy required to produce singlet nitrenes, a spin-allowed process, is 40-50 kcal $mole^{-1}$ (Reiser and Wagner, 1971), corresponding to light at 600-700 nm. Light absorbed in most of the visible spectrum therefore is able to photolyse aromatic azides.

Mercury lamps frequently used, emit u.v. light mainly at 253.7, 313 and 366 nm in intensity proportions depending on the construction of the lamp. Xenon lamps produce a significant amount of light between 225-400 nm, even though most of the emission is at longer wavelengths (Murov, 1973). Liquid or glass filters with controlled spectral transmittance can be used with either lamps when photolysis at a certain wavelength is required.

The popularity of aryl azides as photoaffinity reagents is due to their chemical stability under physiological conditions in the dark (Bayley and Knowles, 1977). However, it has been found that they are rapidly reduced to their corresponding amines by dithiothreitol (DTT) at physiological pH and temperature and that the rate of the reduction is pH dependent (2nd order rate const. = 0.41 $M^{-1}s^{-1}$ pH 8.0, 30°C). The initial rate of reaction with the monothiols, glutathione (GSH) and 2-mercaptoethanol is 0.002 and 0.001 times that of DTT respectively (Staros, 1978). This observation is important in photolabeling studies with biological systems that require thiols for stability or activity and when electron deficient phenyl azides are used, since they are reduced even faster (Staros, 1978). On the other hand, this property may be utilized in decreasing the level of nonspecific labeling, since nontightly bound ligands may be reduced by DTT immediately prior to photolysis. In addition, if thiols are required for activity, low concentrations of monothiols can be used as efficient scavengers.

D. Peptide Hormone Iodination

Several factors were considered that led to the synthesis of radioiodinated glucagon and glucagon-NAPS. A ligand with high specific

radioactivity was required so that the receptor binding studies could be monitored in much greater detail. Secondly, there was a need for a means of detecting the covalently labeled protein(s) by the photoaffinity probe glucagon-NAPS and thirdly, several improved methods for the radioiodination of glucagon have been investigated (Jorgensen and Larsen, 1972; Holohan et al. 1973; Desbuquois et al. 1974; Desbuquous, 1975; Von Schenck et al. 1976; Von Schenck and Jeppson, 1977).

Iodination of polypeptide hormones may influence their binding to antibodies (Berson and Yalow, 1966; Hunter, 1971; Von Schenck et al. 1976) and cell membrane receptors as well as in the case of glucagon it may influence its potency to activate adenylate cyclase (Rodbell et al. 1971; Bromer et al. 1973; Desbuquois, 1975; Lin et al. 1976).

One possibility for the discrepancies reported in the kinetics of binding and action of ^{125}I-glucagon (see Chapter II) is the recent finding that the radioiodinated peptide is a mixture of hormone molecules that has differing biological potency from native glucagon (Bromer et al. 1973; Desbuquois, 1975; Lin et al. 1976). Furthermore, the method of iodination, the amount and localization of iodine incorporated and storage conditions may influence the quality of the labeled hormone (Von Schenck et al. 1976).

Theoretically eight different types of iodotyrosine-glucagon are possible since two iodine atoms can be substituted into each tyrosine and since glucagon has two tyrosines (Tyr 10 and Tyr 13). The degree of iodine substitution of the tyrosine residues of glucagon is pH dependent with a predominance of monoiodotyrosine (MIT) and restricted diiodotyrosine (DIT) formation at pH 10.0, (<5% at 0.3 g atomI/mol glucagon)

(Von Schenck et al. 1976). The reasons for this pH dependence of the iodine distribution on the tyrosine residues are not clear. The random coil conformation of glucagon at pH 6.0-7.5 (Gratzer et al. 1968; Patel 1970) may explain the predominance of DIT formation. At pH 10.0, the pK of the individual tyrosines play a greater role in limiting the rate of the reaction. Iodination from monoiodotyrosine (pK 8.2) to diiodotyrosine (pK 6.4) proceeds more completely at neutral pH than at pH 10.0 (Von Schenck et al. 1976).

Studies on the kinetic relationship of receptor binding between glucagon and the glucagon-NAPS derivative required adaptation of a radioiodination method that would result in minimal changes in the hormone structure. Furthermore, the integrity of the azide moiety of glucagon-NAPS during iodination had to be secured. Therefore, iodination catalyzed by lactopetoxidase at pH 10,0 in the presence of propyleneglycol was chosen since the enzyme is active at this pH. The reaction conditions are relatively mild and the production of radioiodinated glucagon with high specific activity is possible (Von Schenck and Jeppson, 1977). 78% of the radioactivity incorporated by this method was shown to be predominantly Tyr-13 iodination. The monoiodotyrosine-13-glucagon can be concentrated and separated almost completely from other iodoglucagon species as well as native glucagon by DEAE-cellulose chromatography as reported by Desbuquois (1975) and Von Schenck and Jeppson,(1977).

Iodoglucagon is often stored and used in the presence of 1-2% bovine plasma albumin (BSA) so as to minimize nonspecific adsorption of the microgram quantities of the hormone to glass walls, cellulose, etc. Most significantly, the excess of BSA provides a sink for the radicals generated by gamma rays which react with BSA rather than the hormone. However, proteolytic degradation of the hormone has been associated with BSA (Wilson and Foster, 1971) and when used, the presence of a protease inhibitor is required, such as Trasylol (Eisentraut et al. 1968), benzamidine (Ensinck et al. 1972) or gelatine (Jurcovicova and Klimes, 1978). Recently it has been reported that storage in n-propanol-water (1:1 v/v) can stabilize radioiodoglucagon so that it can be used for radioimmunoassays even after six weeks (Jironsek, 1979).

E. Immunoreactivity and photolabeling properties of ^{125}I-glucagon-NAPS peptide

Before attempting the photolabeling of the glucagon receptor in liver plasma membrane, the ability of ^{125}I-glucagon-NAPS to covalently label macromolecules upon photolysis had to be demonstrated, and the conditions required for photolysis defined.

Bovine serum albumin, which has often been used in studies of protein behaviour in solution because of its commercial availability, was selected for the nonspecific photolabeling of macromolecules by ^{125}I-glucagon-NAPS. In addition, a glucagon specific antibody that had been raised against glucagon covalently bound to albumin (Tager et al. 1977) was chosen for the demonstration of specific photolabeling.

The production of glucagon antisera and the development of a

radioimmunoassay for the hormone were first reported by Unger et al. (1959). Since then a lot of information about the immunoreactivity of glucagon has been obtained and a number of radioimmunoassays have been developed (Heding, 1971; Holst and Aasted, 1974; Unger et al. 1976, Von Schenck, 1977 ; Holst, 1978; Foe et al. 1978).

Investigations of the structural requirements for glucagon binding to its antibodies have often given complex results due to the variability of glucagon antisera produced, the majority of which cross-react with gut glucagon (Unger et al. 1966; Eisentraut et al. 1968). Accumulated evidence has suggested that specific antisera of pancreatic glucagon are directed towards the -COOH terminus of the hormone, whereas those crossreacting with gut glucagon are directed towards the NH_2 terminus and mid-portion of the peptide (Senyk et al. 1971; Assan and Slusher, 1972; Tager, 1977; Von Schenck, 1977). Senyk et al. (1972) have explained the variability in glucagon antisera produced. They have suggested that the NH_2-terminal portion of glucagon is haptenic and the -COOH terminal immunogenic when free glucagon is the antigen. However, the -COOH terminal becomes haptenic when the peptide is covalently bound to an immunogenic macromolecule such as albumin.

In agreement with previous reports (Vinik and Hardcastle, 1974), the NH_2-terminal tryptic fragment of glucagon (1-17 residues) did not react with the antisera tested by Tager et al. (1977), proving that this region is not haptenic when glucagon conjugated to a large protein is used as an antigen. But it was also shown that the 19-29 -COOH terminal fragment was not always sufficient for reactivity, while fragment 1-27 residues reacted well (Tager et al. 1977). Furthermore, equimolar

mixtures of 1-17 and 18-29 tryptic fragments of pancreatic glucagon did not restore total immunoreactivity (Vinik and Hardcastle, 1974) suggesting that either the antigenic determinant is located between residues 17-19 or a more complex three-dimensional conformation of the hormone is required for antigenicity (Tager et al. 1977).

Considering the above, testing the ^{125}I-glucagon-NAPS immunoreactivity would not only provide a specific protein complex for photoaffinity studies, but it might also give further indication of any large structural differences from native glucagon.

Protein A, a cell wall polypeptide 40,000 daltons, produced by Staphylococcus aureus, binds the Fc portion of certain types of immunoglobulins, primarily IgG, and therefore can be used as an immunoabsorbent (Kessler, 1975, 1976; Goding, 1978; O'Keefe and Bennett, 1980). Protein A covalently bound to agarose has been used conveniently to purify rabbit, human or murine IgG (Goding, 1978). Because of its fast sedimentation characteristic, it was considered to be used for absorption and separation of the glucagon and glucagon-NAPS antibody complexes in solution, thus providing a quick and convenient way of assaying the immunoreactivity of these hormone peptides.

F. Conformational studies

Comparatively little is known about the secondary and tertiary structures of glucagon under physiological conditions, and interpretation of what is known is controversial as a result of its conformational polymorphism.

The physical techniques used to determine the secondary

structure of glucagon in solution have defined at least three conformational states of high, low and no α-helical content. The conformations with high α-helical content includes:

a) a 55% α-helical structure found in the crystalline state (crystals grown at pH 9.2, studied at pH 7.0), by x-ray analysis at ≃ 3.0Å resolution (Sasaki et al. 1975). Stabilization of this conformation is contributed to by hydrophobic interactions between trimers involving the hydrophobic region around Tyr 10 and Tyr 13 at one end and Phe 22 and Trp 25 at the other.

b) a conformation containing 35% α-helix as determined by circular dichroism (C.D.), and optical rotatory dispersion (O.R.D.) measurements (Blanchard and King, 1966; Panijpan and Gratzer, 1974); found in freshly made concentrated basic solution (5 mg/ml, pH 10). The concentration dependence of CD and sedimentation equilibrium studies define a monomeric ⇌ trimeric associated state thus equating this trimeric form to the trimers which make up the asymmetric unit in the crystal (Gratzer et al. 1972).

c) a conformation with as much as 100% α-helix adopted in the helix-inducing solvent 2-chloroethanol (Srere and Brooks, 1969). In certain other organic solvents (ethylene or propylene glycol/water mixtures), glucagon also folds in an α-helical structure (Contaxis and Epand, 1974). It also becomes more α-helical when bound to lipids, i.e. cationic detergents (Bornet and Edelhoch, 1971), phospholipid micelles (Schneider and Edelhoch, 1972) or bilayers (Epand et al. 1977b).

The major conformational state of low helicity is that of 10-15% α-helix in dilute solutions (0.5 mg/ml) at pH 2-10, increasing

to 35% in more concentrated solutions (10 mg/ml) (Srere and Brooks, 1969). This phenomenon has been explained in terms of increased stabilization by hydrophobic interactions of the aromatic amino acid residues (Srere and Brooks, 1969; Gratzer and Beaven, 1969).

The third conformational state is a largely antiparallel β-structure in old concentrated solutions at pH 2-10 detected by the large viscosity in the solution and gel formation (Beaven et al. 1969; Epand, 1971; Yu and Liu, 1972).

The Chou-Fasman empirical procedure for predicting regions of secondary structure in proteins (Chou et al. 1975) suggests two major highly structured conformations of glucagon in a delicately balanced equilibrium: a conformation with β-turns located at residues 2-5, 10-13, 15-18, a α-helix from 19 to 27 and a β-sheet from 5 to 10 (31% α, 21% β), and a conformation with two regions, residues 5-10 and 19-27 in β-sheets (0% α, 52% β).

CD studies of the concentration and time dependence of $\alpha \rightarrow \beta$ transition of glucagon in 0.01 N HCl or at pH 9.2, showed that the most thermodynamically stable state in dilute or concentrated solutions, contains at least 50% β-structure in contrast to the structure preferred in the crystal lattice ($\simeq$ 55% α-helix) (Moran et al. 1977).

Comparative studies of protein conformation in the crystal and in solution have indicated that there is little structural differences for molecules of medium and large size (Rupley, 1969). However, as indicated above, this does not seem to be the case for small polypeptides, like glucagon, whose conformation seems to be susceptible to the local environment and can be altered by solvent perturbations.

Studies with glucagon fragments and chemically modified glucagon on adenylate cyclase activation and binding to the receptor (discussed in Chapter III) indicate that at physiological pH and concentration $\simeq 10^{-11}$M, glucagon must exist as a monomer and it is unlikely a) that glucagon trimers or higher oligomers have any role in receptor binding or b) that the crystal defined conformation is responsible for the activity of glucagon. It is possible, however, that the association of monomers may be involved on storage of glucagon in pancreatic granules and protection against enzymatic proteolysis (Blundell et al. 1972). Therefore, if glucagon in low concentrations as those found under physiological conditions is free from interactions with other glucagon molecules, two important questions arise: a) whether the molecule assumes a random structure in dilute solutions, and b) what conformation is the preferred one at its hormone binding site?

There has been considerable controversy concerning the conformation of glucagon in dilute solutions. It has been stated by a number of workers (Schwyzer, 1963; Gratzer et al. 1968; Gratzer and Beaven, 1969; Edelhoch and Lippolt, 1969; Formisano, 1977; Rothgeb et al. 1978), that in dilute solutions glucagon is largely unstructured with a few stable intramolecular bonds and that the structure of the molecule under these conditions may be taken as the structure of the monomeric glucagon. However, there is substantial evidence to the contrary that glucagon does have a more structured conformation. The viscosity of glucagon in dilute solutions is similar to that of a globular protein as opposed to a random coil (Epand, 1971); fluorescence quenching has given a distance of 12Å between Trp 25 and one of the tyrosines, compared

to the average distance of 29Å expected for a random coil (Epand, 1972); tritium exchange rates indicate that there are 8.5 ± 2.0 hydrogen bonded amide hydrogens (McBride-Warren and Epand, 1972); proton magnetic resonance studies have indicated a number of structured regions (Epand, 1972).

Recently, a model of glucagon has been proposed with a tertiary structure where the three dimensional arrangement of the amino acid residues create an empty space bounded by aromatic side groups (Tyr 10, Phe 22) into which a hydrophobic aromatic moiety of the glucagon receptor may bind (Korn and Ottensmeyer, 1978). This model is in agreement with the results obtained from physical and chemical studies which indicate that the monomeric glucagon in dilute aqueous solutions may have a globular conformation rather than being largely unstructured.

Taking into consideration the available information about the conformation of glucagon and its polymorphism in solution and the possibility that there are conformational requirements for interactions with the receptor, it was important to demonstrate that the glucagon-NAPS derivative assumed a comparable conformation to that of native glucagon under similar conditions. Furthermore, the ability of glucagon-NAPS to covalently label neighbouring molecules upon irradiation was considered for the investigation of the oligomeric forms of glucagon in solution.

Conformational models proposed in the Literature on the conformation requirements for glucagon binding to the receptor and biological activity are reviewed in Chapters II and III.

METHODS

1. Synthesis of 4-chloro-3-nitroacetanilide (II):

Product II was prepared according to the method for acetylation of aromatic amines (Vogel, 1956a). 14.0 g of 4-chloro-3-nitroaniline (I) were transferred into a 100 ml round bottom flask containing 30 ml solution of 1:1 v/v acetic-anhydride-glacial acetic acid and 0.15 g zinc powder as a catalyst. The reaction was exothermic, resulting in a dark brown solution. For completion of the reaction the mixture was further heated in a boiling water bath for 45 min. The hot solution was poured in a thin stream into a beaker containing about 200 ml crushed ice. A light brown precipitate was immediately formed. The mixture was left at room temperature for half an hour and then it was filtered and washed twice with ice cold distilled water. The product was recrystallized from methanol or 95% ethanol, until very light brown, almost white, needle shape crystals were obtained. Recrystallization from 95% ethanol (twice) gave a 76% yield. The product was identified from the structural information obtained from NMR, IR and Mass Spectra. The product melted at 148-9°C and had a mol. wt. 214.6 by M.S.

2. Synthesis of 2,2'-dinitro-4,4'-diacetaminodiphenyl disulfide (III):

III was synthesized from II by a method analogous to that used to prepare bis(0-nitrophenyl)disulfide (Bogert and Stull, 1941). 20.0 g of sodium sulfide (Na_2S-$9H_2O$) were dissolved in a 20 ml solution of water-95% ethanol (1:1 v/v) while warming and stirring the solution. When all

of the sodium sulfide had dissolved, the solution was filtered while still hot and 3.0 g of sulfur were slowly added to the filtrate with continued stirring and warming. The dark yellow solution obtained was added over 5–10 min, while still hot through the condenser to a vigorously stirred, refluxing solution of 13.0 g of 4-chloro-3-nitroacetanilide in 20 ml 95% ethanol in a 100 ml round bottom flask.

The mixture was then heated on a steam bath gently at first and then at full heat for 2 hours. After cooling in ice, the mixture was added in a thin stream to a beaker containing 600 ml crushed ice and distilled water, with stirring. The mixture was left stirring for 1 hr. The organic disulfide was then filtered and washed twice with cold distilled water and once with 50 ml 95% alcohol to remove any unreacted 4-chloro-3-nitroacetanilide. The product was recrystallized from dimethyl formamide (DMF) to give orange needles (yield 50%). The product was identified from the spectral information obtained from NMR, IR and Mass Spectra. It had a mol. wt. of 422.0 and decomposed above 200°C.

3. Synthesis of 2,2'-dinitro-4,4'-diaminodiphenyl disulfide (IV):

IV was synthesized by the method for deacetylation of derivatives of aromatic amines (Vogel, 1956b). 7.0 g of III were placed in a 50 ml round flask containing 20 ml 70% sulfuric acid. The flask was heated with a reflux condenser, and while warming and shaking the flask 10 ml of concentrated sulfuric acid were added. The flask was heated further until a reddish-brown solution was obtained and a drop of the solution diluted in 1 ml water remained clear.

The hot solution was then poured in a thin stream with stirring

into a beaker containing 100 ml crushed ice and was left to cool for half an hour. 50% NaOH was slowly added, with stirring and cooling until the solution became alkaline. The reddish-yellow product was left to stand in ice for one hour. It was then filtered off, washed and dried in the oven (110°C). The reddish flaky crystals were recovered in 79% yield. The product was identified from the spectral information obtained from NMR, IR and Mass Spectra. It had a mol. wt. of 338.4 and decomposed above 150°C.

4. Synthesis of 2,2'-dinitro-4,4'-diazidodiphenyl disulfide (V):

V was synthesized by reacting the diazonium salt of IV with hydrazoic acid according to the procedure of Fleet et al. (1972). 3.5 g of IV were added to a 40 ml solution of concentrated HCl-H_2O (3:1 v/v) and the mixture was stirred for 15 min in a methanol-dry ice bath while maintaining the temperature of the reaction solution at -10 to -20°C. 3.2 g of $NaNO_2$ (≃ 100% excess) dissolved in 5 ml distilled water were slowly added with stirring. Care was taken at this and subsequent steps that the temperature did not rise above 0°C. The reddish solution thus obtained was filtered using prechilled equipment. The filtrate was collected in a large conical flask. 3.0 g NaN_3 (100% excess) dissolved in 5 ml distilled water were added dropwise, with stirring. A light yellow-green precipitate appeared and extensive frothing occurred owing to the evolution of N_2 gas; care had to be taken that the mixture did not overflow. The mixture was then removed from the methanol-dry ice bath and allowed to warm up with continued stirring. When no more N_2 gas was evolving, the product was filtered, washed twice with distilled water and

recrystallized from DMF. The dark yellow needle shaped crystals gave an 86% yield. The product was identified from the spectral information obtained from NMR, IR and Mass Spectra. It had a mol. wt. of 390.4 and decomposed above 130°C.

5. Synthesis of 2-nitro-4-azidophenylsulfenyl chloride (NAPSCl):

NAPSCl was synthesized by chlorolysis of the disulfide (Kuhle, 1973). 3.6 g of V were placed in a 100 ml conical flask containing 25 ml of 1,2-dichloroethane. 10 ml of sulfuryl chloride were slowly added and the mixture was stirred and heated to 30-35°C, until all the disulfide had dissolved and a reddish solution was obtained. The solution was dried under vacuum in a rotary evaporator, and the product was redissolved in 25 ml ethyl acetate by warming it up in a hot water bath. Bright yellow needle-shaped crystals were obtained upon cooling in ice. The product was filtered and washed with ice cold ethyl acetate. It gave a 96% yield and was identified from the spectral information obtained from NMR, IR and Mass Spectra. It had a mol. wt. of 230 and its melting point 75-78°C.

Anal. Calcd. for $C_6H_3N_4O_2SCl$: C, 31.24; H, 1.31; N, 24.24; S, 13.90; Cl, 15.37.

Found: C, 31.35; H, 1.47; N, 24.19; S, 13.61; Cl, 15.16. The product was stored at -20°C in the dark.

6. Reaction of NAPSCl with Amino Acids

1 ml of a standard amino acid mixture containing 2.5 μmoles/ml of all common amino acids was left to react with 100% excess NAPSCl in glacial acetic acid (1 ml), in the dark at room temperature. After 2 h

the reaction was stopped by adding 5 ml water and the precipitated unreacted reagent was removed by centrifugation in a bench centrifuge at top speed for 10 min. The supernatant was filtered, washed with 2 ml water and dried under vacuum. The dried amino acid mixture was redissolved in 1 ml citrate buffer pH 2.2 and an amino acid analysis was run on a Beckman 120C analyzer. The amino acid mixture was also exposed to glacial acetic acid in the absence of NAPSCl as a control.

NAPSCl was incubated under similar conditions with either methionine, tyrosine, phenylalanine or histidine for 30 min. At regular time intervals, 0.1 ml aliquots were withdrawn and diluted to 0.5 ml with water. These samples were centrifuged in a bench centrifuge at top speed for 10 min. Samples (50 µl) of the supernatant were spotted on Whatman #1 chromatography paper. The chromatograms were run for 20 hrs with Butanol-acetic acid-water mixture (20:5:9 v/v) (ascending chromatography). Amino acid solutions containing no reagent were similarly treated and spotted. R_f values were compared with controls after spraying with ninhydrin.

7. Kinetic measurements of the reaction of NAPSCl with Tryptophan

The rate of the reaction of NAPSCl with tryptophan was determined by titration of the unreacted NAPSCl using the Orr and Kharash (1953) method as modified by Scoffone et al. (1968), or by fluorescence measurements.

At time zero, a solution (30 ml) of tryptopham (5×10^{-4}M) was mixed with a 10 ml solution of reagent (10^{-1}M) in glacial acetic acid. Samples (5 ml) of the reaction mixture were removed

at recorded time intervals and added to a 100 ml flask containing 10 ml of 5% KI solution. 50 ml of water were also added and the solution was titrated with 0.01 N $Na_2S_2O_3$ to the disappearance of free iodine. The same procedure was also followed in the absence of tryptophan as a control. Because of the need to observe the end point of titration, the titration was carried out in the light, but the tryptophan-NAPSCl reaction solution was kept in the dark to avoid any reaction products due to azide photolysis.

For fluorescence measurements 10 µl of the reaction solution were removed at recorded time intervals and diluted to 5 ml with water in order to precipitate any unreacted reagent. The diluted samples were centrifuged as described above, filtered, and the decrease in tryptophan fluorescence emission excited at 295 nm was measured using a Perkin-Elmer MPF-44 fluorimeter. 10 µl of an equimolar tryptophan solution were diluted to 5 ml with water and its fluorescence emission was taken as that of zero time.

8. Reaction of Glucagon with NAPSCl

Glucagon-NAPS was prepared in the dark according to the method of Veronese et al. (1970) with the exception that the reaction was carried out with a 2:1 molar ratio of reagent to protein, to avoid nonspecific labeling.

To a solution of 5 mg (1.4 µmoles) of glucagon dissolved in 0.8 ml of 50% acetic acid, 0.6 mg (2.8 µmoles) of NAPSCl reagent in 0.2 ml of glacial acetic acid were added with vigorous stirring. After standing for 1 min at room temperature the entire solution was applied to a Sephadex G-25 fine column (1.5 x 50 cm) equilibrated with 0.2 N acetic acid. Glucagon-NAPS was eluted with 0.2 N acetic acid. 3.0 ml fractions

were collected at a flow rate of 35.0 ml/hr. The elution pattern was monitored by absorption measurements at λ_{max} = 280 nm. Glucagon-NAPS was eluted in the void volume. The fractions were pooled, lyophilized (4.8 mg, yield >95%) and the product was stored at 4°C, in the dark and in a desiccator.

9. Purification and Characterization of Glucagon-NAPS

Glucagon-NAPS was purified by partition chromatography according to Hruby and Grosinsky (1971).

3.0 mg of glucagon-NAPS derivative were dissolved in .1 ml of 50% acetic acid. To this were added 0.4 ml of the upper phase of a Butanol-Ethanol-Benzene-0.2 M NH_4OH, (5:2:1:8 v/v), mixture that had been equilibrating overnight. The sample was applied to a Sephadex G-25 fine column (1.5 x 90 cm) which had been previously equilibrated with the lower phase of the above solution mixture after adjusting the pH to 9.4 with 50% acetic acid. Fractions (2.0 ml) were collected at a rate of 8-10 ml/hr. The elution pattern was monitored by the Lowry et al. (1951) protein determination, using bovine serum albumin as a standard and by absorption measurements at λ_{max} = 395 nm.

The presence of any unmodified glucagon in the product obtained after gel filtration through Sephadex G-25 was detected by measuring the fluorescence emitted by glucagon at 350 nm upon excitation at 295 nm in 7 M guanidine-HCl, 10 mM glycine pH 8.1.

The purity of the glucagon-NAPS derivative was also checked using 10% acrylamide gel electrophoresis according to Davis (1964).

The gels were stained with .025% Brilliant Blue-G in 10% TCA according to Bromer et al. (1971).

The purified glucagon-NAPS derivative was hydrolyzed *in vacuo* with 6 N HCl for 22 hrs, at 110°C, in the dark and the hydrolysate was subjected to an amino acid analysis.

Conformational changes in the glucagon-NAPS derivative were studied by circular dichroism measurements using a Cary Model 61 spectropolarimeter calibrated according to the values given by Cassim and Yang (1969). The temperature of the sample was maintained by a thermostable cell holder connected to a circulating water bath at constant temperature.

10. Photolysis of NAPSCl and Glucagon-NAPS

A 0.3 - 0.4 mM solution of NAPSCl in glacial acetic acid was photolysed for up to an hour in a thermally regulated jacketed glass cell, 10 cm away from the light source (High Pressure Xenon Lamp XBO-150W). The solution was bubbled with water-saturated N_2 gas 5 min prior to photolysis and water-saturated gas was blown through the solution during photolysis at a rapid enough rate to ensure mixing of the solution. The circulating water bath contained approximately 3 mm thick 0.01% (w/v) potassium phthalate as a filter for ultraviolet light. The temperature of the cell, monitored with a thermistor was maintained at 25°C. At recorded time intervals, aliquots were removed, their absorbance at the λ_{max} of 420 nm were recorded and were then dried under vacuum. IR spectra of these samples dissolved in chloroform were subsequently recorded.

Glucagon or glucagon-NAPS solutions in glacial acetic acid (10 μM) were photolysed for 8-10 min under the same conditions as NAPSCl. Photolysis was monitored by absorption measurements at λ_{max} = 395 nm.

11. Photolysis of ^{125}I-Glucagon and ^{125}I-Glucagon-NAPS

^{125}I-glucagon or ^{125}I-glucagon-NAPS in 0.2 M glycine pH 6.5-8.8

(5,000 - 4,000 cpm) were photolysed under the above conditions with the addition of a glass filter (Corning CS No. 7-51, 4.9-5.1 mm) fitted between the light source and the phthalate filter. Photolysis was carried out for 15-20 min. The integrity of the photolysed hormone derivatives was checked by gel filtration of the denatured peptides in a Sephadex G-75 column (50 cm x 0.5 cm) using 0.2 M glycine pH 2.6, 9 M urea, as eluant. The elution profile was monitored by measuring the radioactivity of the fractions collected using a well type γ-counter (Beckman Gamma-300). Nonphotolysed samples were run under similar conditions as controls.

12. Photochemical properties of NAPSCl

Various concentrations (10^{-5} - 10^{-4}M) of NAPSCl in glacial acetic acid were prepared in duplicate. One set was kept in the dark, the other was exposed to ordinary fluorescent room lighting. Decomposition of the azide derivative with time was monitored by changes in OD_{420}. The percent change in OD_{420} was compared with similar sets photolysed immediately and 40 hrs (kept in the dark) after preparation.

2,2'-dinitro-4,4'-diazidodiphenyldisulfide (10^{-4}M) was photolysed for 15 min under the same conditions as NAPSCl and the products of photolysis were analyzed by thin layer chromatography on silica gel plates with fluorescent indicator in methylene chloride-acetic acid (9:1 v/v, solvent A), or ethyl acetate (solvent B), at room temperature (23°C). The spots were visualized under short U.V. light. 2,2'-dinitro-4,4'-diaminodiphenyldisulfide was used as a standard for the detection of any diradical formation of nitrene upon irradiation which is likely to result in the formation of a primary amine (Hanstein et al. 1979).

13. Iodination of glucagon and glucagon-NAPS

Glucagon and the glucagon-NAPS derivative were iodinated by the lactoperoxidase method at pH 10.0 in the presence of propylene glycol (Von Schenk and Jeppsson, 1977). The amount of H_2O_2 was equivalent to that of $Na^{125}I$ (0.3 gatom ^{125}I/mol glucagon). The reaction was stopped by the addition of 1.0 ml of 0.1 M phosphate buffer pH 7.5 containing 0.05% NaN_3. The radioiodinated peptide was purified from unreacted ^{125}I by the talc absorption method (Goldstein and Bletcher, 1976). The hormone was eluted from talc with 50% ethanol and lyophilized immediately. The lyophilized material was redissolved in 1 mM NH_4HCO_3, pH 9.5 and applied to a DEAE-cellulose column (1 x 17 cm, DE 52, Whatman) swollen in the same medium. The monoiodopeptide derivative was eluted according to the method described by Desbuquois (1975). The initial 10 mM NH_4HCO_3 pH 9.2 was followed by a linear gradient to 100 mM NH_4HCO_3 pH 8.0. Both elution buffers contained 6 M urea. The radioactivity of the fractions (0.8 - 1.0 ml) was monitored by counting 5-10 µl samples. The fractions containing the monoiodopeptide were pooled and dialyzed against 100 mM NH_4HCO_3 pH 7.9 at 4°C. After removal of the NH_4HCO_3 by lyophilization the monoiodopeptide was stored in propanol-water (1:1) solution at -20°C (specific activity ~ 2.47 µCi/pmole assuming one g atom ^{125}I/mole of glucagon).

14. Photoaffinity labeling of bovine serum albumin and a glucagon specific antibody by ^{125}I-glucagon-NAPS

^{125}I-glucagon or ^{125}I-glucagon-NAPS (10,000-20,000 cpm, 1.8 µCi/pmole) were incubated in 5 ml solution of 0.2 M glycine pH 6.5-7.0, 2.5 mg/ml BSA, 750 KIU/ml Trasylol ± cold glucagon in the dark for 10 min at room temperature. The samples were then photolysed as described above and

subsequently lyophilized. The protein was denatured with 0.2 M glycine-HCl pH 2.6, 9 M urea and the noncovalently bound radiolabeled hormone was removed through a Sephadex G-75 column as previously described. The elution profile was monitored by UV absorption at λ_{max} = 278 nm and by counting the fractions collected. Nonphotolysed samples similarly treated were also run in the dark.

Equivalent amounts of radiolabeled peptides were also incubated with glucagon specific antiserum or normal rabbit serum in a 1:10 dilution. These sera prepared according to the method of Tager et al. (1977) were generously donated to us by Dr. J. Gauldie, McMaster University. Their affinity for glucagon was measured by Dr. N. Track, University of Toronto by radioimmunoassay and found suitable for this purpose.

The total volume of samples was 0.25 ml containing 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10.0% (w/v) glycerol, 2.0 mg/ml BSA, 750 K.I.U./ml Trasylol. After incubation at 4°C for 72 h in the dark, the samples were diluted 10 X with the same buffer, their pH was adjusted to 6.5-7.0 with dilute HCl and they were photolysed under the above conditions for 20 min.

After photolysis the radiolabeled IgG was purified in a Sephacryl S-300 column (80 cm x 2 cm) using the same incubation buffer as eluant. The pooled IgG fractions were denatured with 9 M urea after lyophilization and the noncovalently bound radiolabeled hormone was removed by gel filtration through a Sephadex G-75 column as described above. Nonphotolysed samples similarly treated in the dark were used as controls.

15. Radioimmunoprecipitation of ^{125}I-glucagon and ^{125}I-glucagon-NAPS

^{125}I-glucagon and ^{125}I-glucagon-NAPS (10,000-20,000 cpm, 1.8 µCi/pmole) were incubated with glucagon specific antiserum and normal serum and subsequently photolysed as described above. As controls other samples were similarly treated but not photolysed. After photolysis 200 µl of settled protein A - Sepharose CL-4B swollen in the same buffer were added to each sample. The suspensions were shaken for 24 h at 4°C. The immunoprecipitate protein A - Sepharose complex was then washed once with ice cold buffer containing 250 mM LiCl, 50 mM Tris-HCl pH 9.0, 1% (v/v) 2-mercaptoethanol, and three times with 125 mM LiCl, 25 mM Tris-HCl pH 9.0, 0/5% (v/v) 2-mercaptoethanol according to the method of Schauffhausen (1978). The precipitate was solubilized with 60 mM Tris- HCl pH 6.8, 6% (w/v) sodium dodecyl sulphate, 10% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol and counted.

16. Spectrophotometric Studies

All absorption measurements and spectra were recorded using a Cary 118 spectrophotometer.

17. Gel electrophoresis

The purity of glucagon and glucagon-NAPS peptide hormones was checked by gel electrophoresis using crosslinked 10% acrylamide cylindrical gels (10 x 0.6 cm), at pH 4.0, prepared according to the method of Davis (1964).

The covalently radiophotolabeled oligomeric species of glucagon

and glucagon-NAPS were separated by gel electrophoresis in crosslinked 12.5% acrylamide cylindrical gels (14 x 0.6 cm) containing 8 M urea, 0.1% SDS prepared according to the procedure described by Swank and Munkres (1971). Irradiated or nonirradiated samples were dried with N_2 gas, redissolved in 0.1 ml 8M urea, 10% SDS, heated at 60°C for 30 min and left at room temperature overnight. Mercaptoethanol 1.0% in 0.01 M phosphate buffer (10 µl), pH 6.8 and 0.1% bromophenol blue (5 µl) were added to each sample. The samples were electrophoresed for 18-20 h at 5-10 mA/gel. After electrophoresis, the gels were fixed with 10% trichloroacetic acid for no less than 3 h, frozen, cut into 2 mm slices with a Bio-Rad gel slicer (model 190) and counted in a Searle γ-counter. Protein bands were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 dye, 10% glacial acetic acid, 50% methanol for 3-4 h and destained with 8% acetic acid, 25% methanol. The marker proteins used for molecular weight determinations were: ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme, cytochrome c, bovine trypsin inhibitor, insulin, glucagon and Trasylol.

For the detection of radiolabeled aggregates of glucagon with a molecular weight higher than 40,000, as well as the covalently labeled BSA with ^{125}I-glucagon-NAPS samples were electrophoresed on cylindrical gels (14 x 0.6 cm) by using a modification of the method of Laemmli (1970). Separating gel (≃ 14 cm) contained 10% (w/v) acrylamide, 0.27% (w/v) N,N'-methylene-bisacrylamide, 0.1% (w/v) SDS, 0.03% (v/v) N,N,N',N'-tetramethylethylene-diamine, 0.01% (w/v) ammonium persulphate, 0.1% (w/v) glycerol, 0.5 M urea and 0.38 M Tris-pH 8.8. Stacking gels (2 cm long) contained 5% (w/v) acrylamide, 0.15% (w/v) N,N'-methylbisacrylamide, 0.01% (w/v) SDS, 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine, 0.06% (w/v) ammonium persulphate,

0.05% (v/v) glycerol, 0.5 M urea and 0.12 M Tris-HCl pH 6.8. The electrode buffer was 25 mM Tris, 0.192 mM glycine, 0.1% (v/v) SDS. Protein samples were applied in the gels in 6 M urea, 10% SDS pretreated as described above. Gels were run for 20 h at 60 V. They were fixed with 10% trichloroacetic acid for 3-4 h, frozen, cut into 1 mm slices and counted. Molecular weights were determined from protein standards run in parallel gels stained with 0.25% (w/v) Coomassie Brilliant Blue R-250, 50% ethanol, 10% acetic acid for 3-4 h and destained with 25% ethanol, 10% acetic acid for about 24 h. The marker proteins used were: myosin (H-chain), phosphorylase b, bovine serum albumin, ovalbumin, α-chymotrypsinogen, β-lactoglobulin and cytochrome c; their molecular weights were: 200,000, 92,500, 68,000, 43,000, 25,700, 18,400 and 12,300 respectively.

RESULTS

1. Reaction of NAPSCl with Tryptophan

The specificity of the sulfenyl chloride moiety of the synthesized NAPSCl reagent was established by reaction of the reagent with a standard mixture of amino acids (Table II), as well as with glucagon (Table III). The results indicated that tryptophan and to a small extent methionine are the only amino acid residues, in the absence of cysteine, to undergo reaction. Methionine may undergo oxidation rather than reaction with the sulfenyl chloride moiety. Paper chromatography of the NAPSCl reaction mixture with tyrosine, phenylalanine, methionine and histidine gave R_f values indistinguishable from those of the unmodified amino acids, indicating that the electrophilic NAPSCl reagent remains largely unreactive towards those amino acids.

The kinetics of the reaction with tryptophan was followed by determining the rate of disappearance of the sulfenyl chloride via the reaction: $2\ \text{NAPS-S-Cl}+2\text{I}^- \rightarrow \text{NAPS-S-NAP}+\text{I}_2+2\text{Cl}^-$. By titrating the free iodine, the percent of reacted tryptophan was calculated on the basis of the consumption of the reagent (Fig. 1). Similar results were obtained by measuring the fluorescence of the remaining tryptophan (Fig. 1). The rate constant of the reaction was calculated on the basis of the titration data only since fluorescence data was more subject to experimental error. In both cases, 50% of tryptophan had reacted within 5-6 min. NAPSCl, therefore, reacted with tryptophan approximately eight times faster than NPSCl (Scoffone et al. 1968).

[a] A standard amino acid mixture (2.5 μmoles/ml) of all common amino acids was reacted in duplicate with 100% molar excess NAPSCl in glacial acetic acid in the dark at room temperature for 2 h.

[b] Average values of duplicate determination.

[c] The amino acid mixture was exposed to glacial acetic acid in the absence of NAPSCl.

[d] Half-cystine.

TABLE II

Recovery of Amino Acids Reacted

with 2-nitro-4-azidophenylsulfenyl chloride[a]

Amino Acid	Fraction recovered	
	Experimental[b]	Control[c]
Asp	1.1	1.1
Thr	1.1	1.0
Ser	1.2	1.0
Glu	1.0	1.0
Pro	1.1	1.2
Gly	1.1	1.0
Ala	1.1	1.0
Cys[d]	0.9	1.0
Val	1.1	1.0
Met	0.9	1.0
Ile	1.0	1.0
Leu	1.0	1.0
Tyr	1.0	1.0
Phe	1.0	1.0
His	1.0	1.0
Lys	1.0	1.0
Arg	1.0	1.0
Trp	0.0	1.2

TABLE III

Amino Acid Composition of Glucagon-NAPS

Residues/mole of Peptide

Amino Acid	Found[a]	Expected[b]
Asp	4.3	4
Thr	3.0	3
Ser	3.6	4
Glu	3.2	3
Gly	1.3	1
Ala	1.1	1
Val	0.9	1
Met	0.8	1
Leu	2.0	2
Tyr	1.9	2
Phe	1.9	2
His	0.9	1
Lys	1.0	1
Arg	2.0	2
Trp[c]	-	1

[a] Peptide purified by partition chromatography values given are for 22 h hydrolysis at 110° in 6 N HCl calculated on the basis of 28 residues.

[b] Based on known amino acid composition (Bromer et al., 1957). Aspartic and glutamic values include production from asparagine and glutamine respectively.

[c] Destroyed on acid hydrolysis, not determined.

Fig. 1. Rates of reaction of 2-nitro-4-azidophenylsulfenylchloride (NAPSCl) with tryptophan

Tryptophan was reacted with NAPSCl in a 2:1 molar ratio in glacial acetic acid. The rate of the reaction, in aliquots removed at each time point, was followed by either decrease in tryptophan fluorescence or by titration of the unreactive reagent with free iodine as described in "Methods". Symbols indicate the mean ± SEM of three determinations. (A) Percent loss of tryptophan fluorescence with time (o); percent of reacted tryptophan with time calculated on the basis of the remaining unreactive reagent titrated with free iodine, (•). (B) Linear plot of the kinetic equation for a second order reaction from the titration data of the NAPSCl-tryptophan reaction. (a) Initial concentration of NAPSCl; (b) initial concentration of tryptophan; (a-x), (b-x) concentrations remaining at a given time.

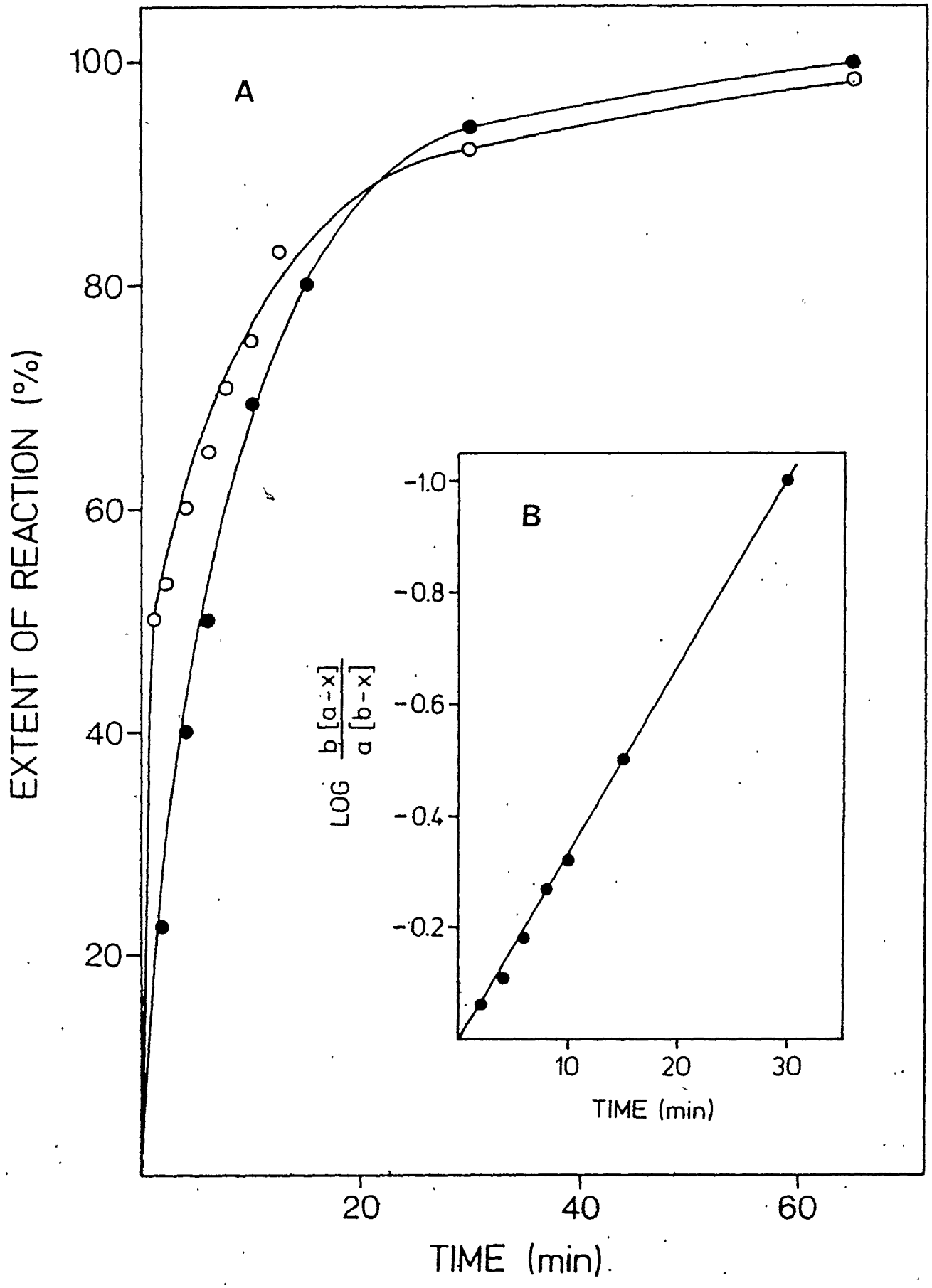

A
B
100
80
60
40
20
EXTENT OF REACTION (%)
20
40
60
TIME (min)
-1.0
-0.8
-0.6
-0.4
-0.2
LOG b[a-x] / a[b-x]
10
20
30
TIME (min)

Figure 1

Faster reaction rates of sulfenyl chlorides have been reported by Scoffone et al. (1968) with tryptophan containing peptides as compared to the free amino acid.

2. Synthesis and Characterization of Glucagon-NAPS

Reaction of NAPSCl with glucagon in a 2:1 molar ratio resulted in quantitative labeling of tryptophan-25. Only one major species of glucagon-NAPS was observed by both partition chromatography (Fig. 2) and polyacrylamide gel electrophoresis (data not shown). The small contaminant (>5%) observed in both native and modified glucagon could be due to deamidated glucagon since it only differed by one charge as was indicated by gel electrophoresis.

Fluorescence studies of the modified derivative in 7 M guanidine-HCl, 10 mM glycine pH 8.1, at 350 nm where glucagon fluoresces with an excitation wavelength 295 nm indicated that there is less than 4.3×10^{-2}% contamination ($\pm 0.1 \times 10^{-2}$% error of the noise level of three determinations) with unmodified glucagon calculated on the basis of the detection limits of the technique used. (Data not shown).

3. Photoreactivity of NAPSCl and Glucagon-NAPS

The photoreaction of the azide moiety of NAPSCl was followed by changes in OD at λ_{max} = 420 nm upon irradiation with light (Fig. 3). These data and the disappearance of the IR band at 2120 cm^{-1} (data not shown) indicated that photolysis was completed within 6 min. When the glass filter No. 7-51 was added the photolysis was completed within 25 min (Fig. 4). The percent change in OD_{420} was also used to determine the stability of NAPSCl under ordinary fluorescent room light in the

absence of any day light (Table IV). For solutions kept in the dark, there was only a 15% change in OD_{420} after 60 hr, while there was more than 50% change for those exposed to light. Over a 50% decrease in OD_{420} was also observed for the solutions photolysed immediately after preparation, whereas there was greater than 70% decrease in absorbance for those kept in the dark and photolysed 48 hr later, indicating that some decomposition of the reagent may take place upon standing in acidic solutions at room temperatures over long periods of time. This decomposition may be due to the hydrolysis of the sulfenyl chloride group by water molecules in the solution or moisture being picked up from the air. Hydrolysis (100%) of NPSCl and DNPSCl has been shown to occur with water in ≈2.5-3.0 min (Veronese et al. 1970). The 5.2% change in absorbance after a 3 hr exposure to light may not reflect decomposition of the azide alone, suggesting that the reagent is not very susceptible to photolysis under laboratory light conditions over short time periods.

Thin layer chromatography of the irradiation product mixture of 2,2-dinitro-4,4'-diazidodiphenyldisulfide indicated that no product comigrated with the authentic sample of 2,2-dinitro-4,4'-diaminodiphenyl-disulfide (within the detection limits of the technique used). The rest of the photolysis products detected were not characterized any further.

Glucagon-NAPS and the photolysed derivative gave characteristic absorption spectra and are compared to that of glucagon (Fig. 6). The decrease in OD_{395} was characteristic of the photoreaction of the aryl azide moiety. The absorption maxima and the molar extinction coefficients of the sulfenylated tryptophan with the monofunctional reagent 3-nitro-phenyl-sulfenyl chloride (NPSCl) and with NAPSCl as well as glucagon-NAPS and native glucagon, both before and after photolysis are compared in Table V.

Fig. 2. Partition chromatography of glucagon and glucagon-NAPS on Sephadex G-25

The Sephadex G-25 column (1.5 x 90 cm) was equilibrated with the lower phase of the solvent system used: butanol-ethanol-benzene-0.2 N NH_4OH (5:2:1:8 v/v) pH 9.4. Samples 3.0 - 5.0 mg were applied and eluted with the upper phase at 8-10 ml/hr in 2.0 ml fractions. The elution pattern was monitored by the Lowry et al. (1951) protein determination. V_H: hold up volume; V_E: elution volume; $R_f = V_H/V_E$. Glucagon (▲-▲); Glucagon-NAPS (●--●).

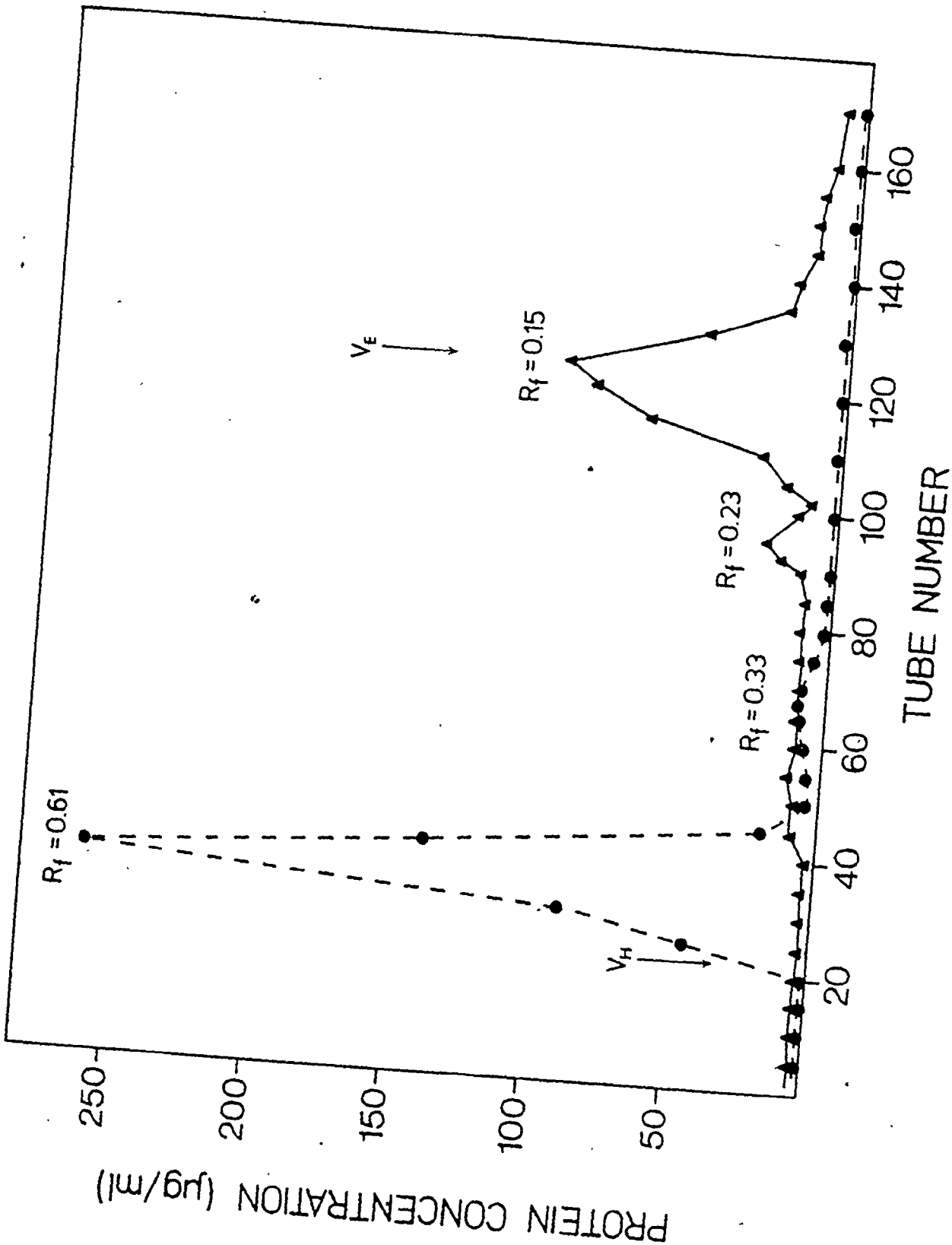

PROTEIN CONCENTRATION (µg/ml)
250
200
150
100
50
20
40
60
80
100
120
140
160
TUBE NUMBER
R_f = 0.61
R_f = 0.33
R_f = 0.23
R_f = 0.15
V_H
V_E

Figure 2

Fig. 3. Rate of photolysis of 2-nitro-4-azidophenylsulfenylchloride (NAPSCl) at λ >300 nm

A solution of 0.3-0.4 mM NAPSCl in glacial acetic acid at 25°C, were irradiated 10 cm away from a High Pressure Xenon Lamp XBO-150W, with light at wavelengths >300 nm, as described in "Methods" using the potassium phthalate filter. At designated time intervals, irradiation was stopped, 1.0 ml aliquots were removed and their absorbance at λ_{max} 420 nm were measured. The results are the average of two determinations.

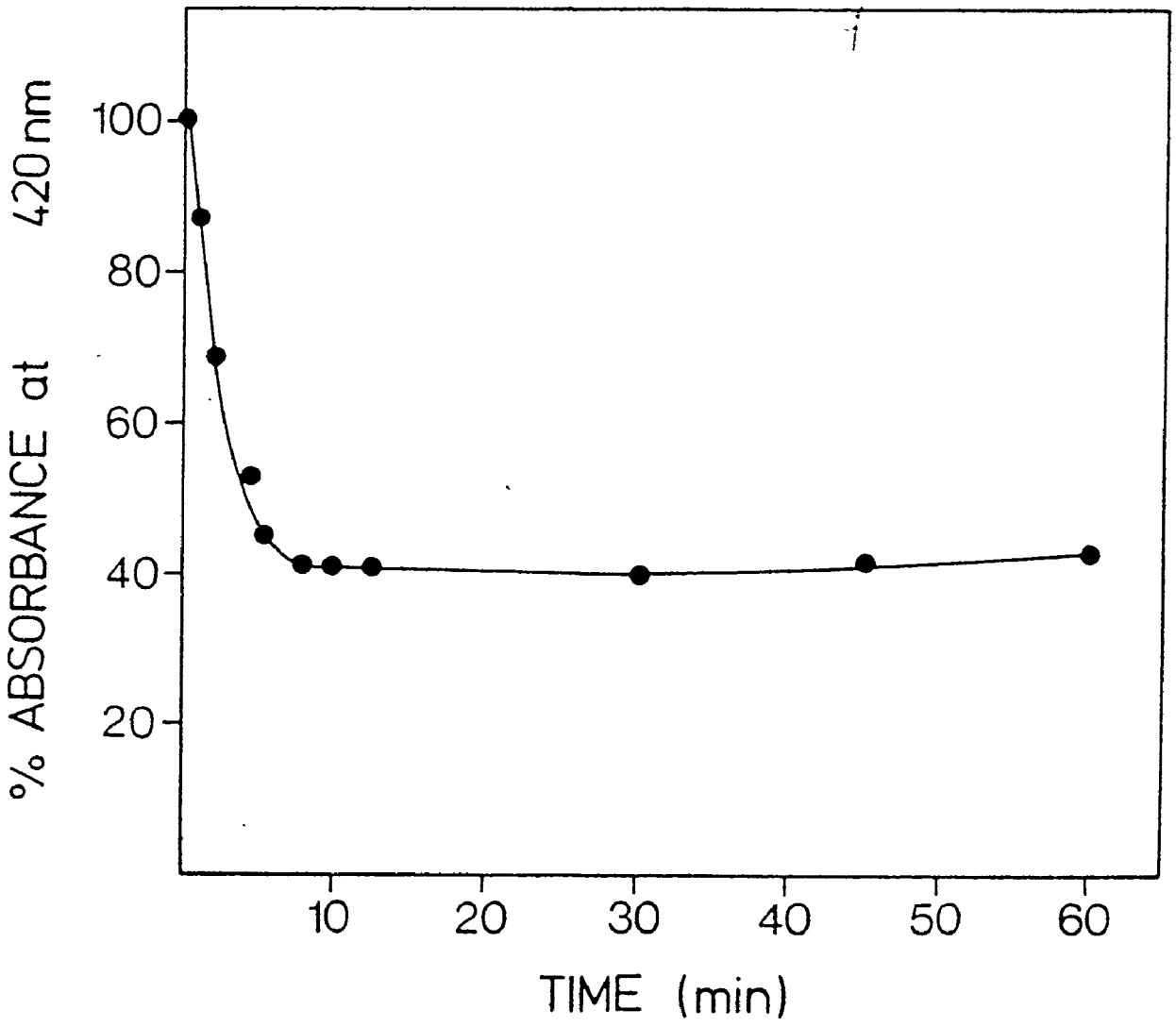


Figure 3

Fig. 4. Rate of photolysis of 2-nitro-4-azidophenylsulfenylchloride (NAPSCl) at λ = 310 - 390 nm

The rate of photolysis of a solution of 0.5 mM NAPSCl in glacial acetic acid was measured as described in the legends to Fig.3 and in "Methods". Irradiation was carried under the same conditions with the exception that a 5.1 mm thick ultraviolet transmitting, visible absorbing glass filter (Corning, No. 7-51) was inserted between the light source and the cell ($\simeq$8 cm away from the light). Transmittance $\approx$80% at λ = 385 nm. (Fig. 5). The results are the average of two determinations.

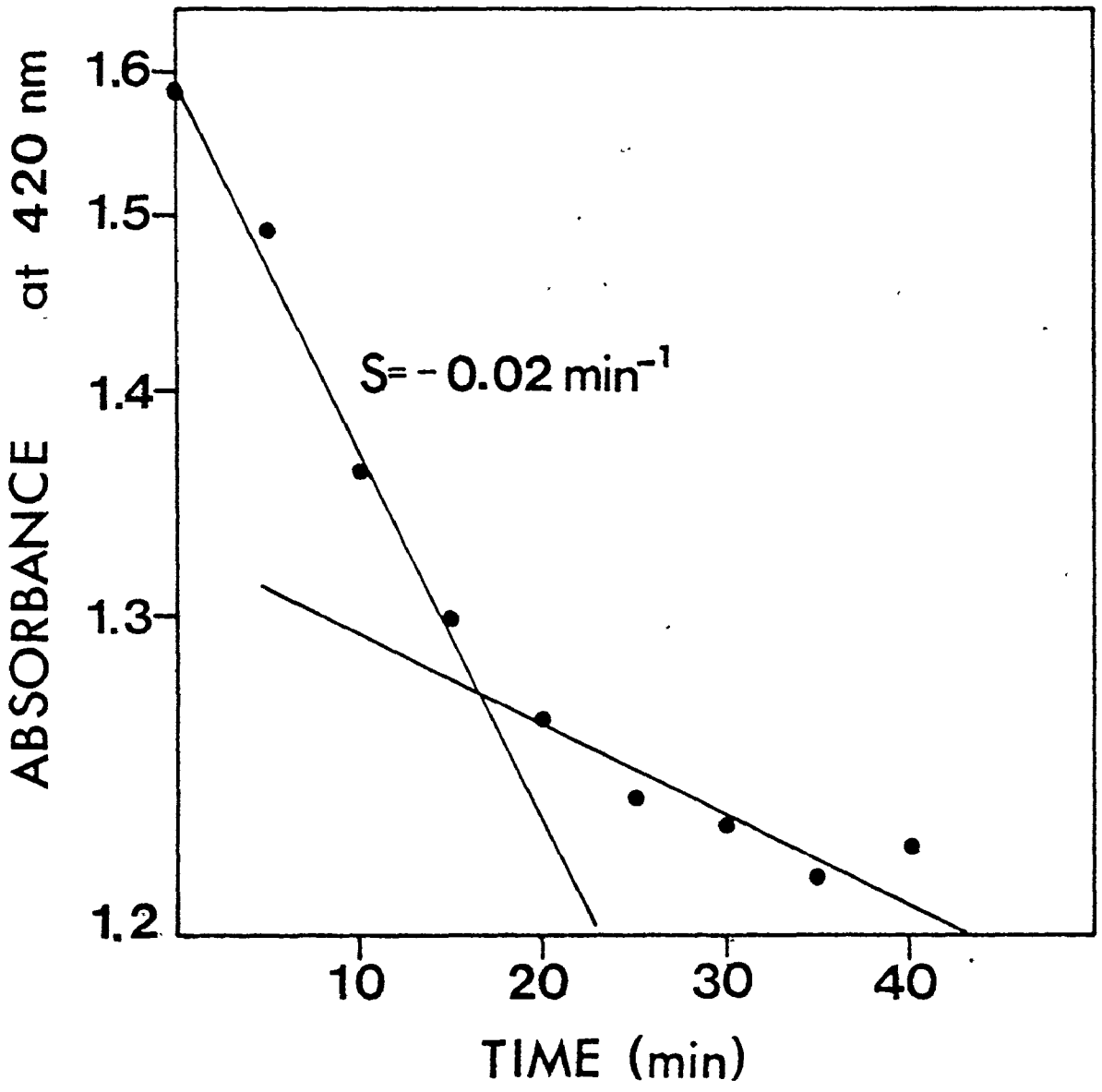

ABSORBANCE at 420 nm
1.6
1.5
1.4
1.3
1.2
S= - 0.02 min⁻¹
10
20
30
40
TIME (min)

Figure 4

Fig. 5. Transmittance profiles of filters used in irradiation experiments

(-●-●-) Transmittance profile of 0.01% (w/v) potassium phthalate solution recorded in 1 cm pathlength quartz cell; (-o-o-) Control limits for the ultraviolet transmitting, visible absorbing filter (Corning CS No. 7-51, 4.9 - 5.1 mm) as shown in Corning Color Filter Catalog.

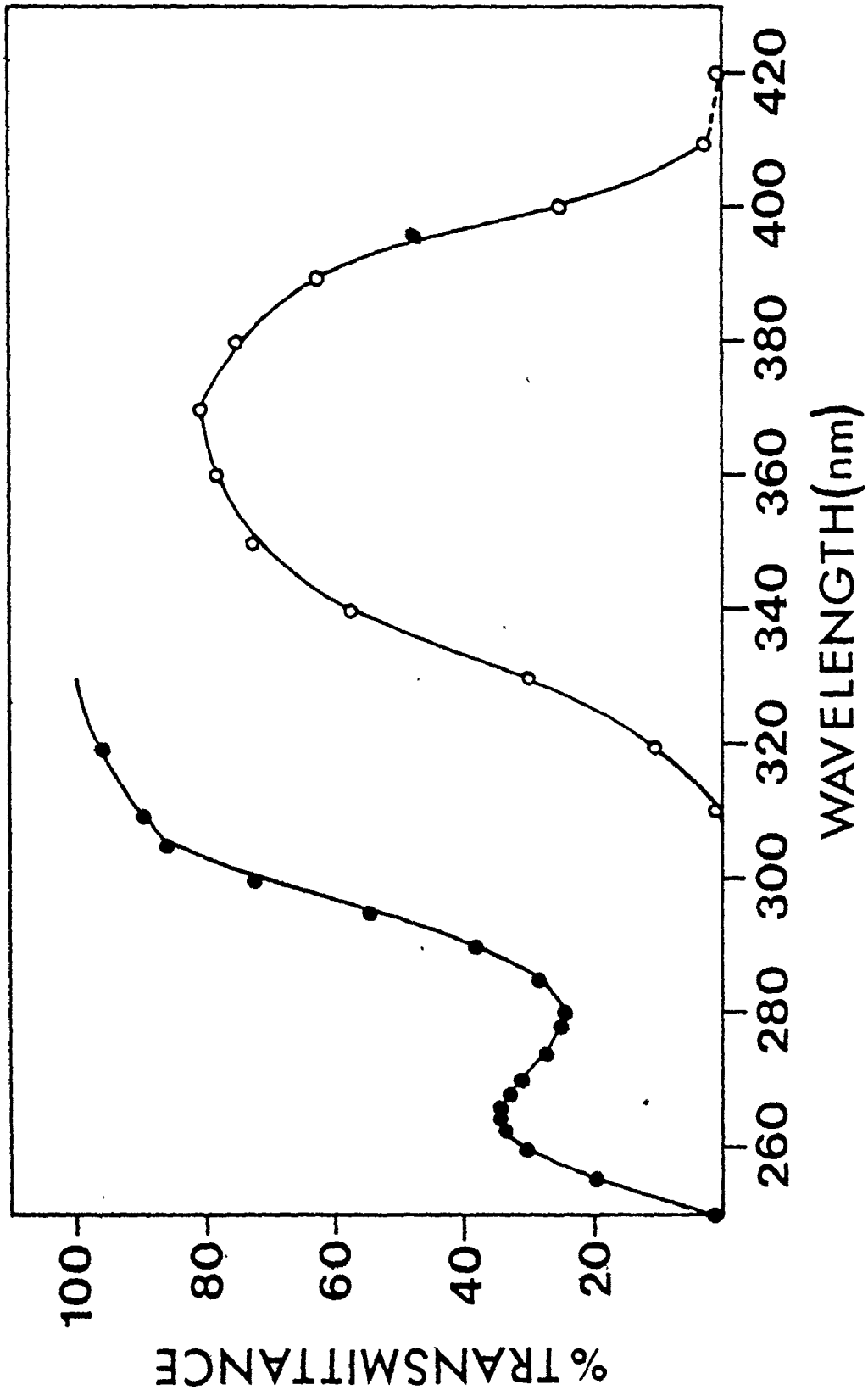

% TRANSMITTANCE
100
80
60
40
20
260
280
300
320
340
360
380
400
420
WAVELENGTH(nm)

Figure 5

TABLE IV

Effect of Room Lighting on the Stability

of 2-Nitro-4-Azidophenylsulfenyl Chloride

Percent OD_{420} Remaining

Time (h)	Dark	Light[a]
0	100	100
3	96.7	94.8
15	96.0	73.0
26	91.7	59.4
60	84.7	37.8

Photolysed[b] at zero time % OD_{420} = 45%

Photolysed[b] 48 h after being in the dark % OD_{420} = 20%

10^{-5} - 10^{-6}M solutions of NAPSCl reagent in glacial acetic acid were prepared in the dark and their absorbance at 420 nm was measured at various time intervals. [a] Ordinary fluorescence light in the absence of any day light. [b] Photolysed refers to exposure of the solution for 10 min at 25°C under N_2 using a 150 watt Xenon arc lamp at wavelengths >300 nm.

Fig. 6. Absorption spectra of glucagon, glucagon-NAPS and photolysed glucagon-NAPS

The absorption spectra of 10^{-6}M glucagon (●–●); glucagon-NAPS, (o--o) and photolysed glucagon-NAPS (▲-▲) in 80% acetic acid were recorded at 25°C in 1 cm path length quartz cells. 10^{-6}M glucagon-NAPS (▲-▲) in 80% acetic acid was photolysed under N_2 for 8 min under the conditions described in "Methods", and the absorption spectrum was recorded as described above.

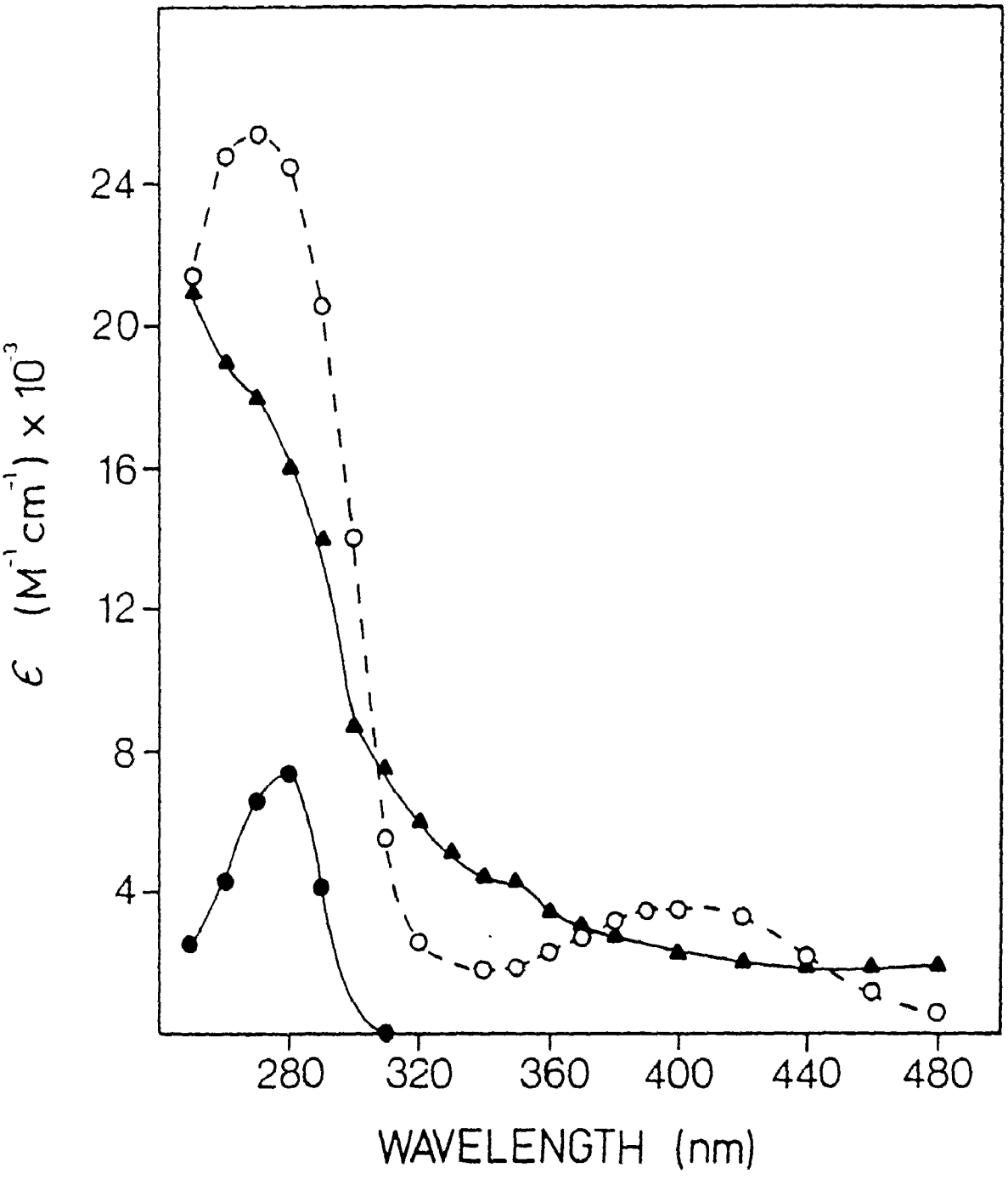

24
20
16
12
8
4
ε (M⁻¹cm⁻¹) x 10⁻³
280
320
360
400
440
480
WAVELENGTH (nm)

Figure 6

TABLE V

Absorption characteristics of compounds used in this work

Compound	Solvent	λ_{max} (nm)	ε (x 10^{-3}) (M^{-1} cm^{-1})
NPS-Cl	HOAc	391	3.20
NAPS-Cl	HOAc	420	2.95
NAPS-Cl[a]	HOAc	420	1.28
Trp-NPS	H_2O	361	1.88
Trp-NAPS	H_2O	395	4.55
Trp-NAPS[a]	H_2O	395	3.25
Glucagon[b]	HOAc	280	8.31
Glucagon-NAPS	HOAc	395	3.60
Glucagon-NAPS	HOAc	275	24.90
Glucagon-NAPS[a]	HOAc	395	1.10

[a] Photolysed at 25°C, under N_2 for 8 min.

[b] Spectrum unaltered by photolysis.

4. Iodination by Lactoperoxidase, H_2O_2, at pH 10.0

Iodination of glucagon and glucagon-NAPS by the lactoperoxidase method at pH 10.0 and with a ratio of reactants of 0.3 g atom ^{125}I/mole hormone resulted in 60-70% incorporation of label. The radiolabeled hormone fractionated on DEAE-cellulose was identified as monoiodoglucagon (MIG) (70-80%)(specific activity ≈ 2.47 μCi/pmole) by comparison of the conductivity elution profile with that previously reported (Desbuquois, 1975; Von Schenk and Jeppson, 1977) (Fig. 7). No radioactivity above background was observed where the diiodoglucagon (DIG) elutes. This is in agreement with Von Schenk et al. (1976), that the formation of DIG or MIG is dependent on pH and the overall degree of iodine substitution of the glucagon. Less than 5% DIG was reported during iodination at pH 10.0 with an iodine substitution of 0.5 g atom ^{125}I/mole glucagon.

The peak called unknown in Fig. 7, possibly represents iodinated glucagon fragments since it was lost after dialysing out the urea, while monoiodoglucagon was retained. Further it was not observed if instead of the glucagon-talc absorption step after iodination for the separation of the hormone from free iodine, the iodinated sample was dialysed before it was applied to DEAE-cellulose.

DEAE-cellulose purified radiolabeled hormone was used for up to two weeks after iodination. After two weeks, high molecular weight aggregates and breakdown products were removed by chromatography on a P-10 column (Fig. 19) and the repurified sample was used within two weeks or less. The high recovery of intact radiolabeled hormone indicated that the peptide remains stable for a long time, at least up to two months, when stored in propanol:w[illegible] v/v) at -20°C.

Fig. 7. Fractionation of crude monoiodoglucagon by chromatography on DEAE-cellulose

^{125}I-labeled glucagon 2.87 nmol, (0.3 g atoms of iodine per mol) was subjected to DEAE-cellulose chromatography as described in "Methods". Incorporation of ^{125}I- was about 60%. Fractions (1.0-0.8 ml) were collected at a rate of 1.8 ml/h. Aliquots of 10 µl were counted. Recovery was about 40-60%. For conductivity measurements, 5 fractions were pooled together. o——o, absorbance profile of glucagon (peak I) eluted under the same conditions. •——•, ^{125}I-cpm of radiolabeled glucagon. Peak II, unknown (≈ 20% of total); Peak III, monoiodoglucagon (≈ 75-80% of total).

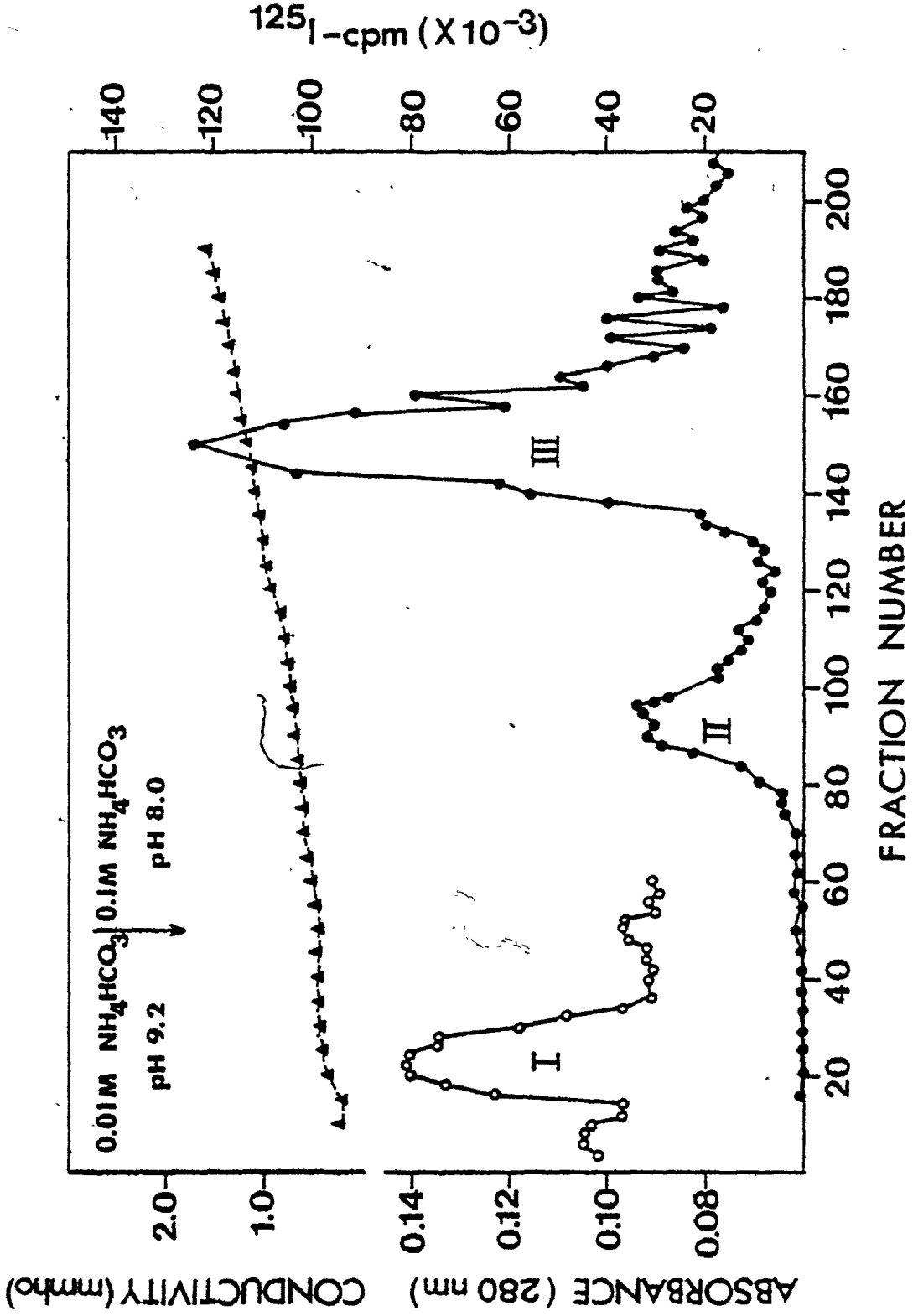

^{125}I-cpm (X10^{-3})
140
120
100
80
60
40
20
0.01M NH$_4$HCO$_3$ pH 9.2
0.1M NH$_4$HCO$_3$ pH 8.0
I
II
III
2.0
1.0
CONDUCTIVITY (mmho)
0.14
0.12
0.10
0.08
ABSORBANCE (280 nm)
20 40 60 80 100 120 140 160 180 200
FRACTION NUMBER

Figure 7

When stock radiolabeled hormone was used, the propanol:water solution was dried with N_2 gas and the hormone was resuspended in the appropriate buffer containing 1 mg/ml BSA and 750 KIU/ml Trasylol to prevent nonspecific absorption to glass and enzymatic degradation by proteases present in BSA.

Decrease in the amount of iodine incorporated in glucagon-NAPS (10-20%) (data not shown) could be attributed to lesser exposure of tyrosine residues to iodine. It is possible that in propylene glycol the presence of NAPS- on tryptophan-25 residues increases the hydrophobicity of the -COOH terminus and thus may cause changes in the tertiary structure of hormone and make the tyrosine residues less accessible.

5. Stability of the Radiolabeled Hormone to Photolysis

Iodination of the tyrosine residues of the hormone results in an increase in their extinction coefficient, a shift of the absorption maximum to the red and a lowering of the pK (Edelhoch, 1962: Katzenellenbogen and Hsiung, 1975). The latter effect results in the products of photolysis being pH dependent in the region pH 7-10 with loss of covalently bound ^{125}I-occurring at higher pH, presumably as a result of direct photolysis of iodotyrosinate. Only 16% of ^{125}I-glucagon was recovered in the fractions where glucagon elutes when photolysis was carried out at pH 8.0. On the other hand, more than 80% was recovered in these fractions when the radiolabeled hormone was photolysed at pH 6.5-7.0 (Fig. 8). As a result, photolysis of the radiolabeled hormone derivatives was carried out at neutral pH. Under these conditions there should be little migration of ^{125}I-iodine.

Fig. 8. Sephadex G-75 profile of irradiated ^{125}I-glucagon

^{125}I-glucagon samples (4,000-5,000 cpm, specific activity 2.0×10^6 cpm/pmole) in 0.2 M glycine pH 6.75 or 8.8 were irradiated under the conditions described in the legends to Fig. 3 for 15 min. After irradiation, the samples were applied on a Sephadex G-75 column (50 x 0.5 cm) and eluted with 0.2 M glycine pH 2.6, 9 M urea. 0.7 ml fractions were collected at a rate of 10 ml/h. The elution profile was monitored by measuring the radioactivity of the fractions collected. The column was calibrated with Dextran-blue and 2,4'-dinitrophenyl glycine. Unphotolysed samples were run under similar conditions as controls. (-●-●-), Photolysed at pH 6.7; (-▲-▲-), photolysed at pH 8.8; (o—o), unphotolysed. Percentages are of total cpm applied on the column.

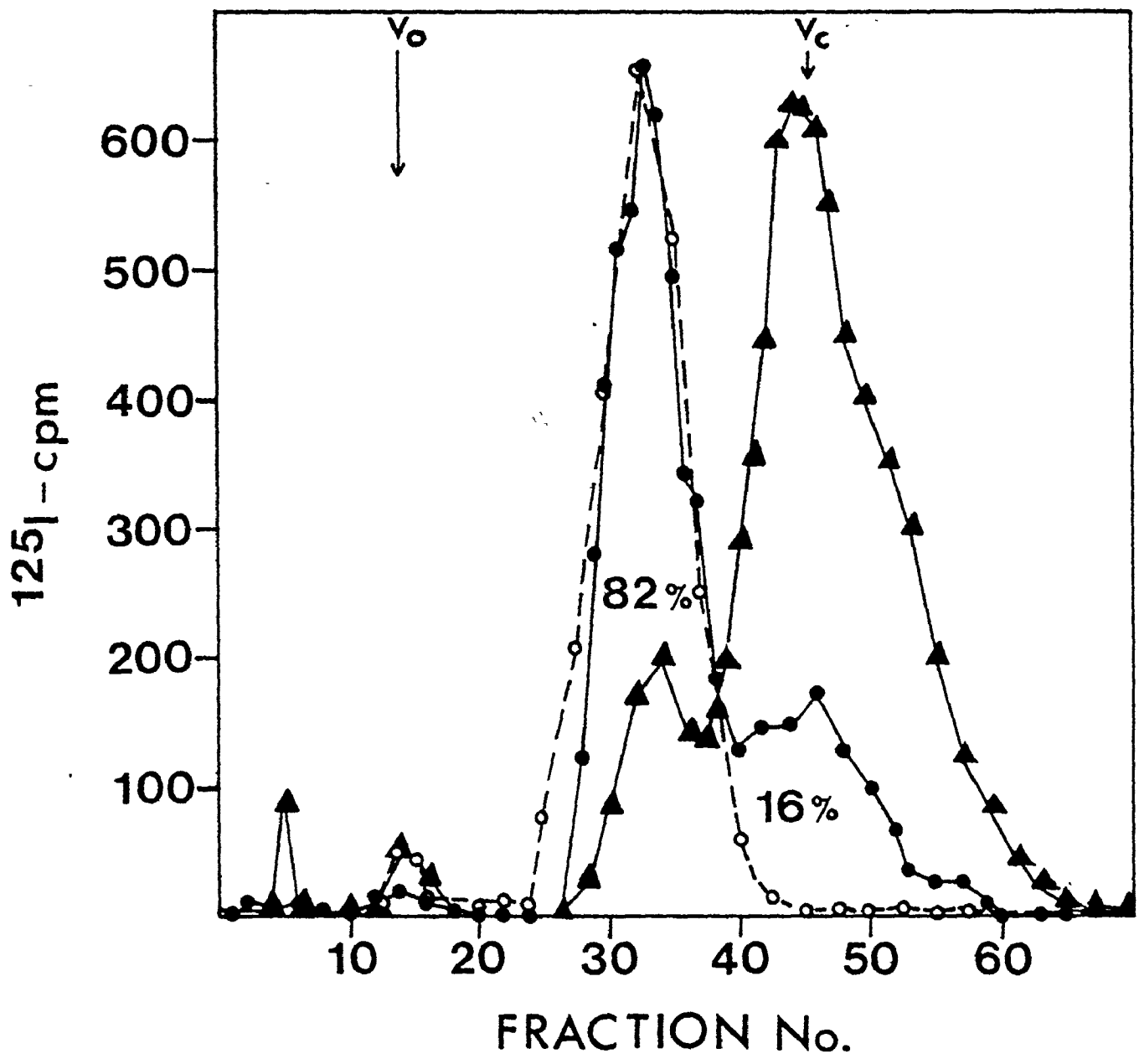

Vo
Vc
600
500
400
300
200
100
125I - cpm
82%
16%
10
20
30
40
50
60
FRACTION No.

Figure 8

6. "Photoaffinity" Labeling of Bovine Serum Albumin (BSA) and Anti-Glucagon-IgG.

The ability of the monoiodinated glucagon-NAPS derivative to be covalently crosslinked to other molecules upon irradiation was tested using BSA. The percent radiolabeling of BSA measured under denaturing conditions was dependent on the concentration of BSA resulting in 20-25% crosslinking at 2.5 mg/ml after photolysis for twice the half life of the photoaffinity labeled hormone. Radiolabeling of the BSA was not displaced by 10,000 fold excess unlabeled glucagon indicating the nonspecific nature of covalent labeling (Fig. 9). Nonphotolysed samples as well as those photolysed in the presence of ^{125}I-glucagon showed only 2.0-2.5% radioactivity associated with BSA by gel filtration on Sephadex G-75. This radioactivity was attributed to high molecular weight aggregates which were spontaneously formed during storage of the radiolabeled hormone peptides, presumably as a result of radiolysis.

Protein A radioimmunoprecipitation as well as gel filtration on Sephacryl S-300 of glucagon and ^{125}I-glucagon-NAPS-glucagon-antibody complexes showed that 75-85% of the radiolabeled hormone was associated with the glucagon specific antiserum, while only 5-10% was associated with the normal serum. Specific immunoprecipitation was approximately 10% lower in the samples photolysed before fractionation (Table VI, Fig. 10).

Covalent labeling of the anti-glucagon-IgG-glucagon-NAPS complex measured by Sephadex G-75 gel filtration under denaturing conditions was 40% of the immunoprecipitated material in the photolysed sample, compared to 17% in the unphotolysed control (Fig. 11). Similarly 15-20% of the ^{125}I-glucagon-NAPS radioactivity, which was associated nonspecifically

Fig. 9. Gel electrophoresis profile of bovine serum albumin (BSA) covalently labeled with ^{125}I-glucagon-NAPS upon irradiation

^{125}I-glucagon-NAPS (1.7×10^{-8}M, specific activity 3.0×10^{6} cpm/pmole) was preincubated with 0.25 mg/ml BSA in 30 mM Tris-HCl, pH 7.0, 750 KIU/ml Trasylol in the presence and absence of 10^{-6}M cold glucagon or glucagon-NAPS, at 30°C for 10 min in the dark. The sample was subsequently saturated with N_2 gas saturated with water (4°C) for one min and photolysed for 2 min under N_2, according to the procedure described in "Methods", Chapter II. The photolysed sample was dried under dry N_2 gas, redissolved in 150 µl 6 M urea, 10% SDS, heated at 60°C for 30 min and left at room temperature overnight. 5 µl of 0.1% bromophenol blue were added and the samples were electrophoresed on 10% (w/v) acrylamide, 0.1% (w/v) SDS cross-linked cylindrical gels (14 X 0.6 cm) prepared and run as described in "Methods". Marker protein standards were run on separate parallel gels. Gels were stained and distained as described in "Methods". The ^{125}I-cpm profile was determined from 1 mm slices of frozen gels. Since the gel electrophoresis profiles were the same under any of the above conditions, only that of BSA preincubated with ^{125}I-glucagon-NAPS and photolysed is presented.

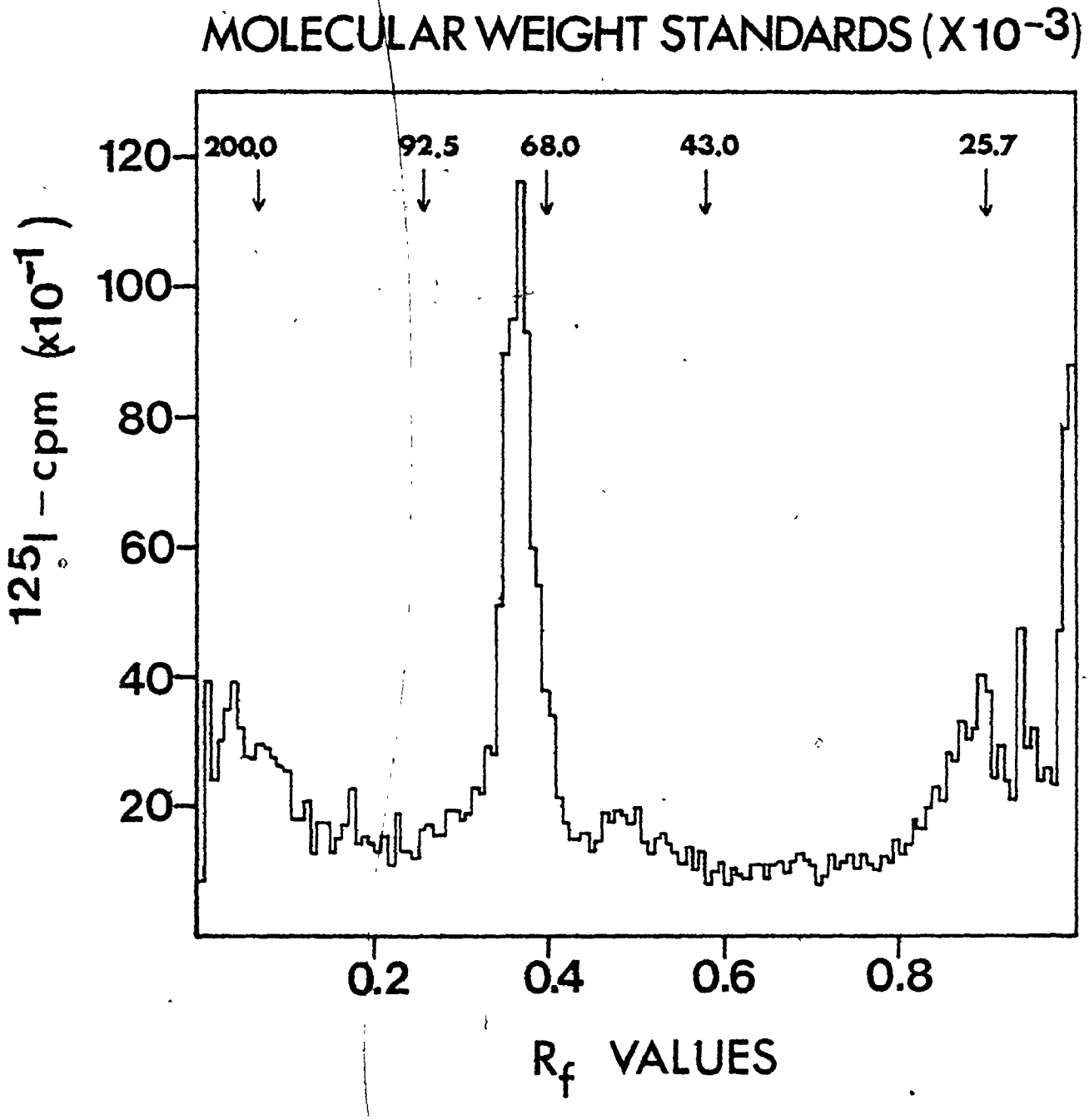

MOLECULAR WEIGHT STANDARDS (X10⁻³)
200.0
92.5
68.0
43.0
25.7
120
100
80
60
40
20
125I – cpm (x10⁻¹)
0.2
0.4
0.6
0.8
Rf VALUES

Figure 9

TABLE VI

Protein A radioimmunoassay of glucagon and glucagon-NAPS

Irradiation	% elution			
	Normal Serum + ^{125}I-glucagon	Antiglucagon Ab + ^{125}I-glucagon	Normal Serum + ^{125}I-glucagon-NAPS	Anti-glucagon Ab + ^{125}I-glucagon-NAPS
+	2.2 ± 0.4	71.5 ± 0.9	7.7 ± 1.0	61.3 ± 0.5
-	5.3 ± 0.2	85.2 ± 0.5	4.8 ± 0.6	76.6 ± 1.0

$1.5 - 2.0 \times 10^{-11}$ M ^{125}I-glucagon or ^{125}I-glucagon-NAPS were preincubated with 1:10 diluted glucagon specific antiserum or normal rabbit serum in 1:10 dilution as described in "Methods". After 72 hr incubation at 4°C, the samples were diluted 10 X, their pH was adjusted to 6.5 - 7.0 and were photolysed for 20 min or kept in the dark at room temperature. 200 µl of settled protein A-Sepharose CL-4B were then added, followed by another 24 hr incubation at 4°C. Free and bound hormone-antibody complex were separated by centrifugation followed by four washings as described in "Methods". Bound Ab complex was eluted from protein A with 60 mM Tris-HCl pH 6.8, 6% (w/v) sodium dodecyl sulphate (SDS), 10% w/v glycerol, 10% (v/v) 2-mercaptoethanol and counted. Values presented are the mean ± SEM of three determinations in parallel.

Fig. 10. Fractionation of ^{125}I-glucagon-NAPS antibody complex on Sephacryl S-300 column

1.5 - 2.0 x 10^{-11}M ^{125}I-glucagon-NAPS was incubated with glucagon specific antiserum or normal rabbit serum (1:10 dilution) in 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10% glycerol, 2.0 mg/ml BSA, 750 KIU/ml Trasylol at 4°C for 72 h in the dark. After incubation the samples were diluted 10 X with the same buffer, the pH adjusted to 6.5 - 7.0 and the samples were either photolysed for 20 min as described in "Methods", or kept in the dark. Radiolabeled IgG was subsequently purified through an 80 x 2 cm Sephacryl S-300 column eluting with the same buffer. A: Samples incubated in the dark; B: photolysed samples. (-●-●-) protein profile of glucagon antiserum (A) and of normal serum (B), respectively; (▲--▲) ^{125}I-cpm profile of glucagon antiserum; (o-o) ^{125}I-cpm profile of normal serum. ^{125}I-glucagon-antibody profile was omitted for brevity. The protein profiles of normal serum and glucagon-antiserum were the same in the dark or irradiated samples but only one of each are plotted in A or B for clarity.

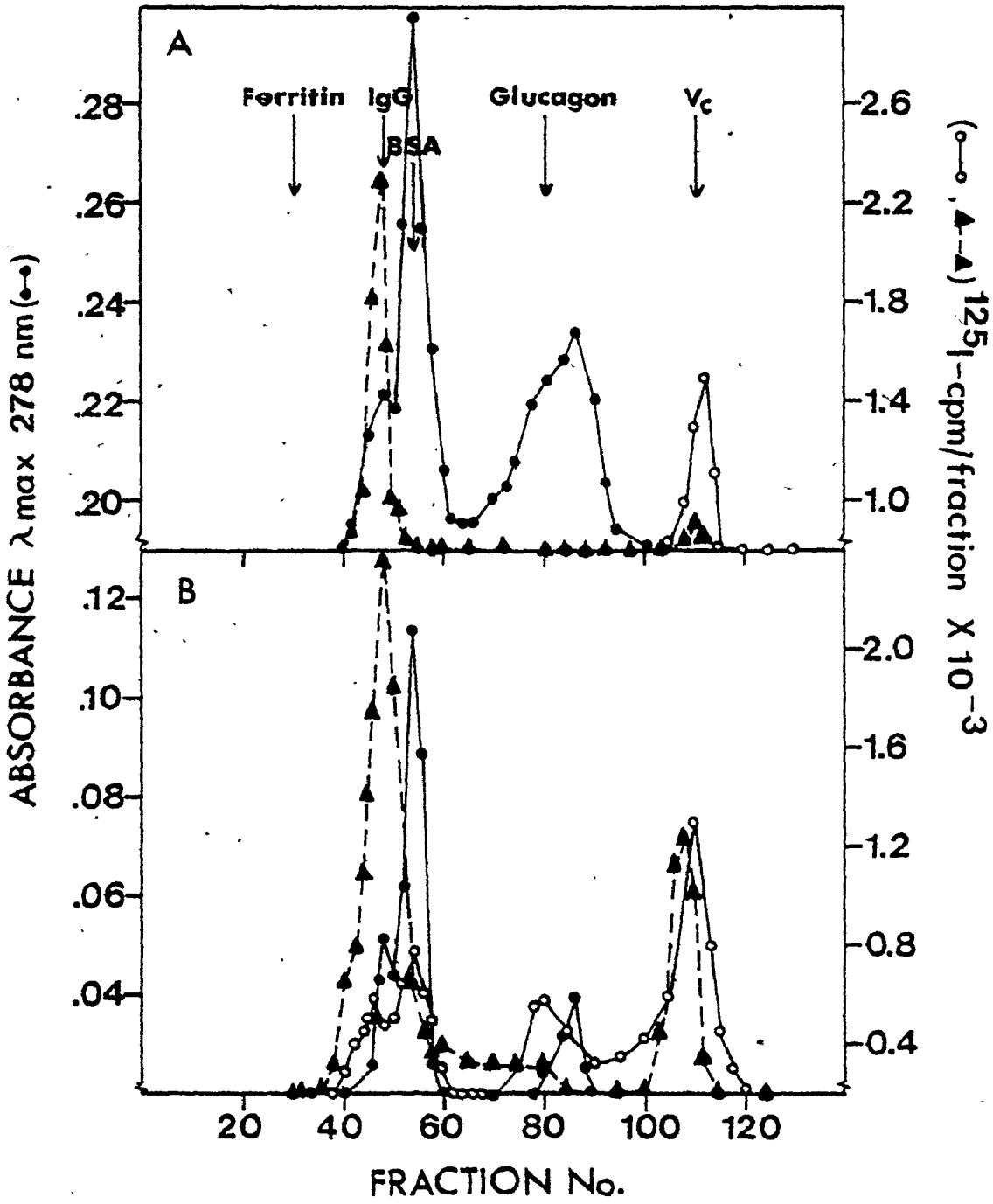

A
Ferritin
IgG
BSA
Glucagon
Vc
B
ABSORBANCE λmax 278 nm(•—•)
(○—○, ▲—▲) 125I-cpm/fraction X 10-3
FRACTION No.
.28
.26
.24
.22
.20
.12
.10
.08
.06
.04
2.6
2.2
1.8
1.4
1.0
2.0
1.6
1.2
0.8
0.4
20
40
60
80
100
120

Figure 10

Fig.11. Fractionation of glucagon anti IgG covalently photolabeled with ^{125}I-glucagon-NAPS, on Sephadex G-75 column under denaturing conditions

^{125}I-glucagon-NAPS-IgG complex purified through Sephacryl S-300 column as described in the legend to Fig.10 was lyophilized, redissolved in 0.2 M glycine pH 2.6, 9 M urea, applied on a Sephadex G-75 column (50 x 0.5 cm) and subsequently eluted with the same buffer. 0.5 - 0.6 ml fractions were collected at a rate of 10 ml/hr. (-●-●-), Protein profile; (▲--▲), ^{125}I-cpm preincubated, photolysed; (o-o), ^{125}I-cpm, preincubated in the dark.

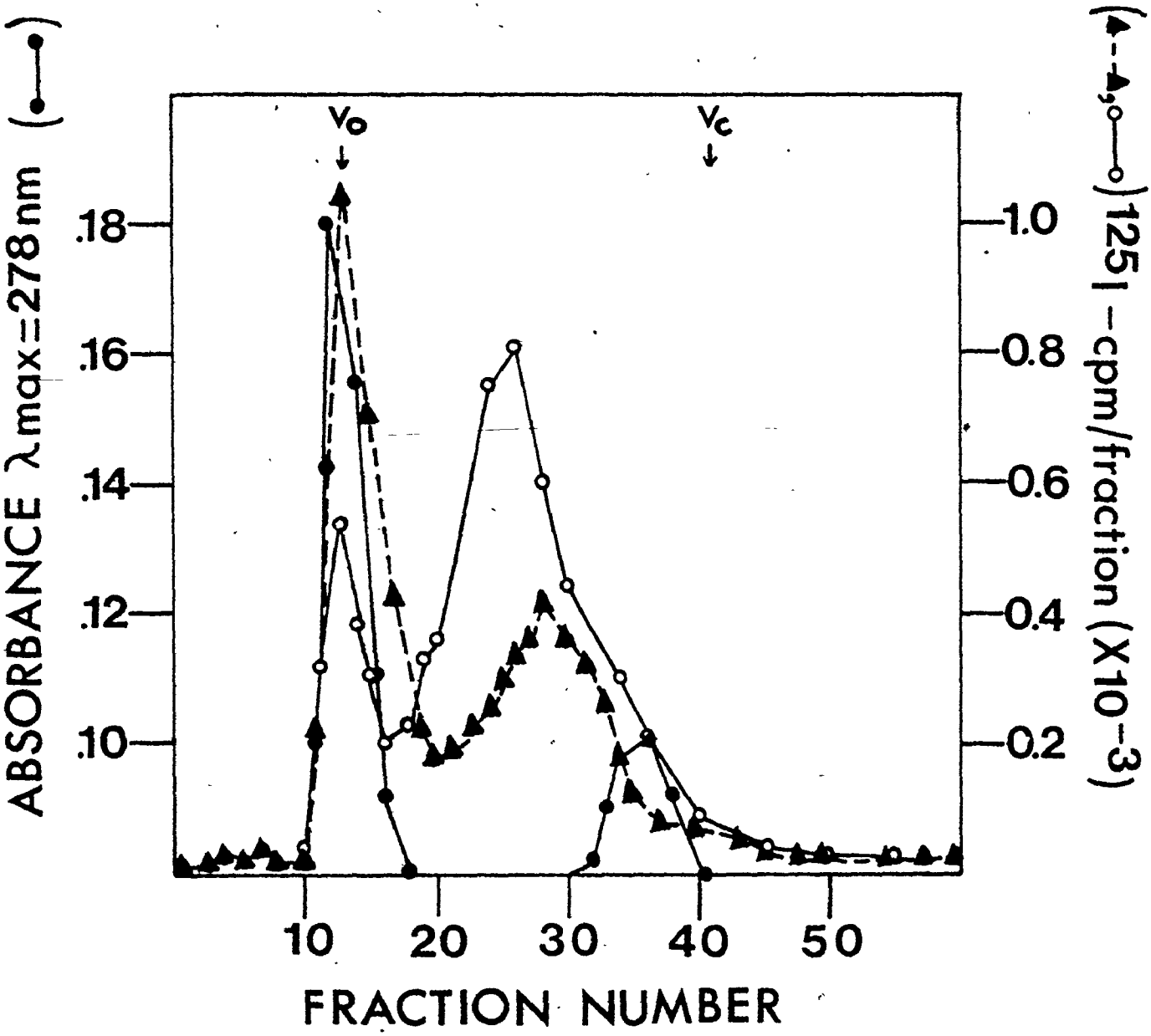

ABSORBANCE λmax=278nm (●—●)
(▲-▲,○—○) 125I – cpm/fraction (X10⁻³)
Vo
Vc
.18
.16
.14
.12
.10
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
FRACTION NUMBER

Figure 11

with the IgG fraction of normal serum, was recovered in the high molecular weight fractions of G-75 from both photolysed and unphotolysed ± 1,000 X excess cold hormone. Unphotolysed and photolysed ^{125}I-glucagon antibody complexes showed only 2.0-3.0% radioactivity associated with IgG (data not shown). The large degree of covalent labeling in the dark was attributed to chemical reactions occurring during the long incubation period (72 h) and the high pH of the incubation solution. It was thus not possible to clearly distinguish between radiolabeling due to chemical reactions occurring in the dark or during irradiation. Irradiation in the presence of 1,000 X excess cold glucagon reduced labeling by 60-70% indicating the specificity of photolabeling.

7. Conformational studies

Studies on the conformational properties of glucagon-NAPS derivative using circular dichroism at 0.2-0.3 mg/ml concentrations under both acidic and basic conditions, indicated that glucagon-NAPS has a greater tendency to form structures of higher helical content than glucagon. Similar results have been obtained with the glucagon-NPS derivative (Epand and Cote, 1976). Since both derivatives show large Cotton effects in the visible and near u.v. region from the modified Trp, it is possible that there is a contribution from extrinsic Cotton effects, in the region of amide chromophore absorption (Epand and Cote, 1976). However, Cotton effects in the range 260-400 nm are sensitive to conformation and in this case may reflect aggregate formation with a higher α-helical content. (Figs. 12,13,14).

Fig. 12. Circular dichroism of glucagon and glucagon-NAPS

Circular dichroism spectra of glucagon and glucagon-NAPS (0.3 mg/ml) in 0.01 M NaOH were recorded at 25°C. (——) glucagon; (---) glucagon-NAPS

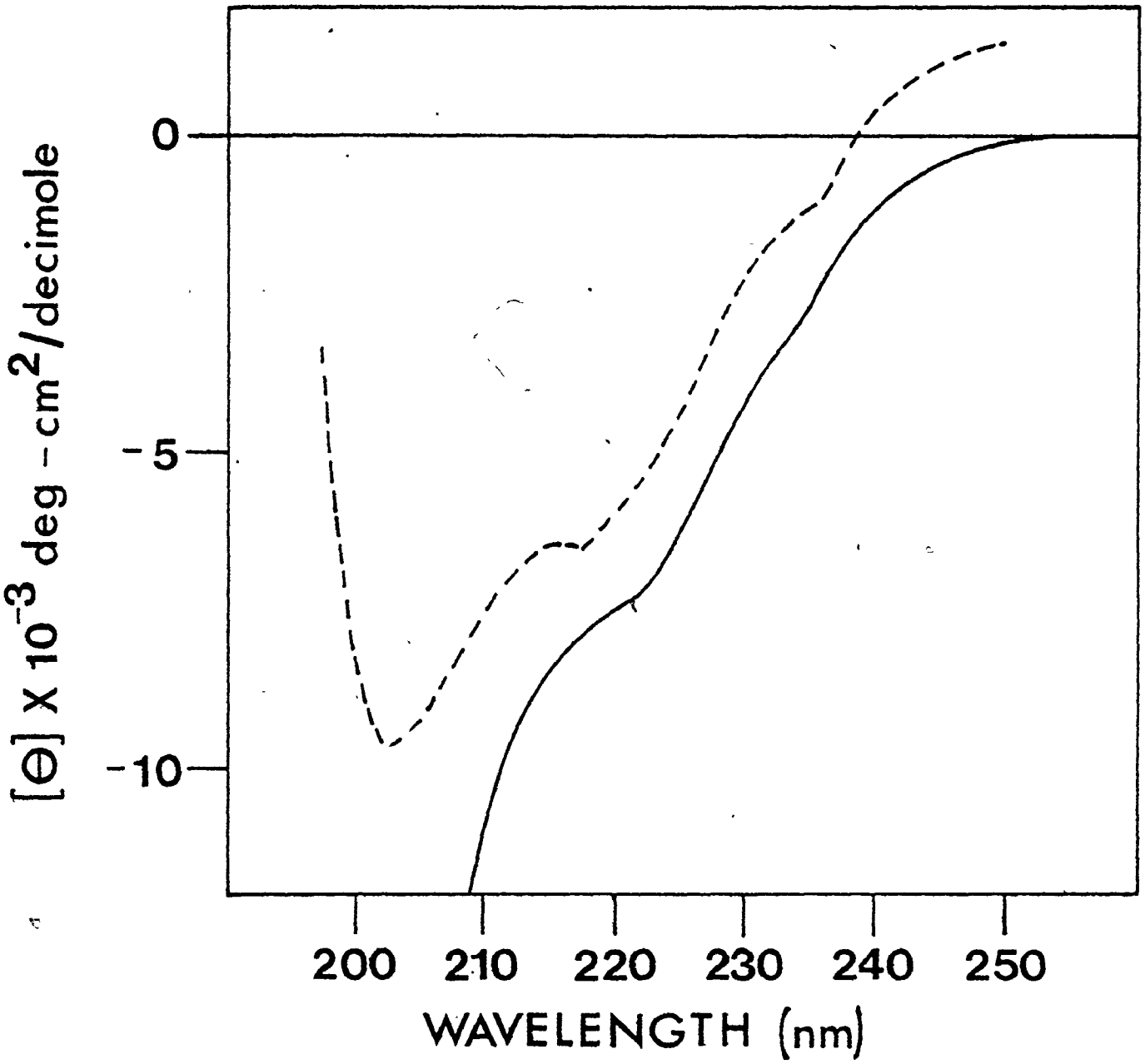

0
-5
-10
[θ] X 10^-3 deg - cm^2/decimole
200
210
220
230
240
250
WAVELENGTH (nm)

Figure 12

Fig. 13. Circular dichroism of glucagon and glucagon-NAPS

Circular dichroism spectra of glucagon and glucagon-NAPS (0.2 mg/ml) in 0.2 M acetic acid were recorded at 30°C. (——) glucagon; (---) glucagon-NAPS.

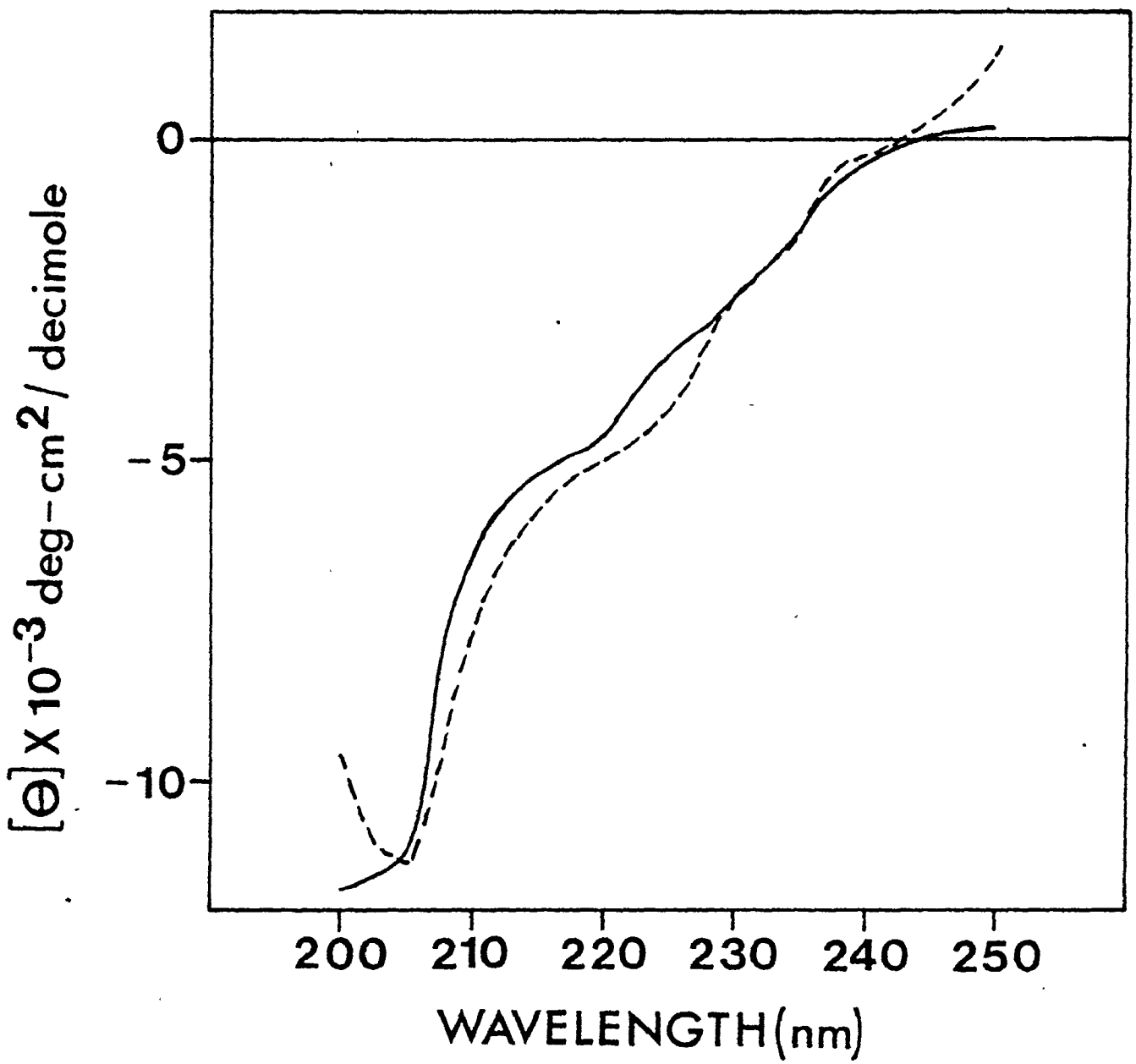

0
-5
-10
$[\theta] \times 10^{-3}$ deg-cm^2/ decimole
200
210
220
230
240
250
WAVELENGTH(nm)

Figure 13

Fig. 14. Circular dichroism of glucagon-NAPS

The circular dichroism spectrum of glucagon-NAPS (0.3 mg/ml) in 0.2 M acetic acid was recorded at 25°C.

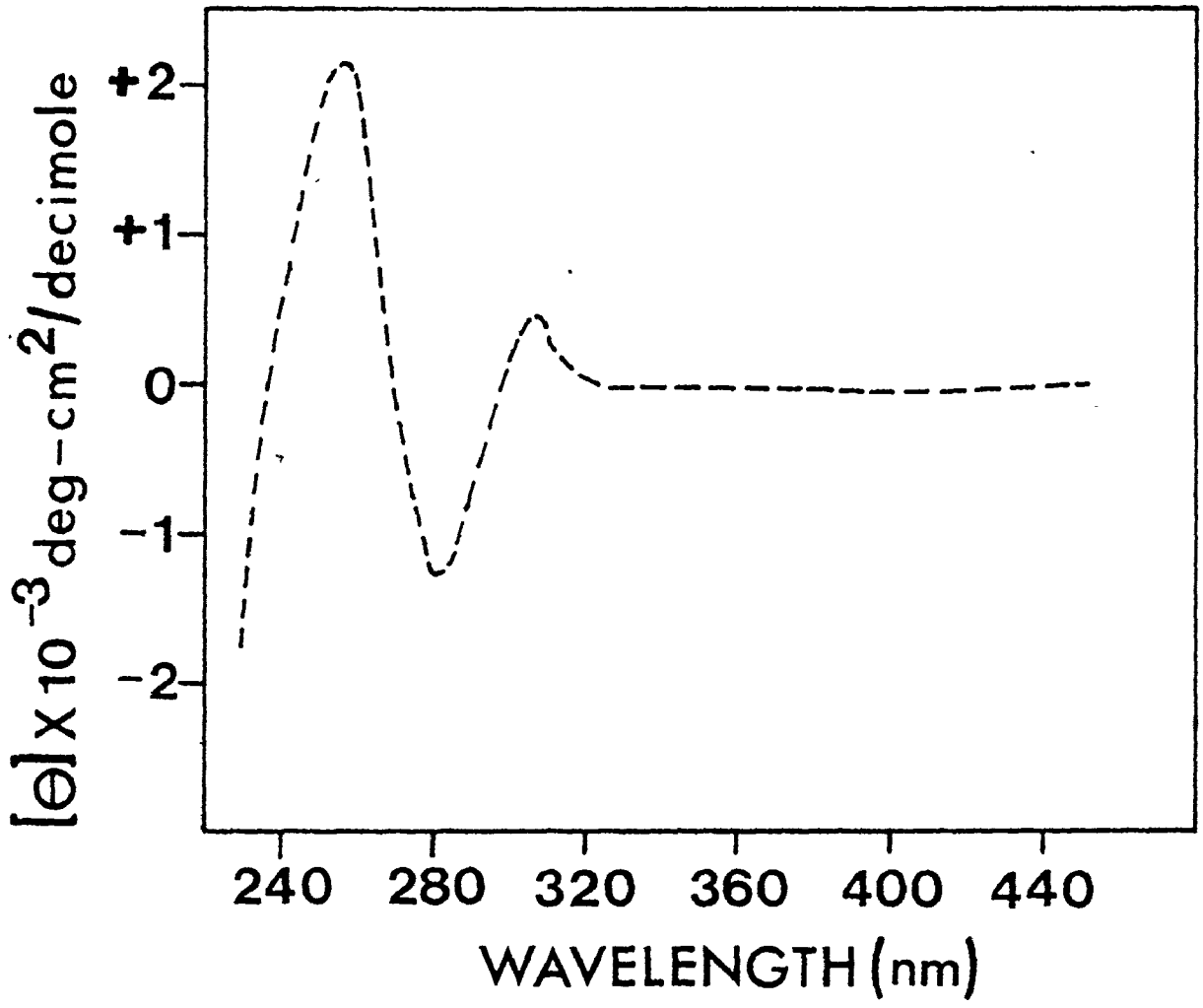

[θ] X 10^-3 deg-cm^2/decimole
+2
+1
0
-1
-2
240
280
320
360
400
440
WAVELENGTH (nm)

Figure 14

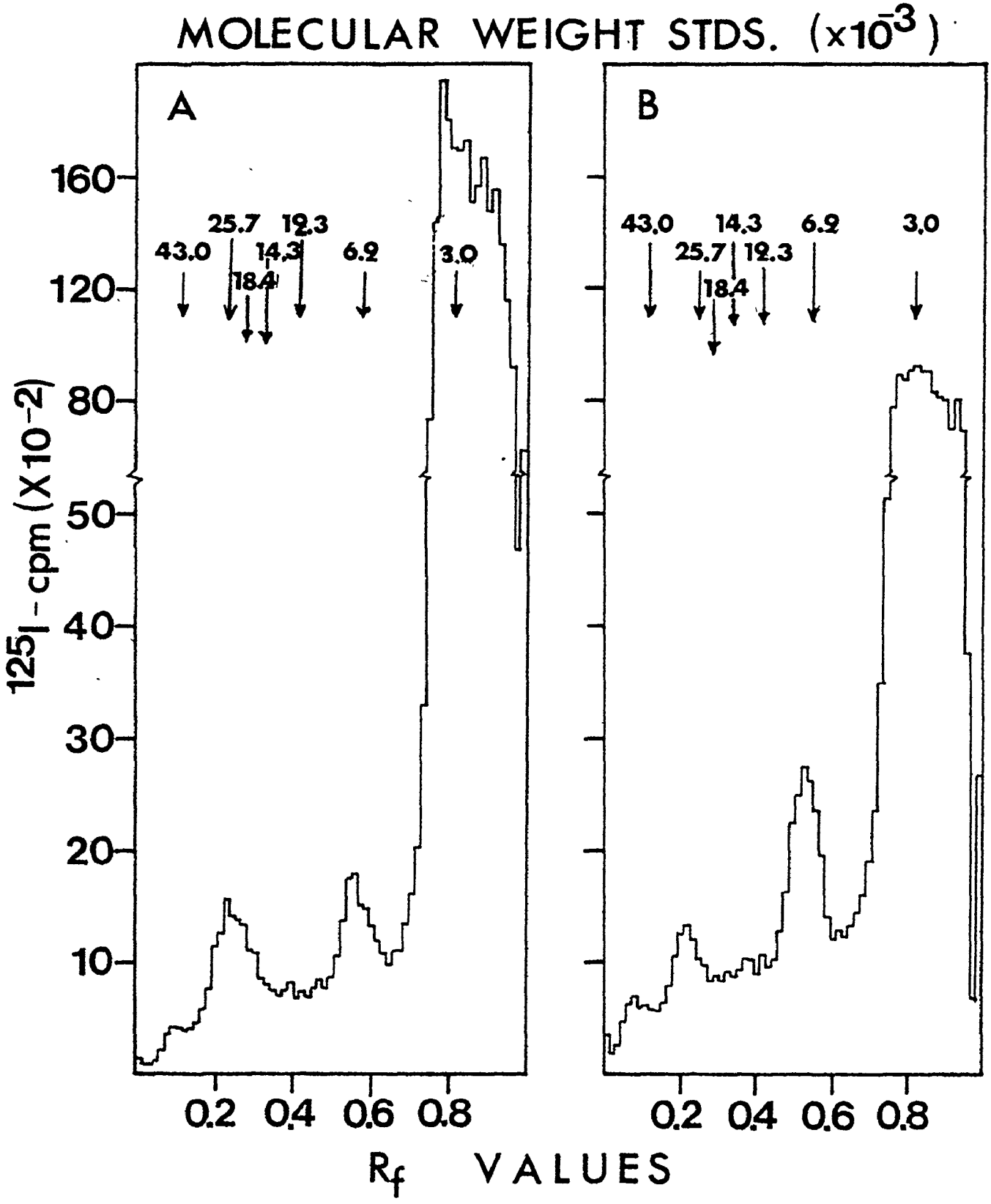
8. Gel Electrophoresis of Covalently Photolabeled Glucagon with ^{125}I-glucagon-NAPS

Polyacrylamide gel electrophoresis in 8 M urea, 12.5% acrylamide of irradiated glucagon (0.10 - 1.0 x 10^{-6}M) in the presence of ^{125}I-glucagon 7.2 x 10^{-10}M (specific activity 2.4 - 2.9 x 10^{6} cpm/pmole), (Fig. 15) identified two radioactively labeled components with molecular weights ≈20,000-26,000 (5%) and 6,000 (5%) and a third, where glucagon eluted, with a MW <3,000. The same components were also observed in unphotolysed ^{125}I-glucagon-NAPS or ^{125}I-glucagon. The 6,000 molecular weight component was labeled 50% more in the presence of cold hormone (10^{-10} - 10^{-6}M), but no appreciable differences were observed for the higher molecular weight species. Other high molecular weight components were also observed if repurified ^{125}I-glucagon or ^{125}I-glucagon-NAPS were used and their amounts varied with the days of storage after repurification. Protein bands stained with Coomassie Blue dye, one >42,000, an 11,000 and a 6,000 were observed in highly overloaded gels, (>200 µg of cold glucagon) constituting less than .01% of peptide. Precipitated protein on top of the gel was also observed. Gels loaded with 50 µg or less did not show any of the higher molecular weight bands (data not shown). The above components were attributed to aggregates spontaneously forming in concentrated glucagon solution. The covalent crosslinking of the radioactive bands in the absence of irradiation was attributed to radiation effects. Indication of aggregates with higher molecular weights than monomeric glucagon were also observed in the irradiation profile of BSA (Fig. 9). These molecular weight species or radiolabeling of BSA were not observed in samples photolysed in 8 M urea which may have

Fig. 15. Electrophoresis profile of irradiated ^{125}I-glucagon-NAPS in 8 M urea, 0.1% SDS, 12.5% acrylamide gels in the presence and absence of cold glucagon

^{125}I-glucagon-NAPS 7.2×10^{-11} M (specific activity 2.78×10^{6} cpm/pmole) was incubated with various amounts of cold glucagon ($0.10\text{-}1.0 \times 10^{-6}$ M) in 30 mM Tris-HCl pH 7.0, 750 KIU/ml Trasylol, at 30°C for 10 min in the dark. The total volume was 0.2 ml. After incubation the samples were cooled at 4°C, saturated with water-saturated N_2 gas for 1 min and subsequently photolysed for 2 min. Irradiation conditions were similar to those described in "Methods" in Chapter II. After photolysis the samples were dried with N_2 gas, redissolved in 0.1 ml 8 M urea, 10% SDS, heated at 60°C and left overnight at room temperature. The samples were subsequently treated and electrophoresed as described in "Methods".

A: ^{125}I-glucagon-NAPS, unphotolysed, control; B: ^{125}I-glucagon-NAPS, photolysed in the presence of 0.05 mg/ml cold glucagon. For brevity only the radioactivity profiles of representative gels are presented.


MOLECULAR WEIGHT STDS. (x10⁻³)
A
B
43.0
25.7
18.4
14.3
12.3
6.2
3.0
160
120
80
50
40
30
20
10
125I - cpm (X10⁻²)
0.2
0.4
0.6
0.8
Rf VALUES

acted as a scavenger as well as a denaturing agent. Monomeric glucagon in 8 M urea gels runs anomalously with a molecular weight of ≈1,800 (Swank and Munkres, (1971). The breadth of the radiolabeled monomeric ^{125}I-glucagon-NAPS (Fig. 15) is probably due to radiolysis fragments with relative mobilities close to that of the intact peptide.

DISCUSSION

The bifunctional reagent 2-nitro-4-azidophenylsulfenyl chloride (NASPCl) was synthesized by a series of five reactions which required inexpensive starting materials and which gave products in good yields. The structure of the final product was determined by proton NMR, IR, Mass Spectra, elemental analysis and spectrophotometric studies.

It has been reported previously that sulfenyl halides such as NPSCl and DNPSCl are highly specific reagents for tryptophan and cysteine residues of polypeptides and proteins under mildly acidic reaction conditions. The product of their reaction with tryptophan is a modified indole with a thioether function in position 2 of the ring (Scoffone et al. 1968; Fontana et al. 1968a,b; Veronese et al. 1968,1970). As expected, NAPSCl also reacts specifically with tryptophan in a mixture of amino acids not containing cysteine. The reaction with tryptophan is characterized by a second-order rate constant of approximately 1.0×10^{2} M^{-1} sec^{-1}. There is also a small loss of methionine, however, which may be caused by oxidation as has been found in the case of NPSCl (Scoffone et al. 1968). In addition to tryptophan, sulfenyl halides can also convert cysteine into an unsymmetrical disulfide under similar reaction conditions. Cysteine can be recovered by treatment with a reducing agent (Fontana et al. 1968a,b). Even though the reaction of NAPSCl with cysteine was not investigated, it is believed that NAPSCl can effectively modify cysteine containing

proteins as well. Another potential bifunctional reagent for a selective modification of cysteine alone in polypeptides and proteins containing both cysteine and tryptophan could be the intermediate 2,2'-dinitro-4,4'-diazidodiphenyldisulfide. Reaction conditions for this reagent would require investigation because of the insolubility of the reagent in protic solvents.

Besides the specificity of NAPSCl for tryptophan containing polypeptides and proteins that lack sulfhydryl groups, the greatest advantage is that it can be used in photolabeling studies of ligand receptor interactions. Photolabeling is possible because of the presence of the azide moiety at the para position of the aromatic ring. As a nitrosulfenyl compound with absorption maxima in the visible region (NAPSCl λ_{max} = 420 nm; $\varepsilon = 2.95 \times 10^{3} M^{-1} cm^{-1}$), the NAPS-derivative can be photolysed at long enough wavelengths to keep radiation damage to their targets minimal. The characteristic absorption spectra of NAPSCl and its derivatives before and after photolysis indicated that NAPS-derivatives have the necessary properties for effective photolabels. The fact that the aniline derivatives were not detected in the irradiation products of NAPSCl or 2,2'-dinitro-4,4'-diazidodiphenyldisulfide suggests that the major product of photoreaction is a singlet rather than a triplet state so that migration of the label through free radical formation becomes less likely.

NAPSCl was shown to be specific and selective for the modification of the tryptophan residue in the hormone glucagon. Only one major species of glucagon modified at the single tryptophan-25 residue was obtained. The high yields of the product of the reaction of NAPSCl with glucagon indicated that NAPSCl can be an equally effective tryptophan-modifying

reagent as NPSCl and DNPSCl (Scoffone et al. 1968; Epand and Cote, 1976). The characteristic absorption spectra of glucagon-NAPS (λ_{max} = 395 nm; $\varepsilon = 3.6 \times 10^{3} M^{-1} cm^{-1}$) and its destruction by photolysis indicated that it can be used for photoaffinity labeling studies.

The iodination method at pH 10 using lactoperoxidase, H_2O_2, propylene glycol provided radiolabeled glucagon and glucagon-NAPS with a high specific activity so that they could be used effectively for binding studies with the glucagon receptor and for photoaffinity labeling. Radioimmuno-precipitation of ^{125}I-glucagon and ^{125}I-glucagon-NAPS with a glucagon specific antibody demonstrated that there are no gross alterations in hormone structure related to the modification of glucagon by NAPSCl. However, the circular dichroism conformational studies of the derivative indicated that the peptide has a higher α-helical content compared to native glucagon contributed possibly by the enhancement of hydrophobic interactions in the conformers of the derivative due to the presence of NAPS reagent. This higher helical content was observed with glucagon-NPS (Epand and Cote, 1976); extrinsic Cotton effects in the range of 260-400 nm were also observed with Trp-25 trinitrophenylated glucagon along with a slow formation of aggregates in acidic solutions (Panijpan and Gratzer, 1974). 1974). Both glucagon-NPS (Epand and Cote, 1976) and the glucagon-NAPS derivatives activated the adenylate cyclase enzyme system of liver plasma membranes even more effectively than the native hormone. Glucagon-NAPS was shown to bind to plasma membrane receptor sites with an enhanced affinity (see Chapter II). It seems, therefore, that a nonpolar modification of the hydrophobic tryptophan residue does not decrease the

biological activity and binding of the hormone to the receptor. These results suggest that the -COOH terminal region of glucagon may interact with membrane receptor sites by relatively nonspecific hydrophobic forces, The hydrophobic properties of the -COOH terminal region of glucagon were shown to be essential for the binding of glucagon to its receptor sites in the plasma membranes, and for binding to phospholipids; as well as for the monomeric associations of glucagon in aqueous solutions (Pohl et al. 1971; Sasaki et al. 1975; Epand, 1980).

The ability of glucagon-NAPS to covalently label neighbouring macromolecules upon irradiation was demonstrated with the specific covalent crosslinking of a glucagon-specific antibody and the nonspecific labeling of bovine serum albumin.

The irradiation studies of covalently crosslinked oligomeric species of glucagon in the presence of ^{125}I-glucagon-NAPS indicated that in dilute solutions 10^{-6} - 10^{-11}M, the peptide is possibly in a monomeric state. Covalently crosslinked dimers may be due to species kinetically trapped in a β-structure during irradiation. The dimeric as well as hexameric species (20,000-26,000, assuming an average molecular weight of peptide 3580; glucagon 3483, glucagon-NAPS 3678) observed in unphotolysed samples are most likely crosslinked by radical formation. In samples with higher peptide concentrations or protein content (i.e. in the presence of BSA) (Fig. 9) oligomeric species of higher molecular weight may form due to a mass action effect and these aggregates formed will be covalently crosslinked upon irradiation. This would be in agreement with the concentration dependence of CD, sedimentation and thermodynamic equilibrium studies (Gratzer and Beaven, 1969; Swann and Hammes, 1969; Formisano et al. 1977). In those

studies the association of glucagon to higher oligomers was attributed to hydrophobic interactions. In the case of ^{125}I-glucagon-NAPS both the iodohydroxyl phenoxy group of tyrosine and the presence of NAPS on tryptophan-25 may contribute to such interactions. The possibility, however, that the higher molecular aggregates observed in the presence of BSA or at high peptide concentrations are due to contaminants or artifacts can not be excluded. A more thorough investigation using other analytical techniques would be required to identify covalently crosslinked oligomers of glucagon-NAPS. It is also possible that at high protein concentrations (2.0-3.0 mg/ml; volume applied 0.1 ml) with an SDS/protein ratio <1, SDS may stabilize such aggregates as shown in the studies on the helical conformation of glucagon in surfactant solutions by Wu and Yang (1980). These aggregates may not necessarily be covalently crosslinked.

The involvement of glucagon oligomers in membrane glucagon interactions and their significance in hormone receptor interactions are discussed in Chapter III.

From the above studies it can be concluded that the bifunctional photoaffinity reagent NAPSCl can be used effectively for the modification of tryptophan containing proteins. Modification of the hormone peptide glucagon with NAPSCl and selective iodination of the glucagon-NAPS derivative provided a radioactively labeled peptide with high specific activity capable of covalent crosslinking to macromolecules upon irradiation. Thus, ^{125}I-glucagon-NAPS provides an ideal photoaffinity derivative for the crosslinking and characterization of the membrane components involved in glucagon-receptor interactions. Glucagon-NAPS may also prove to be a useful tool in the studies for the elucidation of

glucagon conformation under various concentrations, pH and temperature as well as in the studies of interactions with phospholipids.

CHAPTER II

Biological Activity and Binding Characteristics of Glucagon-NAPS Derivative; Photoaffinity labeling of Hepatic Plasma Membranes by Glucagon-NAPS

CONTENTS OF CHAPTER II

INTRODUCTION

Before attempting the photolabeling of the glucagon receptor with ^{125}I-glucagon-NAPS derivative it was essential to demonstrate that the derivative shares the same properties as those of native glucagon in its action to stimulate adenylate cyclase and its specificity of binding to the receptor.

Liver plasma membranes were selected for these studies since liver is the main target organ for glucagon. They were isolated according to Neville's procedure (Neville, 1968). Membranes purified by this procedure have been extensively characterized with regard to composition and marker enzymes as well as in terms of their retention of binding and hormone stimulation by glucagon and insulin even after storage for months at -70 to -90°C (Emmelot et al. 1964; Pohl et al. 1971).

A long series of studies in Rodbell's laboratories and of other investigators have documented that the binding of glucagon to liver plasma membranes and the formation of cAMP are related processes, resulting from the interactions of glucagon with a hormone recognizing membrane component, the glucagon receptor.

A. Glucagon-Adenylate Cyclase

Liver membrane adenylate cyclase activity is a function of glucagon concentrations in the range of 10^{-10}-10^{-7}M glucagon, with half maximal stimulation occurring at about 2-4 x 10^{-9}M. Neither basalnor glucagon stimulated adenylate cyclase activity are inhibited by insulin or other

peptide hormones. Enzyme activity requires a divalent cation, either Mg^{++} or Mn^{++} and is inhibited by Ca^{++}. 1 mM EDTA enhances the glucagon stimulated activity while glutaraldehyde, sulfhydryl reagents, phospholipase A and urea treatment completely inactivate the adenylate cyclase system. Fluoride ion stimulates adenylate cyclase activity to a lesser degree than does glucagon (Pohl et al. 1971).

Guanyl nucleotides were shown to stimulate the hormone response of adenylate cyclase at concentrations lower than 0.1 mM indicating that the hormone-activating process is relatively specific for these nucleotides. It was also shown that they do not act competitively with glucagon. The action of these nucleotides is inhibited by sulfhydryl reagents which also inhibit binding of glucagon (Rodbell et al. 1971a,b).

B. Glucagon-Receptor Binding

The use of ^{125}I-glucagon in binding studies with liver plasma membranes has demonstrated an apparent affinity of the hormone (2×10^{-9}M) which is equivalent to that needed for adenylate cyclase stimulation.

The binding sites of glucagon were shown to be finite in number, $\simeq$ 2.6 pmole/mg membrane protein and saturable at 4.8×10^{-8}M glucagon, the same range found for maximal activation of adenylate cyclase. Specificity of the binding sites was demonstrated by using fragments with partial sequences of glucagon and a number of peptide hormones, like insulin and secretin. Secretin shares 14 positionally identical amino acid residues with glucagon (Falk and Cole, 1965). Glucagon binding to liver plasma membranes was not inhibited by any of the peptide hormones tested (Rodbell et al. 1971a,b).

Binding of the radiolabeled glucagon was found to be temperature

dependent and as in the case of adenylate cyclase stimulation, treatment with phospholipase A and denaturing agents abolished binding, suggesting that a lipoprotein and/or lipid components may be essential for binding and action of glucagon.

GTP at concentrations as low as 0.05 μM were shown to increase the rate of glucagon binding to its receptor sites. Steady state was reached within 30 sec in agreement with the rate of adenylate stimulation by glucagon in the presence of GTP and was independent of glucagon concentrations within the range of 0.7-3 nM (Rodbell et al. 1971a,b,. 1974; Lin et al. 1977). Scatchard analysis of binding studies in the absence of GTP showed curvature suggestive of positive cooperativity, or heterogeneity in the binding states. GTP causes the formation of high affinity receptor sites with only about 10% of the binding capacity of the total glucagon receptor sites. The rest of the receptor sites are converted to states with lower affinity for the hormone (Rodbell et al. 1974; Lin et al. 1977). The nucleotide effects are temperature independent and are inhibited by sulfhydryl reagents although binding is only inhibited by 61% (Rodbell et al. 1971a,b).

C. The Role of GTP in the Glucagon Receptor Interactions and Adenylate Cyclase Stimulation

Rodbell et al. (1971a,b, 1973, 1974, 1975);Birnbaumer et al. (1972), Birnbaumer (1973),have demonstrated the importance of GTP in the action of hormones on adenylate cyclase systems and the presence of a nucleotide regulatory site in eukaryotic cells, in addition to the hormone receptor and the catalytic site.

Binding of glucagon to the receptor in the absence of GTP is

slow and the time of equilibrium is inversely proportional to the hormone concentration. In the presence of GTP, equilibrium is reached in less than a minute and the kinetics of binding agree with the kinetics of activation of adenylate cyclase by the hormone as previously discussed for glucagon. GTP fails to cause marked activation of adenylate cyclase systems in the absence of hormones (Rodbell et al. 1974) and in certain cases in the presence of hormones (Lefkowitz, 1974).

Studies with Gpp(NH)p (guanosine-5'-(β,γ-imi o)triphosphate) indicated that a terminal high energy phosphate of GTP is not utilized in the allosteric modulation of adenylate cyclase by GTP (Rodbell et al. 1971b). Gpp(NH)p unlike GTP was found to be a potent activator of adenylate cyclase systems, irrespective of the hormone receptor coupled to the system or even in the absence of a receptor (Londos et al. 1974). However, Gpp(NH)p was considerably less potent than GTP on the hormone binding process (Lad et al. 1977).

GDP was also shown to inhibit competitively Gpp(NH)p action on adenylate cyclase; as well as the basal activity of the enzyme and that stimulated by low concentrations of glucagon (Salomon et al. 1975).

Binding and effects of guanyl nucleotides were inhibited in the presence of sulfhydryl reagents suggesting the involvement of -SH groups (Salomon and Rodbell, 1975).

Later studies with GTP, GDP and Gpp(NH)p showed that all nucleotides competed for a common site and their markedly different potencies were due to their different susceptibilities to hydrolysis when bound to membrane sites (Salomon and Rodbell, 1975).

Studies by Kimura and Nagata (1977), in the absence of a nucleotide

regenerating enzyme system reported a different observation to that of Salomon et al. (1975) concerning the action of GDP. GDP was shown to enhance liver adenylate cyclase activity with a similar affinity to that of GTP, and to stimulate the enzyme to the same extent as GTP or Gpp(NH)p in the presence of 1 μM glucagon. A similar GDP stimulation was also reported for epinephrine (Hanoune et al. 1975). Furthermore, the stimulation of adenylate cyclase was found to be a function of the concentration of GDP or GTP but not of Gpp(NH)p and this effect was contributed partly by the GDP produced by nucleotidase hydrolysis of GTP in the membranes. Their conclusion was that GDP rather than GTP may actually be involved in the coupling process of hormone receptor to adenylate cyclase.

Further studies by Hanoune et al. (1975) using nonhydrolysable ATP and GTP analogues, showed that the structural requirements for a glucagon response appears to be the presence of a free hydroxyl group at the 3' position of the ribose. They also showed that the GDP and GTP stimulatory effects on adenylate cyclase when ATP is present as substrate, may be due to phosphorylation of GDP which is enhanced by the glucagon action on receptor sites. This was supported by the fact that the presence of nucleoside triphosphates other than GTP restored the GDP inhibitory effects (Kimura and Nagata, 1979).

Hydrolysis of GTP by the putative action of GTPase, which is associated with the GTP-regulatory protein in the adenylate cyclase systems (Cassel et al. 1977), has been associated with the turning off of the GTP-activation process. GTP and GTP analogues interact with the GTPase enzyme, resulting in an active adenylate cyclase system. The GDP action, however, may result in an enzymatic state with low activity,

suggesting that the affinity and the release rate of GDP may be the limiting step regulating enzyme activity (Cassel and Selinger, 1978).

It has been suggested that cholera toxin which inhibits GTPase and results in the enhancement of cAMP production, may act by preventing GTP hydrolysis on the GTP regulatory protein, thus maintaining the adenylate cyclase system in an active state (Cassel and Selinger, 1977; Cassel and Pfeuffer, 1978; Pfeuffer, 1979).

Subsequent studies (Iyenger and Birnbaumer, 1979; Iyengar et al. 1979) of adenylate cyclase stimulation by glucagon after pretreatment with Gpp(NH)p, which can not be hydrolyzed by GTPase, GDP stimulated adenylate cyclase with a K_a of glucagon of 0.1-0.15 μM. This effect was not attributed to transphosphorylation reactions when ATP is the substrate. If the membranes were not preactivated by Gpp(NH)p, GDP inhibited Gpp(NH)p adenylate cyclase stimulation competitively but did not affect the glucagon stimulation. These observations were accounted for by suggesting two sites of regulation, one dependent on the occupancy of the receptor site by the nucleotide (either GDP or GTP) for activation and the second dependent on the GTPase activity, being inhibited by GDP and nonhydrolysable nucleotide analogues of GTP. It was suggested that both levels may coexist regulating external or internal stimuli (Iyenger and Birnbaumer, 1979; Iyenger et al. 1979).

Indeed, further studies on the effects of GTP and Gpp(NH)p on adenylate cyclase stimulation and receptor affinity for glucagon in liver plasma membranes indicated that the nucleotide sites responsible for altering receptor conformation and enzyme activity may be functionally distinct (Lad et al. 1977). Detergent solubilization of the glucagon-receptor adenylate cyclase activity by the nonionic detergent Lubrol-PX and

fractionation by chromatography showed that both hormone-receptor and adenylate cyclase activity are each associated with a guanyl nucleotide site, thus indicating two functionally and probably structurally distinct regulatory sites (Welton et al. 1977).

Radiation-inactivation techniques (target site analysis) of the adenylate cyclase and glucagon receptor in isolated hepatic plasma membranes showed that the glucagon receptor is associated with a nucleotide regulatory unit in a multimeric complex of subunits. Interactions of these subunits were suggested as being responsible for the apparent cooperativity observed in the binding of glucagon in the absence of GTP (Schleger et al. 1979).

The above glucagon and other peptide hormone receptor-adenylate cyclase interactions, along with the radiation inactivation observations and the role of the GTP regulatory site are consistent with Rodbell's (1980) aggregation-disaggregation model discussed in the "Introduction".

In contrast to the above, irradiation-inactivation studies by Martin et al. 1979, introduced a different model where, in the presence of glucagon the receptor associates with the GTP-regulatory protein-adenylate cyclase catalytic subunit. In the presence of GTP or Gpp(NH)p the complex dissociated resulting in the release of a fully activated catalytic subunit. Both glucagon and a guanine nucleotide are needed to fully dissociate the catalytic subunit from the complex. Therefore, according to this model the role of the hormone receptor is the facilitation of the GTP action on the catalytic subunit of the adenylate cyclase.

Similar irradiation studies by Houslay et al. 1977, have

demonstrated that in the absence of glucagon the receptor and catalytic unit are functionally distinct mobile entities; on hormone binding to the receptor, the complex couples with the catalytic unit, activating it and forming a multicomponent system spanning the bilayer.

Transient and steady state kinetics in the interactions of guanyl nucleotides with the adenylate cyclase system from rat liver plasma membranes were simulated on the basis of a two-state model assuming an allosterically regulated enzyme (Birnbaumer et al. 1980a,b). The two-state model when tested for the dose response curves of hormone in four adenylate cyclase systems, including glucagon, showed that hormones may activate the adenylate cyclase system by potentiating the effect of guanyl nucleotides isomerization of active, inactive enzymatic state. This model also predicts that in the presence of an active receptor, hormonal stimulation may result in the absence of a nucleotide or in the presence of an ineffective one, like GDP. The model was independent of a GTPase enzyme associated with the system (Iyengar et al. 1980; Birnbaumer et al. 1980a,b).

D. The Role of Phospholipids in Glucagon Activated Adenylate Cyclase

The attempts for purification of a solubilized glucagon-sensitive adenylate cyclase system have been unsuccessful until now, suggesting that membrane lipids may be involved in the system for basal and hormone-stimulated activities. The use of detergents and phospholipase A under appropriate conditions have been shown to result in increased fluoride stimulation of adenylate cyclase but loss of glucagon stimulation

(Birnbaumer et al. 1971). Partial restoration of glucagon response was observed after incubation of phospholipase-treated membranes with aqueous micellar lipid dispersions (Pohl et al. 1971b).

Specificity in phospholipid requirements for glucagon-adenylate cyclase was demonstrated by Levey et al. (1975), (Levey, 1975), in a solubilized adenylate cyclase system of particulate fraction from rat heart. The role of acidic phospholipids required for glucagon adenylate cyclase stimulation (Levey et al. 1975) was also demonstrated in liver plasma membranes by the use of specific phospholipases (Rubalcava and Rodbell, 1973).

Diet supplementation with ethanolamine and/or fatty acids in LM mice, was shown to alter basal as well as fluoride and hormone-stimulated adenylate cyclase activity of fibroblast cells (Engelhard et al. 1976).

Studies on the temperature dependence of adenylate cyclase activity in hamster liver plasma membranes gave Arrhenius plots of uncoupled adenylate cyclase with a single break at 25°C, but those of coupled adenylate cyclase activity with glucagon exhibited two breaks at 25°C and 13°C (Houslay, 1979). GTP abolished the glucagon-conferred sensitivity to the lipid phase separation occurring in the outer half of the bilayer in both hamster and rat liver plasma membranes but Gpp(NH)p did not. These results were explained in terms of two guanine nucleotide binding sites as separate proteins, one regulating adenylate cyclase activity and localized in the inner half of the bilayer and a transmembrane protein occupied in the presence of hormone for regulating coupling. In the presence of GTP and glucagon, a transmembrane complex exists, resulting

in two lipid phase separations. When GTP is present alone lipid phase separation occurs in the inner half of the bilayer only, while in the presence of Gpp(NH)p a stable complex is produced sensitive to lipid phase separation in both halves of the bilayer. When Gpp(NH)p alone is present, the activated complex is sensitive to lipid phase separation in the inner half of the bilayer only. This model suggests that in the presence of GTP a number of catalytic units can be activated by a single occupied receptor, thus explaining the GTP effects on glucagon-adenylate cyclase stimulation and its noncorrespondence to receptor occupancy (Houslay, 1979).

Introduction of synthetic phospholipids shift the break in Arrhenius plots of glucagon sensitive adenylate cyclase in accordance with the phase separation temperatures of the synthetic phospholipid, but not in the fluoride stimulated system whose Arrhenius plots are linear (Houslay et al. 1976).

The local anaesthetic benzyl alcohol increased glucagon- and fluoride-stimulated adenylate cyclase in a concentration dependent fashion up to 50 mM indicating that increase in membrane fluidity alters the association of membrane bound adenylate cyclase enzyme with the surrounding lipids and suggesting that this association may also occur when the enzyme is coupled to the glucagon receptor (Dipple and Houslay, 1978).

E. Adenylate Cyclase-NaF Stimulation

Glucagon and fluoride ions have been shown to activate the adenylate cyclase enzyme system through processes that have different

characteristics (Birnbaumer et al. 1971). Guanyl nucleotides inhibited the response of adenylate cyclase to fluoride (Rodbell et al. 1971b).

The catalytic component of the adenylate cyclase system as reflected by fluoride activation was either unaffected or enhanced by agents that alter lipids (Pohl et al. 1970; Houslay et al. 1976a).

Fluoride is believed to act directly on the catalytic unit exposed on the inner surface of the membrane bilayer (Houslay et al. 1976b).

Irradiation inactivation studies of the size of adenylate cyclase system showed that activation by fluoride is not temperature dependent. The effect of fluoride was stimulatory up to 10 mM concentrations and then it became inhibitory. If the enzyme was preactivated with glucagon or Gpp(NH)p then fluoride was inhibitory at all concentrations, suggesting that activation by fluoride and guanyl nucleotides requires the dissociation of a GTP regulatory subunit of about 100,000 daltons (Martin et al. 1980).

The importance of GTP in glucagon-binding to liver plasma membrane receptor and its role in the intimate association with the hormone coupled adenylate cyclase enzyme system, as well as the complexity of the system and the controversy in the ideas defining the mechanism of hormone interactions has been presented above.

Presentation of these findings was considered necessary for a better understanding of the parameters and conditions in the studies with the glucagon-NAPS derivative.

The adenylate cyclase stimulation by glucagon and glucagon-NAPS were measured under conditions to optimize enzyme activation. Dose response curves were obtained in order to compare the relative affinities of glucagon

and glucagon-NAPS for the receptor in liver plasma membranes and to determine whether glucagon-NAPS can cause the same maximal response as the native hormone indicative of interactions with the same receptor recognition sites. However, since only limited information can be obtained from dose response curves concerning the receptor sites, they were complemented with binding studies. Considering the effect of GTP on hormone receptor interactions, and the difference in receptor affinity that is displayed by the radiolabeled hormone, binding studies were conducted with both ^{125}I-glucagon and ^{125}I-glucagon-NAPS in the presence and absence of GTP. The dissociation constant of binding isotherm curves were correlated with the concentration of the hormone required for biological action. Competitive binding studies using the radiolabeled hormone and either glucagon or glucagon-NAPS were used to evaluate the characteristics of the receptor system studied. Knowledge of the receptor binding parameters was considered necessary for the interpretations of the covalent labeling of the receptor with glucagon-NAPS upon irradiation.

In binding studies it is critical to distinguish nonspecific binding from that of nonreceptor specific binding and specific binding to the receptor sites. In addition to binding to nonspecific sites, additional nonspecific binding can result from degradation of radiolabeled hormone, transpeptidation of radiolabeled amino acid residues, displacement of the radioactive atom or by other processes, so that saturability of the receptor sites may not be observed, i.e. if the background nonspecific binding is large it may obscure the receptor specific binding sites. Because nonspecific binding is dependent on the concentration of radiolabeled hormone used, it is important that the binding studies are

conducted with low concentration of radioactive ligand having a high specific activity. Both ^{125}I-glucagon and ^{125}I-glucagon-NAPS possessing a high specific activity (≈2.47 μCi/pmole) were used at 10^{-11} - 10^{-10}M concentrations in order to minimize nonspecific binding. Nonspecific binding was measured as the amount of radioactive ligand that was not displaced by a 10,000-fold greater concentration of unlabeled ligand.

Nonreceptor specific binding may manifest itself in the nonlinearity of Scatchard plots used for analyzing the binding data. Hollenberg and Cuatrecasas (1979) have presented the possible sources for nonlinearity in such plots which include, along with underestimated nonspecific binding, positive or negative site-site cooperativity; differences between the affinity of labeled and the unlabeled ligands; binding of the ligand simultaneously to more than one site; binding studies at pre-equilibrium; and inaccurate determinations of the amounts of bound and free ligand. These possibilities were taken into account in the interpretation of the data from binding studies performed.

Specificity of glucagon-NAPS binding to receptor sites was demonstrated in liver plasma membranes radiolabeled by glucagon-NAPS upon irradiation.

METHODS

1. Rat liver plasma membrane preparation

Partially purified rat liver plasma membrane was prepared using male Wistar rats (150 g each), according to the modified Neville procedure (Neville, 1968; Pohl et al. 1971). All steps were performed at 0-4°C, in hypotonic bicarbonate solution.

Step 1: 15 rats were decapitated rapidly and the livers were excised (≈80 g of liver).

Step 2: The excised livers were minced in two lots of 40 g with a pair of scissors.

Step 3: Ten g of minced liver were homogenized in a Dounce homogenizer (piston size A) with 25 ml of 1 mM $NaHCO_3$, pH 7.5 - 7.8, using 10 strokes. This was repeated for the rest of the 40 g lot.

Step 4: The four homogenates were combined into a large beaker and the volume made up to one liter with bicarbonate solution. After mixing for 3-5 min. using magnetic stirring, the homogenate was filtered, first through two layers and then four layers of gauze.

Step 5: The filtered homogenate was transferred into four 250 ml polycarbonate centrifuge bottles and centrifuged in a Sorval centrifuge (RC-5) for 10 min at 2,700 rpm (≈1500 x g).

Step 6: During centrifugation the above procedure was repeated for the rest of the liver.

Step 7: The supernatants were carefully aspirated and the pellets

poured into the cleaned Dounce homogenizer and resuspended with three strokes of the loose pestle. The membrane suspension was poured into a graduated cylinder, 68 ml of 69% (wt/wt) sucrose was added and the volume made to 120 ml with water. After mixing well using magnetic stirring the sucrose concentration was measured using an Abbe refractometer. Additional water or 69% sucrose was added as necessary to adjust the sucrose concentration to 44.0 ± 0.1% (wt/wt).

Step 8: The sucrose-homogenate mixture was distributed equally (≈20 ml) into 6 nitrocellulose centrifuge tubes (1 x 3 inch). Each tube was then carefully overlayed with 12 ml of 42.3 ± 0.1% (wt/wt) sucrose.

Step 9: The tubes were centrifuged in a Beckman ultracentrifuge (SW-27 rotor) at ≈90,000 x g, 4°C for 2 hr.

Step 10: The membrane fraction floating on the surface was removed using a teflon coated spatula, transferred into a 15 ml Corex tube, resuspended in 15 ml bicarbonate solution using a 22 gauze needle and centrifuged in a Sorval centrifuge (SW-34 rotor) at 12,000 x g, 4°C for 10 min. This was repeated three times, each time aspirating the supernatant.

Step 11: For the final washing the resuspended membrane was distributed in 300-400 µl aliquots into microfuge tubes and centrifuged in a Beckman microfuge B, at 4°C at 7,960 x g for 10 min. The supernatant was removed and the membrane pellets frozen at -80°C.

The purity of the final preparation was evaluated by the use of marker enzymes. 5'-Nucleotidase (E.C.3.1.3.5.) was measured by the method of Bodansky and Schwartz (1963), glucose-6-phosphatase (E.C.3.1.3.9.) by the method of Toda et al. (1975). The enzymes were assayed in 1 ml

incubation medium for 15 min. at 37°C. Reactions were stopped by the addition of 1 ml 10% trichloroacetic acid. Liberated inorganic phosphate was measured by the method of Fiske and Subba Row (1925). Succinate-cytochrome c reductase was measured by the method of King (1967).

2. Adenylate cyclase assay

Adenylate cyclase was assayed by the technique of Krishna et al. (1968) using [^{14}C] ATP as a substrate and [^{3}H] cAMP as a tracer. The final assay medium contained 30 mM Tris-HCl pH 7.0 or 7.6, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM mercaptoethanol, 0.2 mM cAMP, 0.1 mM GTP, 0.56 mM [^{14}C]ATP, 5 mM creatine phosphate, 0.64 mg/ml creatine phosphokinase (50-60 units/ml), 1 mM aminophylline and 4 mg/ml bovine serum albumin in a final volume of 100 µl. Glucagon or glucagon-NAPS, 10 or 20 mM NaF in 30 mM Tris-HCl containing 1 mg/ml BSA, pH 7.0 or 7.6 were included as indicated. When testing the activities of epinephrine (10^{-4}M), and isoproterenol (10^{-4}M), glucagon, glucagon-NAPS, NaF, epinephrine and isoproterenol were dissolved in 30 mM Tris-HCl, 1 mg/ml BSA, 0.1% w/v ascorbate pH 7.0. The reaction was carried in 1.5 ml Eppendorff tubes. Liver plasma membranes resuspended in 30 mM Tris-HCl, pH 7.0 or 7.6, and 4 mg BSA/ml were added at zero time into the incubation medium giving a final concn. of 0.1 - 0.3 mg/ml. After 1 min equilibration of the membranes at 30°C, the reaction was initiated by the addition of [^{14}C] ATP (15-20 dpm/pmole). The reaction was stopped after 10 min incubation by the addition of 150 µl 1 N $HClO_4$. An additional 265 µl water and 10 µl [^{3}H] cAMP tracer (3,000-10,000 cpm) were added and cAMP was isolated by the procedure reported by White and Karr (1978) using first Dowex 50

columns (Dowex 50W-X8, 200-400 mesh), followed by alumina columns (Alumina WN-3, Brockman affinity II-III).

Adenylate cyclase assays in the presence of glucagon-NAPS were performed in the dark under red light.

3. Adenylate cyclase activation by glucagon in the presence or absence of GTP

Liver plasma membranes(0.14 mg/ml) were tested in quadruplicate for adenylate cyclase enzyme activation by glucagon (2.88×10^{-11}-10^{-6}M) in the presence or absence of 0.1 mM GTP under the assay conditions described above, at pH 7.0.

4. Adenylate cyclase activation by photolysed glucagon or glucagon-NAPS in liver plasma membranes

0.5 ml of glucagon or glucagon-NAPS (10^{-6}M) in 30 mM Tris-HCl pH 7.0, 1 mg BSA/ml and 750 KIU/ml Trasylol, were first cooled in ice for 5 min, saturated with ice-cold water saturated N_2 gas for 1 min. and subsequently photolysed for 2 min. Photolysis conditions were those described later in "Methods". Aliquots removed at 0, 15, 30, 60, 90 and 120 sec were tested in triplicate for adenylate cyclase activation in liver plasma membranes under standard assay conditions. The final concentrations tested were 10^{-7}M glucagon and glucagon-NAPS.

5. Activation of adenylate cyclase by glucagon in rat liver plasma membranes subjected to photolysis

Liver plasma membranes (0.5 ml of 0.5 mg/ml) in 30 mM Tris-HCl pH 7.0, 1 mg BSA/ml were photolysed under the same conditions as the hormones above. Aliquots removed at 0, 15, 30, 60, 90 and 120 sec were assayed in triplicate for adenylate cyclase stimulation by 10^{-7}M glucagon under standard assay conditions. The final membrane concentration was 0.15 mg/ml.

6. Activation of adenylate cyclase in rat liver plasma membranes subjected to photolysis in the presence of glucagon-NAPS

Liver plasma membranes (1.5-2.0 mg/ml) were incubated with glucagon (2.88×10^{-8}M), glucagon-NAPS (2.72×10^{-8}M) or buffer (30 mM Tris-HCl pH 7.0, 4.0 mg BSA/ml) in 30 mM Tris-HCl pH 7.0, 4.0 mg BSA/ml and Trasylol, 750 KIU/ml at 30°C, in the dark for 5 min. The total volume was 0.5 ml.

After incubation the membranes were photolysed for 2 min under N_2 gas as described below.

Photolysis was followed by dilution of the membranes with 4 ml 0.1 mM GTP, 10 mg BSA/ml, 1 mM EGTA, 2 mM mercaptoethanol in 30 mM Tris-HCl pH 7.0 in a Corex glass tube (15 ml capacity). The samples were vortexed and subsequently incubated for 15 min at 30°C to ensure dissociation of the noncovalently bound hormone.

The membranes were then pelleted by centrifugation in a Sorval centrifuge (SS 34 rotor) at 19,500 x g at 4°C for 10 min. The supernatant was discarded and the pellets were washed once with 1.0 ml

30 mM Tris-HCl buffer pH 7.0. The membranes were resuspended in 30 mM Tris-HCl, pH 7.0 containing 1 mg BSA/ml using a 23 gauge needle. The resuspended membranes (0.3-0.4 mg/ml) were assayed in triplicate for adenylate cyclase stimulation by glucagon or glucagon-NAPS at concentrations shown in the legends to figures and tables. The adenylate cyclase assay was performed at pH 7.0 under conditions previously described. The final membrane concentrations ranged between 0.10 - 0.15 mg protein/ml.

A similar set of membranes was treated as above but instead of the photolysis step the membranes were incubated under the same conditions in the dark for 2 min.

As a control, fresh membranes (0.10 - 0.15 mg/ml) were assayed under similar assay conditions.

7. Adenylate cyclase activity of the glucagon-NAPS photolabeled liver plasma membranes, in the presence of NaF, epinephrine, isoproterenol and glucagon

Two sets of liver plasma membranes (1.5 - 2.0 mg/ml) were preincubated with glucagon-NAPS (2.72×10^{-8}M) or 30 mM Tris-HCl, pH 7.0 containing 1 mg BSA/ml and Trasylol, 750 KIU/ml as previously described. One set was photolysed, the other was kept in the dark. After dissociation with 0.1 mM GTP described above, free hormone was separated by centrifugation and the resuspended membranes were assayed in quadruplicate for adenylate cyclase stimulation by 10^{-2}M NaF, 10^{-5}M epinephrine (bitartrate), 10^{-4}M isoproterenol or 10^{-7}M glucagon. The assay conditions (pH 7.0) were as previously described. The adenylate cyclase activating agents were dissolved in 30 mM Tris-HCl pH 7.0, 1 mg BSA/ml, 0.1% (w/v) ascorbate to prevent oxidation.

As a control, fresh membranes (final concentration 0.14 mg/ml) were assayed for adenylate cyclase stimulation by the above reagents.

8. Basal adenylate cyclase activity of the glucagon-NAPS photo-labeled liver plasma membranes

Two sets of liver plasma membranes 1.5 - 2.0 mg protein/ml were preincubated with 10^{-7}M glucagon-NAPS in 30 mM Tris-HCl pH 7.0, 4 mg BSA/ml, 750 KIU/ml Trasylol, or without the hormone. Another two sets were also preincubated with or without glucagon-NAPS in the adenylate cyclase medium after substituting [^{14}C] ATP with 1 mM cold ATP.

After incubation at 30°C for 5 min the membranes were photolysed or kept in the dark; the noncovalently bound hormone was dissociated and removed by centrifugation as previously described. The resuspended membranes were subsequently assayed for adenylate cyclase activity for periods up to 30 min under standard assay conditions. As controls, fresh membranes were also assayed under the same conditions. Final membrane concentrations ranged between 0.1 - 0.2 mg protein/ml.

9. Measurement of the dissociation of bound hormone from liver plasma membranes in the presence of 0.1 mM GTP

Liver plasma membranes (2.5 mg/ml) were preincubated with ^{125}I-glucagon-NAPS(8.5×10^{-10}-1.7×10^{-8}M specific activity 3.0-2.5×10^{6} cpm/pmole) and photolysed for 2 min or kept in the dark under the conditions described above in section 8, in a final volume 0.5 ml. The amount of radiolabeled hormone bound to the membrane was measured according to the method of

Rodbell et al. (1971). Samples, 50 µl, in triplicate, of the incubated medium were layered on top of 300 µl of an ice cold solution of 2.5 mg BSA/ml in 30 mM Tris-HCl, pH 7.0, contained in a Beckman plastic microfuge tube (400 µl capacity). The membranes were immediately sedimented in a Beckman microfuge B at 4°C by a 5 min centrifugation (≃ 10,000 x g). The supernatant fluid was discarded by aspiration to about 3-4 mm above the pellet which could be visualized by shining a high intensity light on the centrifuge tube against a black cardboard. A Pasteur pipette made to a thin point, attached to a vacuum line, was used for aspiration. The walls of the tube were washed gently first with 400 µl 10% ice cold sucrose, the supernatant was aspirated, 300 µl of 10% (w/v) sucrose were added, followed by vortexing and centrifugation for 5 min at 4°C. The sucrose was aspirated to about 1.2 mm above the pellet and the tubes were inverted to drain any additional sucrose remaining on the walls. The tip of the centrifuge tubes were cut with a razor blade just above the pellet and were transferred into 12 x 75 mm glass tubes for counting in a Searle γ-counter (Model 1285). Samples, 50 µl, in triplicate, were counted directly for the determination of the total amount of radiolabeled hormone present.

The remainder of the incubation medium, 0.2 ml, was diluted with 1.6 ml of 0.1 mM GTP, 10 mg BSA/ml, 1 mM EGTA, 2 mM mercaptoethanol in 30 mM Tris-HCl, pH 7.0 in a Corex tube (15 ml), vortexed and subsequently incubated for 15 min in a 30°C water bath. After incubation, the membranes were pelleted by centrifugation at 12,500 x g at 4°C. The supernatant was discarded and the membranes were resuspended in 30 mM Tris-HCl, pH 7.0 (0.2-0.3 mg protein/ml). The remaining amount of radiolabeled hormone bound

was measured in 50 µl triplicate aliquots by the centrifugation method, as described above.

In order to correct for the quantity of nonspecific ^{125}I-glucagon-NAPS bound to the tube and to the membranes, equivalent amounts of membrane incubation medium containing the same concentration of radiolabeled hormone, in the absence of liver membranes or in the presence of cold glucagon-NAPS 10^{-6}M, were treated in the same way as described for test samples.

10. Assay for the determination of binding of glucagon and glucagon-NAPS to liver plasma membranes in the presence and absence of 0.1 mM GTP

Binding of glucagon and glucagon-NAPS to liver plasma membranes was assayed according to the method of Rodbell et al. (1971), assuming that the glucagon specific receptor sites have an equivalent affinity for both native and iodinated peptide hormones.

For binding studies in the absence of GTP and an ATP-regenerating enzyme system, the incubation medium contained 30 mM Tris-HCl, pH 7.0, 1 mM EGTA, 0.2 mM cAMP, 10 mM $MgCl_2$, 2 mM mercaptoethanol, 1 mM aminophylline, 4 mg BSA/ml, 7 mM bacitracin and Trasylol, 750 KIU/ml in a final assay volume of 0.1 ml. If binding was studied in the presence of 0.1 mM GTP, the incubation medium was similar to that for the adenylate cyclase assay at pH 7.0, with the addition of 7 mM bacitracin and 750 KUI/ml Trasylol; [^{14}C] ATP was substituted with 1 mM cold ATP; the

final assay volume was 0.1 ml. The concentrations of the tracer radiolabeled hormone ^{125}I-glucagon or ^{125}I-glucagon-NAPS added varied from assay to assay between 1.0 - 10.0 x 10^{-11}M (specific activity 2.5 - 3.0 x 10^{6} cpm/pmole). Native glucagon and glucagon-NAPS, as well as radiolabeled hormone added were in 30 mM Tris-HCl, pH 7.0, 1 mg BSA/ml, Trasylol 750 KUI/ml. The final concentrations of hormone are described in the legends to figures and tables. All reagents and incubation media were kept at 4°C before initiation of the assay.

The assay was carried out in triplicate in 1.5 ml plastic Eppendorf tubes. The incubation medium containing radiolabeled or radiolabeled plus cold hormone was equilibrated for 1 min in a 30°C water bath. The assay began with the addition of liver plasma membranes suspended in 30 mM Tris-HCl, pH 7.0 and 4 mg BSA/ml to give a final assay concentration of 0.08 - 0.10 mg/ml. The incubation at 30°C was continued for 10 min. At the end of the incubation, a 90 µl sample of the incubated medium was removed and layered on top of 300 µl ice-cold solution containing 2.5 mg BSA/ml of 30 mM Tris-HCl, pH 7.0, in microfuge tubes. Membrane-bound hormone was separated from free hormone by the method described earlier.

Assay blanks in triplicate containing radiolabeled hormone but no liver plasma membranes were incubated under control conditions for 10 min and treated in the same way as test samples.

The free and specifically-bound hormone in the incubation mixture was calculated from the fraction of radiolabeled hormone bound to membranes, after correction for nonspecific binding to the tube walls and to the

membranes measured in the presence of 10^{-6}M unlabeled hormone, as well as for hormone degradation (5-10%). The total amount of radiolabeled hormone was measured in triplicate 90 µl samples of the incubation medium which were not treated like the test samples but instead were counted directly.

11. Competitive binding studies of glucagon and glucagon-NAPS in the presence or absence of 0.1 mM GTP

Binding of ^{125}I-glucagon or ^{125}I-glucagon-NAPS (1.0-10.0 x 10^{-11}M; specific activity 2.5-3.0 x 10^{6} cpm/pmole) to liver plasma membranes in the presence of increasing concentrations of glucagon-NAPS or glucagon, respectively, was measured under the "cyclase" assay conditions described earlier. The amounts of unlabeled peptide hormone added are presented in the legends to figures and tables. The precent of radiolabeled hormone bound was calculated from the total amount bound in the absence of unlabeled hormone after correcting for nonspecific binding to the tube walls and plasma membranes, as previously described, and for hormone degradation.

12. Time course of binding of ^{125}I-glucagon-NAPS to liver plasma membranes in the presence or absence of 0.1 mM GTP

Binding of ^{125}I-glucagon-NAPS to liver plasma membranes with time (0-20 min) was investigated in the same incubation media ± 0.1 mM GTP as described for steady-state binding studies. The concentration of the radiolabeled hormone was 2.0 - 5.0 x 10^{-11}M (specific activity 2.0 - 3.6 x 10^{6} cpm/pmole). Samples removed at each time point were centrifuged for a total of 6 min at 2 min intervals in

order to accomodate all samples in the microfuge. Since effective separation of the free from bound hormone is achieved after 5 min centrifugation (Pohl et al. 1971) and some of the bound glucagon might have dissociated during the time between centrifugations, although dissociation is minimized at 4°C, binding of the radiolabeled hormone with time was determined by another method.

Free labeled hormone was separated from bound hormone by filtration of 0.1 ml samples (in triplicate) of the incubation medium (1.0 - 1.5 ml) at room temperature, using glass filters (Whatman GF/B 2.4 cm), followed by one wash with 0.5 ml 30 mM Tris-HCl, pH 7.0, containing 2.5 mg BSA/ml. In order to minimize nonspecific binding of the radiolabeled hormone to filters, they were previously soaked for 10 min at room temperature in 10% BSA in 30 mM Tris-HCl, pH 7.0, and washed once by filtration with 0.5 ml 2.5 mg BSA/ml in 30 mM Tris-HCl, pH 7.0, before sample application. Nonspecific binding to filters was determined in the absence of membranes and nonspecific binding to the membranes in the presence of 10^{-6}M unlabeled hormone.

13. Inactivation of ^{125}I-glucagon by plasma membranes with respect to binding to membranes in the presence and absence of GTP

^{125}I-glucagon (1.56×10^{-10}M; specific activity 2.31×10^{6} cpm/pmole) was incubated in binding assay incubation media ± GTP described above, in the presence and absence of 0.12 mg membranes/ml, (total volume 0.5 ml), in Eppendorf tubes (1.5 ml) at 30°C for 10 min. After incubation the samples were centrifuged at 4°C in a Beckman microfuge (≃ 10,000 x g). Samples, 0.1 ml, of the supernatant were transferred

into new tubes containing 0.025 ml of the appropriate incubation medium. Fresh membranes in 30 mM Tris-HCl pH 7.0, containing 4 mg BSA/ml were added to each tube, giving a final concentration of 0.07 mg/ml (total volume 0.15 ml). Samples were then incubated at 30°C for 10 min. At the end of the incubation, 0.1 ml aliquots were removed and layered on top of 300 µl ice-cold in 30 mM Tris-HCl, pH 7.0 containing 2.5 mg BSA/ml. Bound and free hormone were separated by the centrifugation method previously described. Blanks without membranes or with membranes plus 10^{-6}M unlabeled glucagon were included in the entire course of the assay. Specific binding was calculated as pmole/mg after correcting for nonspecific binding to tube walls and membranes, and for hormone degradation.

14. Degradation of glucagon by liver plasma membranes in the presence and absence of GTP.

Increasing concentrations of glucagon (10^{-10}-10^{-6}M) were incubated in the "cyclase" (+GTP) or "noncyclase" (-GTP) assay media, in the presence or absence of 0.10 - 0.12 mg liver plasma membranes/ml in a total volume of 0.1 ml. ^{125}I-glucagon 1.0 x 10^{-10}M specific activity 1.3 x 10^{6} cpm/pmole) was included as a tracer. Incubation was carried out in 1.5 ml plastic Eppendorf tubes at 30°C for 10 min. At the end of incubation, 0.2 ml ice-cold solution 10% BSA in 30 mM Tris-HCl pH 7.0 were added to each sample and the protein was precipitated by the addition of 1.0 ml 15% (w/v) ice cold trichloroacetic acid (TCA). The samples were left in ice for one hr. The precipitated protein was pelleted by centrifugation in the Beckman microfuge at 4°C for 10 min (≃ 10,000 x g). After centrifugation 1.0 ml aliquots of the supernatant were removed and counted. The amount

of degraded hormone was calculated as percent of TCA nonprecipitable counts to total counts added after correcting for TCA nonprecipitable counts found in the absence of membrane.

The above conditions were also used to assay degradation of ^{125}I-glucagon alone with increasing membrane concentrations (0.09 - 1.0 mg/ml).

15. Effects of sulphydryl reagents on binding of glucagon to liver plasma membranes

Samples of liver plasma membranes (0.4 mg/ml) were incubated with 1 mM dithiothreitol, 1 mM iodoacetamide, 1 mM iodoacetic acid or no addition in incubation mixture containing 30 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ 1 mM ATP, at 30°C for 30 min (final volume 0.5 ml). Reduced samples were treated with 1 mM iodoacetamide or iodoacetic acid and the rest with buffer at 30°C for 15 min. All samples received 3.0 ml of 30 mM Tris-HCl, pH 8.0 containing 2 mM 2-mercaptoethanol to quench the alkylating agent treatment and were incubated at 30°C for 10 min. After quenching the alkylating agent, the membranes were centrifuged at 12,500 x g at 4°C for 10 min. The membrane pellets were washed once with 2 ml 30 mM Tris-HCl, pH 7.0, and finally resuspended in 0.5 ml 30 mM Tris-HCl, pH 7.0. The resuspended membranes were assayed for binding of 2.90×10^{-9} M hormone in the presence and absence of 0.1 mM GTP. ^{125}I-glucagon (2.25×10^{-11} M; specific activity 2.37×10^{6} cpm/pmole) was included as a tracer. Specific binding was assayed as previously described. Binding to fresh, untreated membranes was assayed as a control. The final membrane concentrations used in binding ranged between 0.20 - 0.26 mg protein/ml.

16. Assessment of purity of radioiodinated peptide hormones

The purity of radiolabeled hormone, stored in propanol:water (1:1 v/v), at -20°C was routinely checked if the peptide was not used immediately after iodination and purification (refer to "Methods" in Chapter 1). Purification of high molecular weight aggregates and breakdown fragments was achieved by gel filtration through a Bio-Gel P-10 (Bio Rad Labs) column (1.0 x 75.0 cm). The column was equilibrated with 1% (w/v) BSA, 0.01% NaN_3, 0.12% EDTA, 0.9% NaCl pH 7.4 and the same solution was used to apply and elute the peptide. The column was calibrated with Dextran-Blue, glucagon and 2,4-dinitrophenyl-glycine. Fractions 0.8 - 1.0 ml were collected at a rate of approximately 20 ml/hr. Trasylol 150 µl, (10,000 KIU/ml) were included in the tubes into which the radiolabeled hormone was eluted.

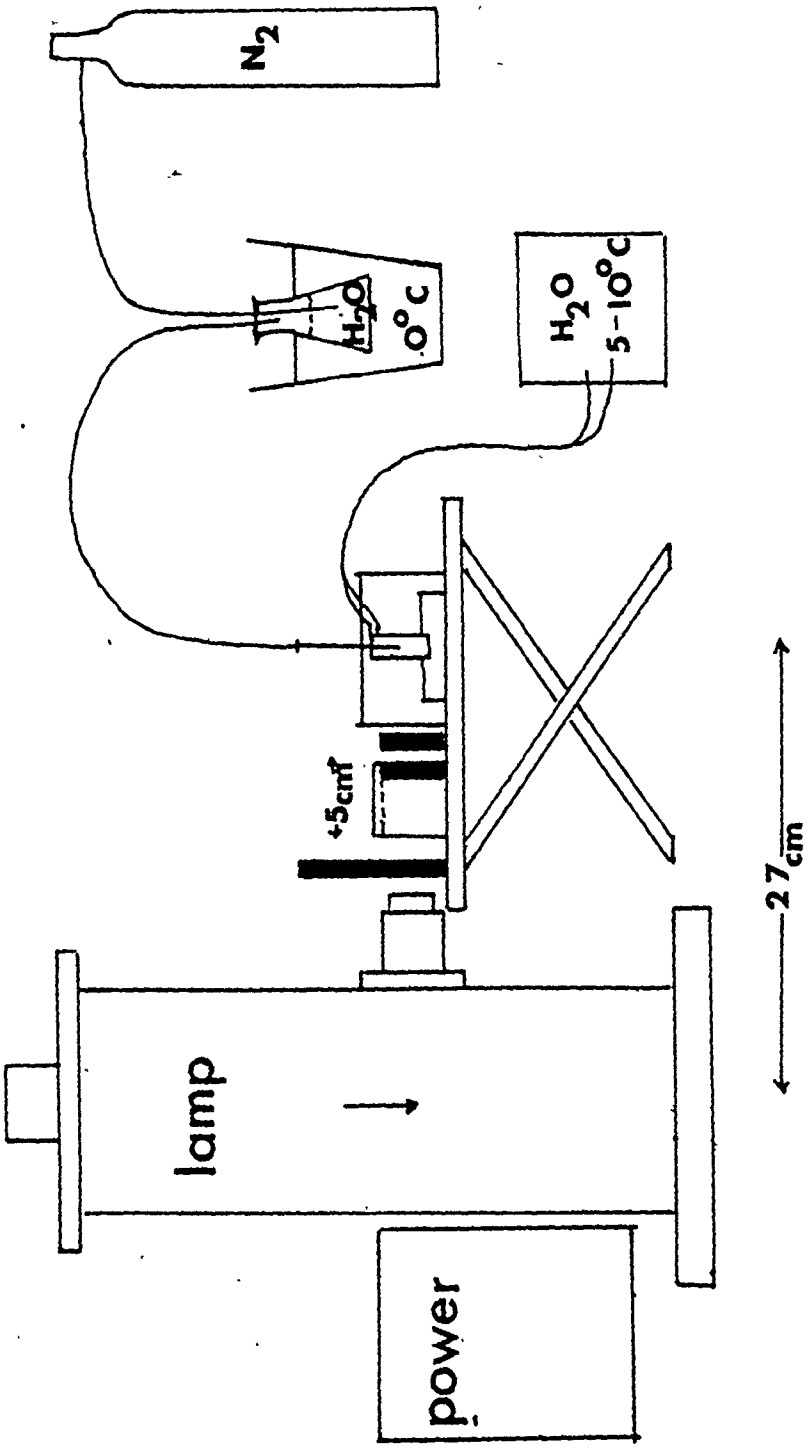
17. Membrane protein determination

Membrane protein was determined by the modified mini-Lowry protein assay according to the method of Hess et al. 1978.

18. Irradiation studies

Irradiation studies were carried out in a temperature controlled quartz cell (1.0 cm path length, 1.5 ml capacity) contained within a cast iron, black cell holder, held at 27 cm from a 7 mm arc length 1,000 watt, Xenon-Mercury lamp stationed in a LH 15N N Lamp Housing connected to a power supply (250-1,000 watts, Model LPS 255 HR), (Shoeffer Instrument Corp), Scheme IV. A 5 cm path length 0.1% (w/v) potassium phthalate filter contained in a pyrex cell (5 x 5 cm) was inserted between the lens and the

Scheme IV



cell holder. Two ultraviolet-transmitting, visible absorbing colour filters (7-51, 4.9-5.1 mm, 4 x 4 cm, Glass No. 5970, Corning: >80% transmittance at λ_{max} = 365 nm) were inserted, one in the K^+-phthalate filter bath and the other in front of the opening of the cuvette holder. The temperature of the cell was kept at 5-10°C by circulating cold tap water. Exposure of samples to light was regulated by a shutter placed in front of the lens. Samples precooled in ice in the dark, were transferred into the photolysis cell and purged with water (4°C)-saturated-N_2 gas for 1 min before irradiation. Samples were normally photolysed for two min unless otherwise stated. N_2 gas was blown through the sample during photolysis at a slow enough rate to ensure mixing of the solution, avoiding foaming due to bovine serum albumin contained in the samples. Irradiation time was monitored using an electric stopwatch readable to 1/100 min (Precision Scientific). The cell was cleaned after irradiation of a sample by three washings with water, then 3 N HCl-methanol (1:1 v/v) followed by water, and finally acetone, and was dried with N_2 gas before the next sample was applied.

RESULTS AND DISCUSSION

1. Membrane purification

The yield of partially purified membranes was approximately 0.8-1.0 mg protein per g wet weight of liver. The marker enzyme data (Table VII) demonstrated a 33-fold increase in specific activity of 5'-nucleotidase in the partially purified membranes relative to the crude homogenate. The glucose-6-phosphatase and succinate-cytochrome c reductase activities indicate some microsomal and mitochondrial contamination respectively, but no increase in their specific activities.

Partially purified membranes were used in these studies since they gave higher maximal adenylate cyclase stimulation by glucagon than fully purified membranes (Pohl et al. 1971).

The partially purified membranes remained stable at -70°C at least 3 months, the period within which the membranes were utilized. Maximal adenylate cyclase activity varied from preparation to preparation, however, 50% stimulation by glucagon and glucagon-NAPS was within the same order of magnitude of hormone concentration.

2. Adenylate cyclase

Dose response curves for the effect of glucagon and glucagon-NAPS on adenylate cyclase activity of liver plasma membranes in the presence of 0.1 mM GTP are presented in Fig. 16,17. As shown, adenylate cyclase activity is a function of glucagon and glucagon-NAPS concentrations over a range of 10^{-10}-10^{-7}M with half maximal stimulation occurring at

TABLE VII

Activities of marker enzymes in liver homogenates and in partially purified membranes

Enzyme	Specific activity μmoles/min/mg protein in Initial homogenate[a]	Partially purified plasma membranes[b]
5'-Nucleotidase	0.005 ± .003	0.167 ± .020
Glucose-6-phosphatase	0.011 ± .002	0.001 ± .001
Succinate cytochrome c reductase	0.004 ± .001	0.005 ± .001

The activity of the above marker enzymes was measured as described in "Methods".

[a] Initial homogenate refers to homogenized liver membranes in hypotonic bicarbonate solution before centrifugation.

[b] Partially purified plasma membranes refers to membranes purified by the discontinuous sucrose gradient, after the first wash.

Values are the mean ± SEM of three determinations in parallel.

Fig. 16. Glucagon dose response curves of adenylate cyclase activity in liver plasma membranes photolysed in the presence of glucagon and glucagon-NAPS

Liver plasma membranes were preincubated with 10^{-7}M glucagon or glucagon-NAPS at 30°C for 5 min and then photolysed for 2 min or kept in the dark under similar conditions. Noncovalently bound hormone dissociated by incubating with 0.1 mM GTP at 30°C, for 15 min, was removed by centrifugation as described in "Methods". Resuspended membranes were subsequently assayed for adenylate cyclase stimulation by glucagon, with 0.56 mM [^{14}C] ATP as substrate, under standard assay conditions. Adenylate cyclase stimulation of fresh membranes was used as control. Activity calculated as cAMP nmoles/mg/10 min is expressed as fold stimulation. Points and error bars represent the mean ± SEM of three parallel determinations for each hormone concentration. Basal and maximal activities are presented in Table X .

(●——●): Control membranes; (o——o): membranes preincubated with 10^{-8}M glucagon, photolysed; (Δ——Δ): membranes preincubated with 10^{-8}M glucagon-NAPS, photolysed.

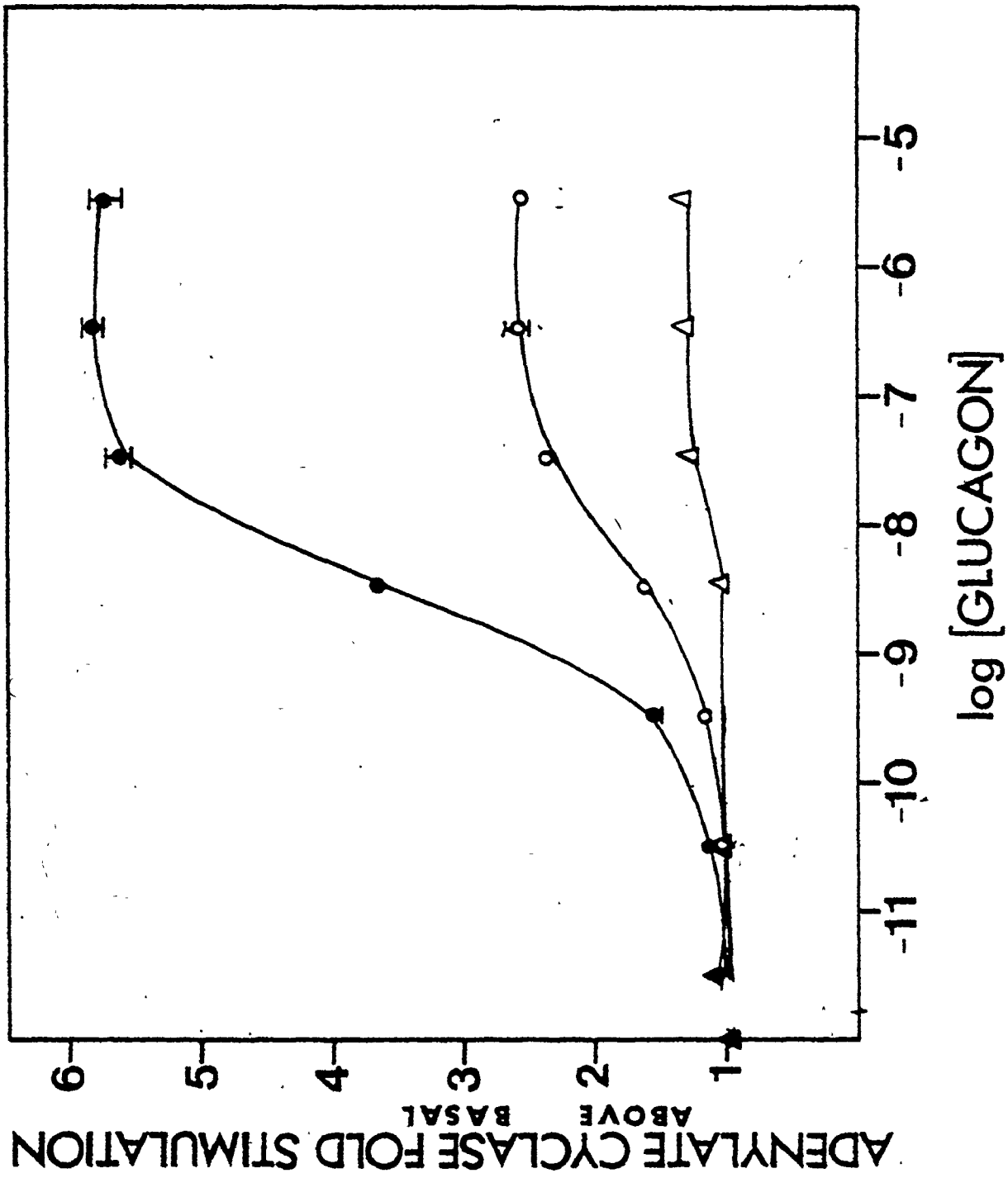

ADENYLATE CYCLASE FOLD STIMULATION
ABOVE BASAL
1
2
3
4
5
6
-11
-10
-9
-8
-7
-6
-5
log [GLUCAGON]

Figure 16

Fig. 17. Glucagon-NAPS dose response curves of adenylate cyclase activity in liver plasma membranes covalently photolabeled with glucagon-NAPS

Liver plasma membranes were preincubated with 10^{-8}M glucagon-NAPS or buffer (30 mM Tris-HCl, pH 7.0, 1 mg BSA/ml, 750 KUI/ml Trasylol) and treated as described in the legends to Fig. 16 and in "Methods". Adenylate cyclase activation by glucagon-NAPS is expressed as fold stimulation above basal. Points and error bars represent the mean ± SEM of three parallel determinations, of experiments repeated at least twice. Basal and maximal activities are presented in Table X .

(●——●): Control membranes; (o——o): membranes preincubated with buffer, in the dark; (Δ——Δ): membranes preincubated with 10^{-7}M glucagon-NAPS, in the dark; (●----●): membranes preincubated with 10^{-7}M glucagon-NAPS, photolysed.

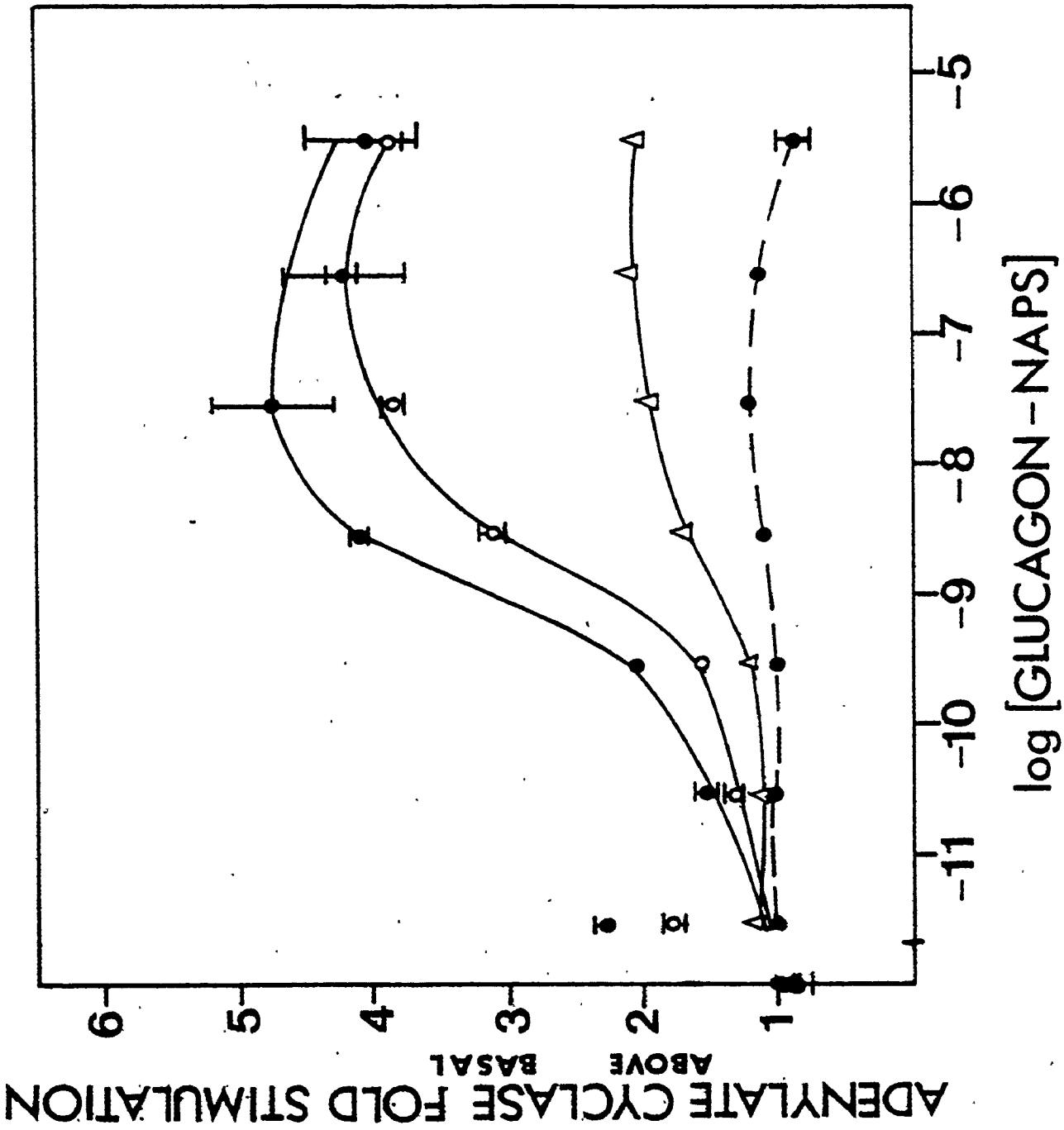

ADENYLATE CYCLASE FOLD STIMULATION
ABOVE BASAL
6
5
4
3
2
1
-5
-6
-7
-8
-9
-10
-11
log [GLUCAGON-NAPS]

Figure 17

approximately 1.6 x 10^{-9}M glucagon and 4.5 x 10^{-10}M glucagon-NAPS.

At 30°C, under the standard assay conditions used, adenylate cyclase activity was linear with time and membrane concentration (data not shown).

The complexity of the adenylate cyclase assay conditions demonstrate the sensitivity of the enzyme assay to the various components added. The ATP-regenerating system and the low membrane concentrations were shown to be essential for the linearity of the reaction (Pohl et al. 1971).

Although the optimum conditions for maximal stimulation of plasma membrane adenylate cyclase are at pH 7.6 in the Tris-HCl buffer system (Pohl et al. 1971), approximately the same stimulation by glucagon and glucagon-NAPS were observed at pH 7.0 and pH 7.6 (data not shown). The former was chosen for dose response studies since binding and irradiation studies were conducted at pH 7.0, due to the sensitivity of the radiolabeled hormones to irradiation at high pH as described in Chapter I, thus avoiding differences in the assay conditions.

BSA was included to avoid nonspecific absorption of the hormone to glass and plastic and in order to provide a protein-rich environment for the small amounts of plasma membranes used.

Cyclic AMP phosphodiesterase was inhibited by the addition of amino-phylline, while glucagon degradation by membrane associated peptidase was avoided by the addition of bacitracin. Trasylol 750 KIU/ml was used as an inhibitor of BSA associated proteolytic enzymes.

The effect of GTP on the stimulation of adenylate cyclase by glucagon

is demonstrated in Fig. 18. GTP, 0.1 mM, increased glucagon stimulation over 10-fold. The difference in the stimulation emphasizes the role of GTP on the adenylate cyclase activation as previously discussed (see Introduction).

3. Irradiation effects

Since a new system was used for the irradiation studies with liver plasma membranes than the one used to evaluate the photoaffinity properties of glucagon-NAPS (see Chapter I), the parameters of the system had to be defined. As indicated in Fig. 19, irradiation of NAPSCl reagent, tested at two different concentrations, was completed within less than 2 min. The use of two colour filters provided an even narrower range of wavelength (see inset Fig. 19) thus excluding any u.v. light and reducing the possibilities of irradiation damage to samples being photolysed. The K^+ phthalate filter acted as a heat absorber and also provided protection of the samples from u.v. light in case of filter damage or breakage. The filters used are not resistant to the high energy light of the 1,000 watt Xenon-Mercury lamp and direct exposure to the light for more than one min resulted in filter damage and breakage. Filters were replaced after 1-2 h. total exposure to light.

The effects of different periods of irradiation of the plasma membranes and of glucagon and glucagon-NAPS were tested by assaying adenylate cyclase activity (Table VIII, IX). Irradiation of plasma membranes resulted in approximately 15% inactivation of the adenylate cyclase enzyme after 2 min. This was attributed to irradiation damage caused by the high energy of the light reaching the sample. The total

Fig. 18. Dose response curves of the effect of glucagon on the adenylate cyclase activity of liver plasma membranes in the absence and presence of GTP

Adenylate cyclase activity was measured with 0.56 mM [^{14}C] ATP as substrate in the absence and presence of 0.1 mM GTP for each glucagon concentration, under the assay conditions described in "Methods". Activity was calculated as cAMP nmoles/mg protein/10 min and is expressed as stimulation above basal level. Values presented are the mean ± SEM of four parallel determinations at each peptide concentration. Basal activities in the presence and absence of GTP were 5.41 ± 0.15 and 0.56 ± 0.07 nmoles/mg/10 min; maximal activities were 19.54 ± 0.25 and 1.40 ± 0.04 nmoles/mg/10 min, respectively.

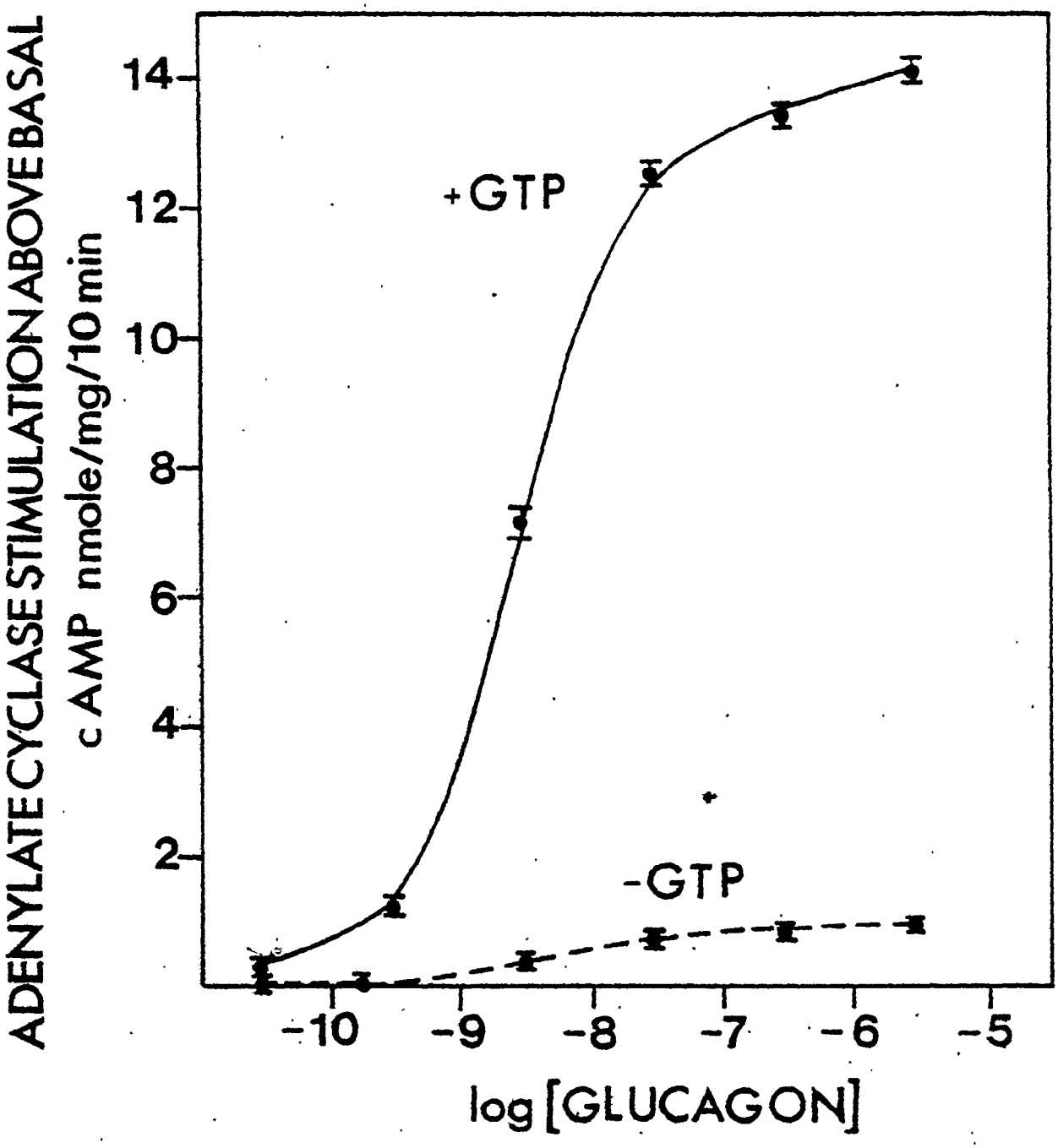

ADENYLATE CYCLASE STIMULATION ABOVE BASAL
c AMP nmole/mg/10 min
14
12
10
8
6
4
2
+GTP
-GTP
-10
-9
-8
-7
-6
-5
log [GLUCAGON]

Figure 18

TABLE VIII

Stimulation of adenylate cyclase by unphotolysed liver plasma membranes

10^{-7}M glucagon (0.001 mg/ml)

Time of photolysis (sec)	Percent stimulation by glucagon (2.88×10^{-7}M)
0	100.1 ± 2.0
15	91.4 ± 5.0
30	93.0 ± 4.4
60	90.0 ± 7.0
90	84.0 ± 7.0
120	85.7 ± 4.4

Liver plasma membranes 0.53 mg/ml in 30 mM Tris-HCl, pH 7.0, 1 mg BSA/ml were photolysed for the times indicated. Aliquots removed at each time point were tested for adenylate cyclase stimulation by 2.88×10^{-7} M glucagon under the assay conditions described in "Methods". % stimulation is expressed

as $$\frac{\text{Activities of photolysed membranes}}{\text{Activities of unphotolysed membranes}} \times 100$$

where activity was calculated as cAMP nmoles/mg/protein/10 min.

Values are the mean ± SEM of triplicate determinations. Basal activity of unphotolysed membranes was 3.50 ± .10 nmoles/mg/10 min. Total glucagon activity was 7.54 ± .31 nmoles/mg/10 min.

TABLE IX

Percent stimulation of liver plasma membrane adenylate cyclase by 10^{-7}M photolysed glucagon and photolysed glucagon-NAPS

Photolysis time (sec)	Percent stimulation: Glucagon 2.88×10^{-7}M	Glucagon-NAPS 2.72×10^{-7}M
0	100.0 ± 2.0	100.0 ± 2.0
15	108.0 ± 5.0	91.0 ± 2.0
30	102.0 ± 6.0	91.0 ± 1.0
60	101.0 ± 5.0	88.0 ± 2.0
90	110.0 ± 6.0	84.0 ± 2.0
120	101.0 ± 2.0	85.0 ± 2.0

10^{-6}M glucagon and glucagon-NAPS were photolysed for the times indicated in 30 mM Tris-HCl, pH 7.0 containing 1 mg BSA/ml and 750 KIU/ml Trasylol, as described in "Methods". Aliquots removed at each time point were tested for adenylate cyclase stimulation under standard assay conditions. Adenylate cyclase activity calculated as cAMP nmole/mg/10 min is expressed as percent of that obtained with unphotolysed hormone. Values are the mean ± SEM of three and four determinations for glucagon and glucagon-NAPS, respectively. Basal and maximal activities with glucagon were 3.46 ± .09 and 8.40 ±0.23 nmoles/mg/10 min; and with glucagon-NAPS 2.62 ± .04 and 12.69 ±0.03 nmoles/mg/10 min, respectively.

Fig. 19. Rate of photolysis of glucagon-NAPS irradiated with a 1000 watt Xenon-Mercury lamp and % transmittance profile of filters used

A) Glucagon-NAPS(1.59×10^{-3}M (a); 8.0×10^{-4}M (b)) in 0.5 ml glacial acetic acid as irradiated for the amount of time indicated. The absorbance of the irradiated samples was measured at λ_{max} 420 nm. Points represent the mean ± SEM of three determinations for each concentration.

B) Control limits for two ultraviolet transmitting, visible absorbing filters inserted between sample and light source (Corning CS No. 7-51, 4.9-5.1 mm) calculated from the % transmittance profile in Corning Color Filter Catalogue.

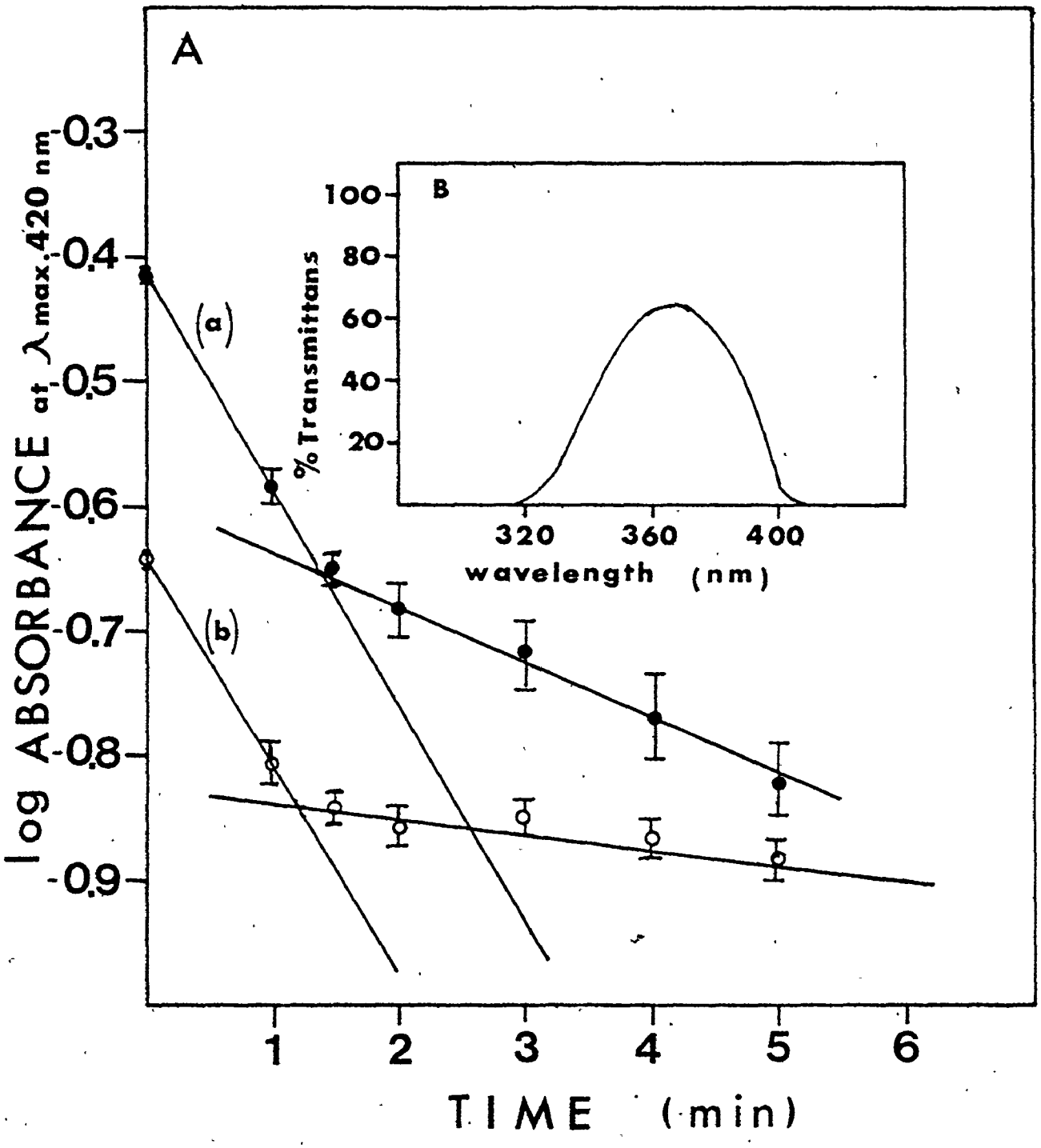

A
log ABSORBANCE at λmax.420 nm
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
(a)
(b)
1
2
3
4
5
6
TIME (min)
B
100
80
60
40
20
% Transmittans
320
360
400
wavelength (nm)

Figure 19

power available within the spectral region of irradiation was calculated to be 75 watts at 50 cm from the light source (calculated from the spectral characteristic of the lamp, Kratos, Schoeffel, Inst.), at 100% transmittance. The use of two filters reduces the percent of light transmitted to ≈32% which is equivalent to 24 watts. Since the sample was irradiated at ≈27 cm away from the lamp the optical power reaching the sample was higher. Membrane samples treated under the same conditions but not exposed to light were not affected, indicating that irradiation was responsible for the inactivation observed. This inactivation of membranes was not considered significant for the evaluation of irradiation studies since appropriate controls were included. Thus a 2 min irradiation was selected as the standard amount of time for photolysis.

Irradiation did not affect the ability of glucagon to stimulate the enzyme; however, inactivation of glucagon-NAPS occurred and increased with time of photolysis, reaching approximately 15% after 2 min. Inactivation was most likely due to intermolecular crosslinking of glucagon-NAPS to BSA and Trasylol, as well as intramolecular crosslinking. Intermolecular crosslinking was observed during irradiation of ^{125}I-glucagon-NAPS with BSA and of ^{125}I-glucagon-NAPS alone, as described in Chapter I.

4. Biological activity of radiolabeled peptides

The biological activity of ^{125}I-glucagon and ^{125}I-glucagon-NAPS was tested at 10^{-11} - 10^{-10}M concentrations, as calculated from their specific activity (2.45 μCi/pmole), after further purification of the peptides (Fig. 20). The activity of the radioiodinated peptides was compared to that of an equimolar concentration of unlabeled hormone (data not shown). Both ^{125}I-glucagon and ^{125}I-glucagon-NAPS stimulated adenylate cyclase

Fig. 20. Fractionation of radiolabeled hormone by chromatography on Bio-Gel P-10 column

Radiolabeled stock ^{125}I-glucagon-NAPS, 66 days after iodination, (remaining specific activity 1.92 x 10^6 cpm/pmole) stored in propanol:water (1:1 v/v), was evaporated to dryness with N_2 gas. The peptide was redissolved in 0.5 ml 1% (w/v) BSA, 0.01% NaN_3, 0.12% EDTA, 0.9% NaCl pH 7.4 and applied to a 1.0 x 75.0 cm P-10 column equilibrated with the same buffer. The peptide was eluted with buffer at a rate of 18 ml/hr. 0.9 ml fractions were collected in tubes containing 150 µl Trasylol (10,000 KIU/ml) where the peptide eluted. The above purification was carried out in the dark. Peak I: high molecular weight aggregates (12.0% of total); Peak II: monoiodopeptide (78-80%); Peak III: low molecular weight fragments (11.0%). For experimental purposes, the fractions of Peak II with less than 5.0% TCA nonprecipitable counts were used.

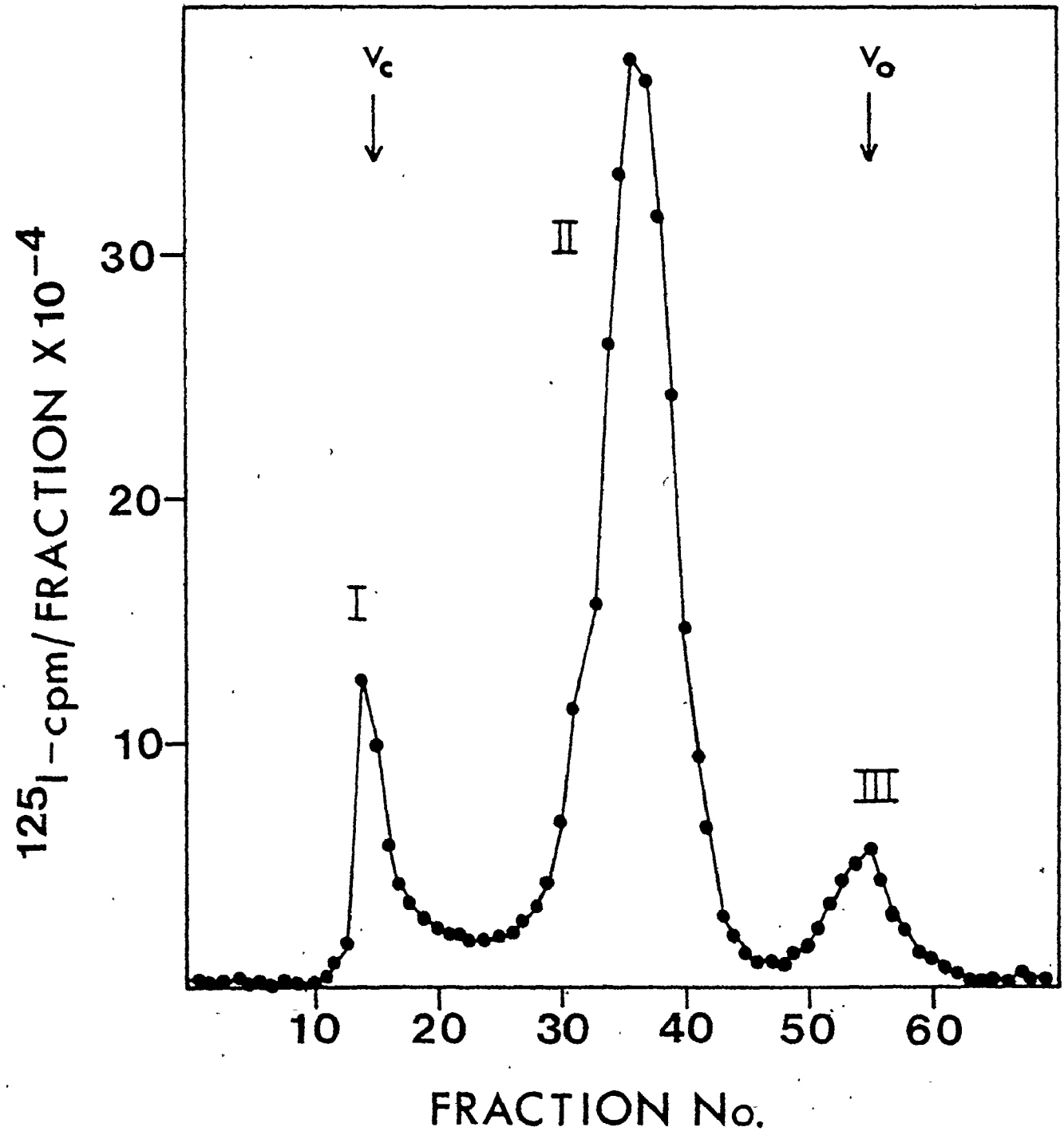

V_c
V_o
I
II
III
30
20
10
^{125}I-cpm/FRACTION X10^{-4}
10
20
30
40
50
60
FRACTION No.

Figure 20

100% and 140% more than native glucagon and glucagon-NAPS, respectively. Increases in the potency of the radiolabeled peptides have been attributed to an increase in the affinity of the peptides for the hormone receptor, contributed by the iodotyrosyl residue (Lin et al. 1976(also (see Chapter I).

5. Adenylate cyclase activity of irradiated liver plasma membranes in the presence of glucagon-NAPS

The effects of photolysis in the presence of glucagon-NAPS on the adenylate cyclase enzyme activation are shown in Figs. 16,17. Adenylate cyclase stimulation by glucagon and glucagon-NAPS in irradiated samples in the presence of glucagon-NAPS was ≈1.2-fold above basal compared to ≈5.7-fold of control membranes. Membranes irradiated in the presence of glucagon were activated ≈2.5-fold above basal. The lower fold stimulation in irradiated samples in the presence of glucagon-NAPS suggests that part of the decrease in the response observed after photolysis with glucagon-NAPS may be due to covalently labeling of the peptide derivative to functional sites. A 10-15% of the inactivation of photolysed samples can be attributed to irradiation damage to membranes as previously shown (Table VIII). The rest of the inactivation observed in photolysed samples preincubated in the presence of glucagon is only an apparent inactivation due to an elevation of basal stimulation (Table X). Pretreatment with hormone results in a higher basal activity in both the samples preincubated with the hormone and those preincubated with buffer (Table X), suggesting an incomplete dissociation of the bound hormone after incubation with GTP and/or a stimulated state of the enzyme due to pretreatment with the

TABLE X

Maximal adenylate cyclase activation by glucagon and glucagon-NAPS in rat liver plasma membranes irradiated in the presence of glucagon-NAPS

Membranes	Adenylate Cyclase Activity (nmoles/mg protein/10 min)		
	Basal	Glucagon (10^{-7}M)	Glucagon-NAPS (10^{-7}M)
A	1.84 ± 0.25	10.84 ± .07	9.72 ± .34
B	5.92 ± 0.12	13.63 ± .21	12.48 ± .14
C	2.94 ± .09	7.33 ± .13	
D	6.80 ± .12	8.70 ± .08	
E	8.40 ± .14		9.63 ± .19
F	2.84 ± .10		11.99 ± .21

Liver plasma membranes photolysed or kept in the dark in the presence of 10^{-8}M glucagon or glucagon-NAPS, were treated as described in the legends to Figs. 16,17, and subsequently assayed for adenylate cyclase activity, in a final concentration of 0.10-0.15 mg protein/ml. Results are mean ± SEM for three parallel determinations of experiments repeated at least twice. (A): Control membranes; (B): membranes preincubated with 10^{-8}M glucagon or glucagon-NAPS in the dark; (C): membranes preincubated with 10^{-8}M glucagon, photolysed; (D,E): membranes preincubated with glucagon-NAPS, photolysed, (F): membranes preincubated with buffer in the dark.

hormone and/or the nucleotide. Inhibition to hormonal stimulation of covalently crosslinked membranes is not due to membrane inactivation due to degradation of the glucagon receptor or other membrane components. This is supported by the fact that membranes treated under the same conditions in the dark in the absence of hormone displayed even greater stimulation than control membranes which had not undergone any pretreatment.

The results of the epinephrine and isoproterenol stimulation of adenylate cyclase enzyme in membranes irradiated in the presence of glucagon-NAPS (Table XI) suggest specificity in the loss of enzyme stimulation by glucagon-NAPS when the fold stimulation of samples incubated in the dark are compared with irradiated samples incubation with glucagon-NAPS. However, because of the changes in basal activity due to pretreatment with the hormone peptide or GTP, the effect is not clearly demonstrated. In contrast to what was expected, NaF stimulation is largely affected by the covalent crosslinking of membranes with glucagon-NAPS (Table XI). NaF response in irradiated samples with glucagon-NAPS was only two-fold above basal compared to four-fold in nonirradiated samples preincubated with glucagon-NAPS, and six-fold in control membranes. The inactivation of NaF observed was suggestive of a common component involved in both glucagon, adenylate cyclase and F^- stimulation. In these sets of experiments (Table XI) glucagon-NAPS stimulation of irradiated membranes in the presence of the derivative was ≈1.50-fold above the basal compared to 4.4-fold in samples preincubated in the dark.

TABLE X I

Adenylate cyclase activation by glucagon, epinephrine, NaF and isoproterenol in liver plasma membranes photolabeled covalently with glucagon-NAPS before assay

	adenylate cyclase activity (nmol/per mg of protein/10 min)				
	Basal	Glucagon 10^{-7}M	Epinephrine 10^{-5}M	NaF 10^{-2}M	Isoproterenol 10^{-4}M
1	2.12 ± .06	15.00 ± .45	3.10 ± .06	12.63 ± .13	2.83 ± .09
2	1.21 ± .06	9.55 ± .40	1.95 ± .03	8.64 ± .15	1.80 ± .05
3	2.39 ± .09	10.47 ± .26	2.99 ± .03	9.67 ± .14	2.78 ± .20
4	0.98 ± .09	5.29 ± .10	1.23 ± .05	6.21 ± .14	1.15 ± .03
5	4.81 ± .22	7.04 ± .31	5.17 ± .06	9.29 ± .05	5.26 ± .09

Liver plasma membranes were preincubated with 10^{-8}M glucagon-NAPS or buffer, and subsequently photolysed or kept in the dark. These membranes were assayed for adenylate cyclase after dissociation of the noncovalently bound hormone as described in the legends to Fig. 16. Final membrane concentrations ranged between 0.08-0.20 mg/ml. Results are mean ± SEM for four parallel determinations.

1: Control membranes; 2: membranes preincubated with buffer in the dark; 3: membranes preincubated with 10^{-8}M glucagon-NAPS, in the dark; 4: membranes preincubated with buffer, photolysed; 5: membranes preincubated with 10^{-8}M glucagon-NAPS, photolysed.

6. Binding studies

In order to demonstrate that glucagon-NAPS binds to the same receptor sites as glucagon and to quantitate the binding characteristics of glucagon-NAPS to liver plasma membranes, binding studies were carried out under "non-cyclase" conditions (absence of substrate, GTP and a regenerating system) and under "cyclase" conditions in the presence of 0.1 mM GTP and 1 mM ATP.

The kinetic characteristics of hormone binding as a function of time are illustrated in Fig. 21. In the absence of GTP the time required to attain equilibrium ranged from 10-15 min as observed by both centrifugation and filtration methods. In the presence of the nucleotides, constant levels of bound hormone were achieved faster, in less than one minute when binding was measured by the filtration method. The time course of ^{125}I-glucagon binding at equimolar concentrations displayed approximately the same kinetic behaviour (data not shown). These results are similar to the kinetic characteristics of adenylate cyclase stimulation by glucagon (30 sec - 1 min) (Rodbell et al. 1974). Discrepancies in the kinetics of binding and action of ^{125}I-glucagon have been reported (Lin et al. 1976) and attributed to heterogeneity of ^{125}I-glucagon molecules and the effect of iodine incorporation into the tyrosyl residues of glucagon (see Chapter I). A linear relationship was demonstrated with ^{3}H glucagon

Fig. 21. Time course of binding of ^{125}I-glucagon-NAPS to liver plasma membranes in the presence and absence of GTP

Binding of the radiolabeled peptide to liver plasma membranes with time was assayed under the conditions described in "Methods". Bound from free hormone was separated in A by the centrifugation method while that in B by the filtration method. The concentrations of ^{125}I-glucagon-NAPS were 5.0×10^{-11}M and 2.0×10^{-11}M for A and B, respectively, (specific activity $2.0\text{-}3.6 \times 10^{6}$ cpm/pmole); membrane concentrations 0.08-0.10 mg/ml. Error bars indicate the SEM of triplicate determinations in parallel except where smaller than symbols used.

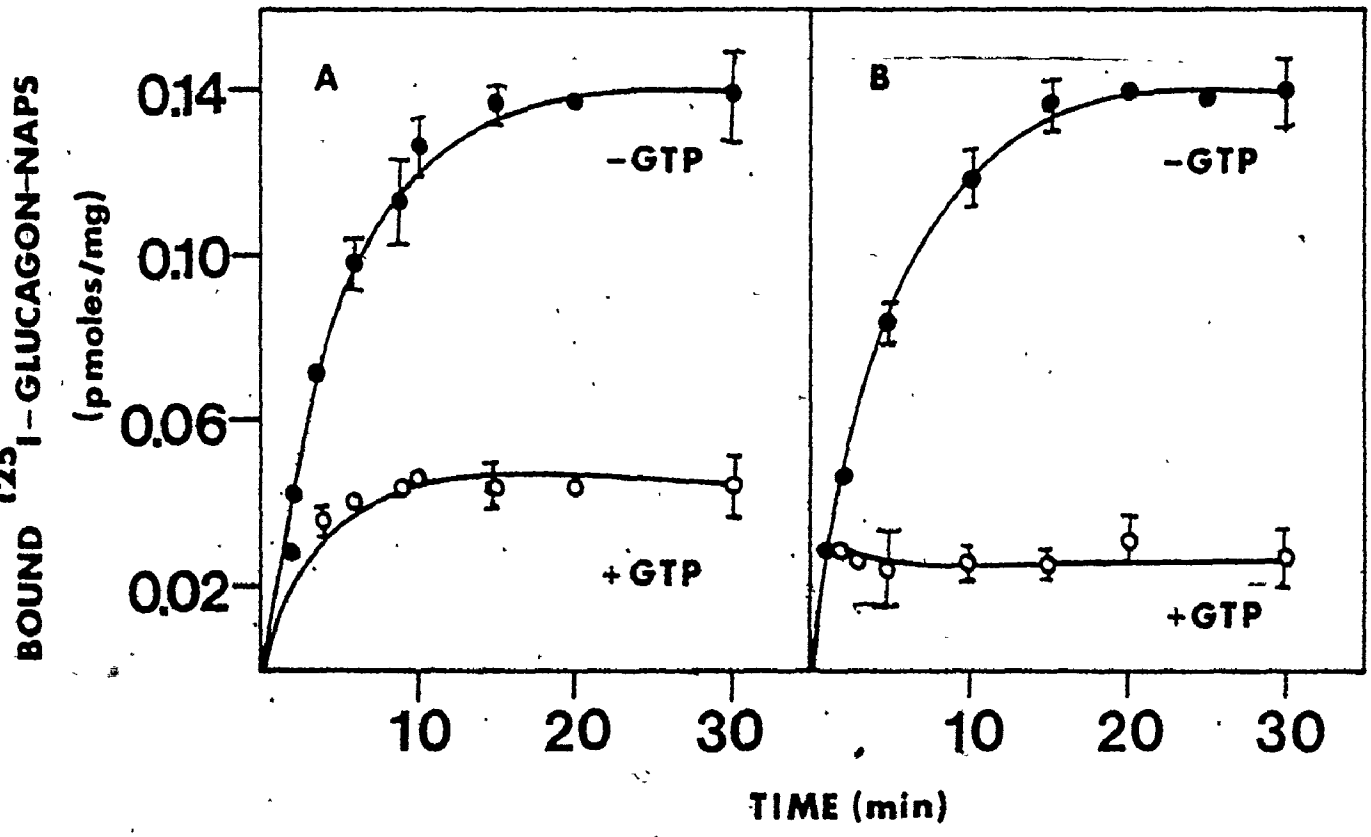

BOUND ^{125}I-GLUCAGON-NAPS (p moles/mg)
0.14
0.10
0.06
0.02
A
B
-GTP
+GTP
-GTP
+GTP
10
20
30
10
20
30
TIME (min)

Figure 21

(Lin et al. 1977) with kinetic characteristics of binding similar to those demonstrated by the monoiodo-labeled peptides used in these studies. Therefore, although ^{125}I-glucagon and ^{125}I-glucagon-NAPS have a slightly higher potency in activating the adenylate cyclase enzyme system, their high purity and specific activity confer an advantage similar to that of ^{3}H-glucagon in binding studies with liver plasma membranes, relative to previous studies where heterogeneous species and high concentrations of radiolabeled peptide have been used.

In addition to the acceleration of the attainment of the equilibrium state, GTP decreases the amount of labeled hormone bound by nearly 80% at hormone concentrations at least as low as 10^{-11}M. As previously shown (Rodbell et al. 1971b), the decrease in binding is due to acceleration of rates of hormone dissociation by GTP which are not compensated for by increased rates of association, resulting in a decrease in the equilibrium levels of bound hormone.

Specificity of binding of ^{125}I-glucagon was demonstrated by measuring the percent of radiolabeled hormone displaced by glucagon and glucagon-NAPS, in the presence and absence of GTP. Specificity of ^{125}I-glucagon-NAPS was similarly tested (Figs. 22,23). As illustrated, >90% inhibition of ^{125}I-glucagon and ^{125}I-glucagon-NAPS receptor binding occurred at $\approx 10^{-7}$M concentrations of either unlabeled agonist in the absence or presence of the nucleotides. However, in disagreement with the conventional competition curves observed with ^{125}I-glucagon,

Fig. 22. Displacement of ^{125}I-glucagon by unlabeled glucagon and glucagon-NAPS

^{125}I-glucagon (0.8-4.0 x 10^{-11}, specific activity 2.5-3.0 x 10^{6} cpm/pmole) was added to incubation samples containing the indicated concentrations of cold glucagon or glucagon-NAPS. The incubation media GTP have been described in "Methods". The assay was initiated by the addition of plasma membranes in a total volume of 0.1 ml. (Final membrane concentration 0.08-0.10 mg/ml). After 10 min incubation at 30°C, 90 µl samples were transferred to 400 µl microfuge plastic tubes containing 300 µl 2.5 mg BSA/ml in 30 mM Tris-HCl, pH 7.0. Bound from free hormone was separated by centrifugation as described in "Methods". The amount of radiolabeled bound hormone was calculated from the total amount bound in the absence of cold hormone. Points and error bars represent the mean ± SEM of three parallel determinations of two membrane preparations. (●——●) glucagon; (○——○) glucagon-NAPS.

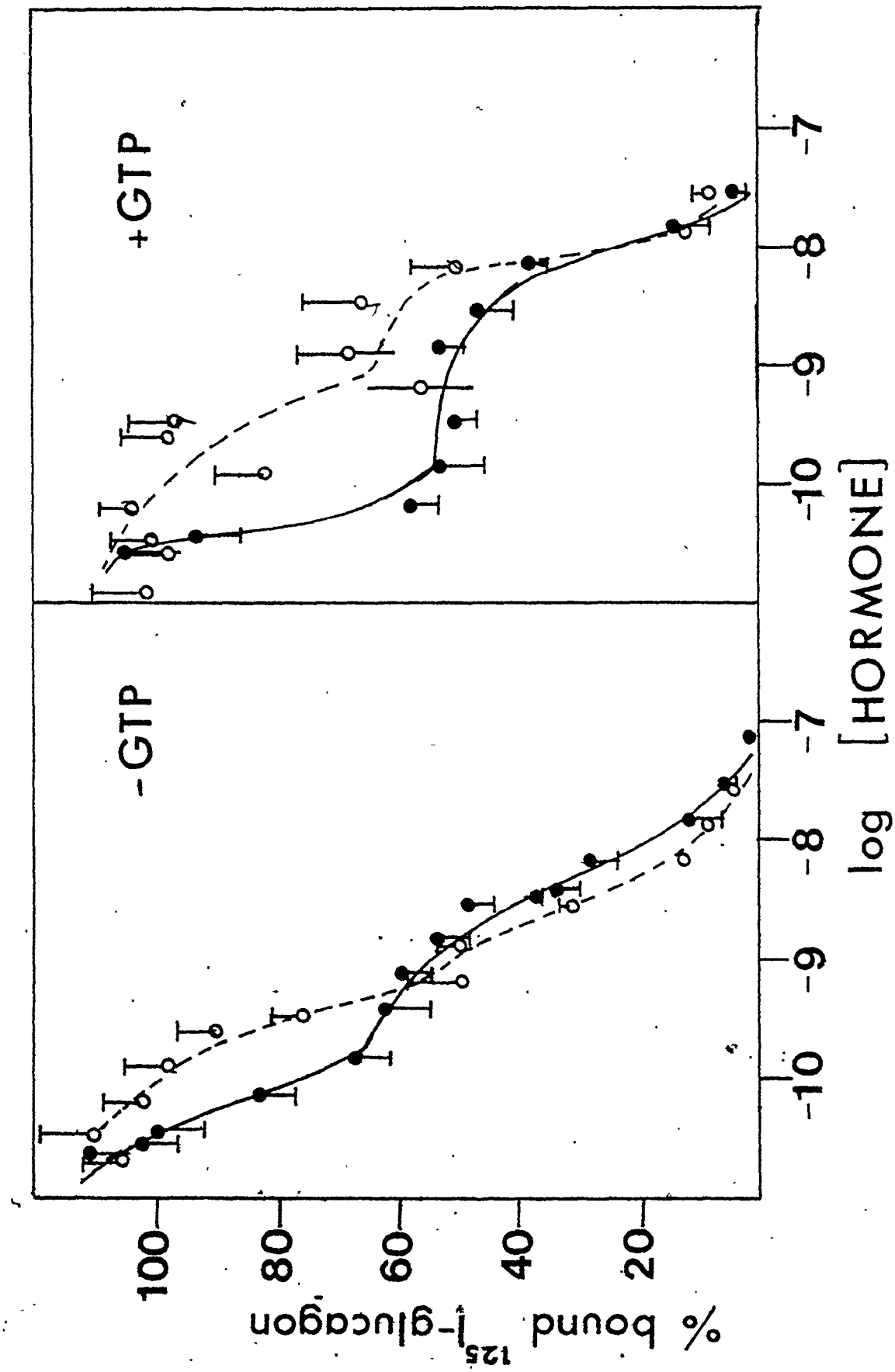
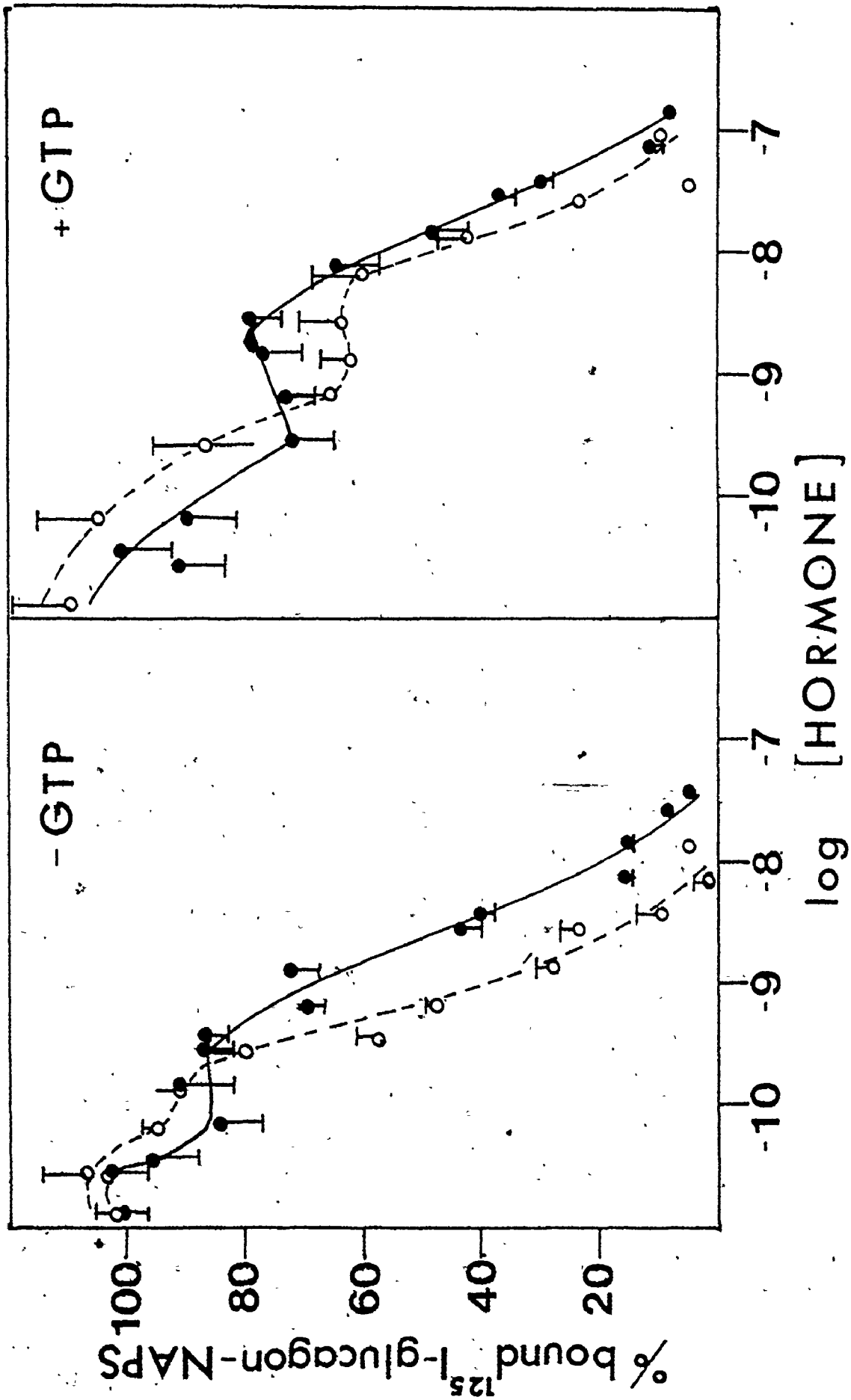


Figure 22

Fig. 23. Displacement of ^{125}I-glucagon-NAPS by unlabeled glucagon and glucagon-NAPS

The percent of ^{125}I-glucagon-NAPS ($1.3-4.0 \times 10^{-11}$, specific activity $2.8-3.0 \times 10^{6}$ cpm/pmole) bound to plasma membranes in the presence of increasing concentrations of cold glucagon and glucagon-NAPS was measured as described in the legend to Fig.22. Points and error bars represent the mean ± SEM of three parallel determinations.

(●–●) glucagon; (o–o) glucagon-NAPS.


+GTP
-GTP
% bound ^{125}I-glucagon-NAPS
100
80
60
40
20
-7
-8
-9
-10
log [HORMONE]

a biphasic curve was observed with an inflection point at about 3.0×10^{-10}M ligand which was independent of the radiolabeled peptide used as a tracer and of the presence of nucleotides (Figs. 22,23). Although glucagon-NAPS could displace bound radiolabeled glucagon from the membranes as effectively as the native hormone, the two peptides displayed different affinities depending on their concentrations (Figs. 22,23). Glucagon competed for the binding sites associated with low hormone concentrations with a greater affinity than glucagon-NAPS as shown in Fig. 22. In contrast to this, glucagon competed for the binding sites associated with high hormone concentration with a lower affinity than glucagon-NAPS (Fig. 23). In the presence of GTP, the difference in the affinities of the two peptides for the sites associated with low hormone concentrations became more pronounced. However, the affinities of the ligands for the sites associated with higher hormone concentrations became almost equivalent to each other (Figs. 22,23).

Inflection points were also observed when the data were corrected for nonspecific binding and hormone degradation and were plotted as binding isotherm curves (Fig . 24). They were more pronounced in the data obtained under "cyclase" conditions. Assuming one site of binding, 50% of the specific membrane receptor sites were occupied at $\simeq 5.62 \times 10^{-10}$, glucagon-NAPS and $\simeq 1.50 \times 10^{-9}$M glucagon, under "noncyclase" conditions (Fig. 24). These values are close to the concentrations of the peptides required for half-maximal stimulation of adenylate cyclase (Figs. 16,17) either in the presence or absence of GTP. Saturation of the binding sites at a concentration of 10^{-8}M also corresponds well with the concentrations of the hormone and the derivative required for maximal stimulation of the

Fig. 24. Concentration dependence curves for specific binding of glucagon-NAPS and glucagon to liver plasma membranes in the presence and absence of 0.1 mM GTP

Binding isotherms, measured by displacement of radiolabeled ligand (see "Methods", Figs. 23,24) were determined either (A) for glucagon-NAPS or (B) for glucagon on the assumption that labeled and unlabeled hormone have the same affinity for the receptor sites. Specific binding was determined by treatment of the data according to Kahn et al. 1974. Nonspecific binding was calculated from the sites which appeared unsaturable over the range of glucagon-NAPS or glucagon used and subtracted from each value of bound hormone. Points represent the mean ± SEM of three independent determinations and experiments were repeated at least twice.

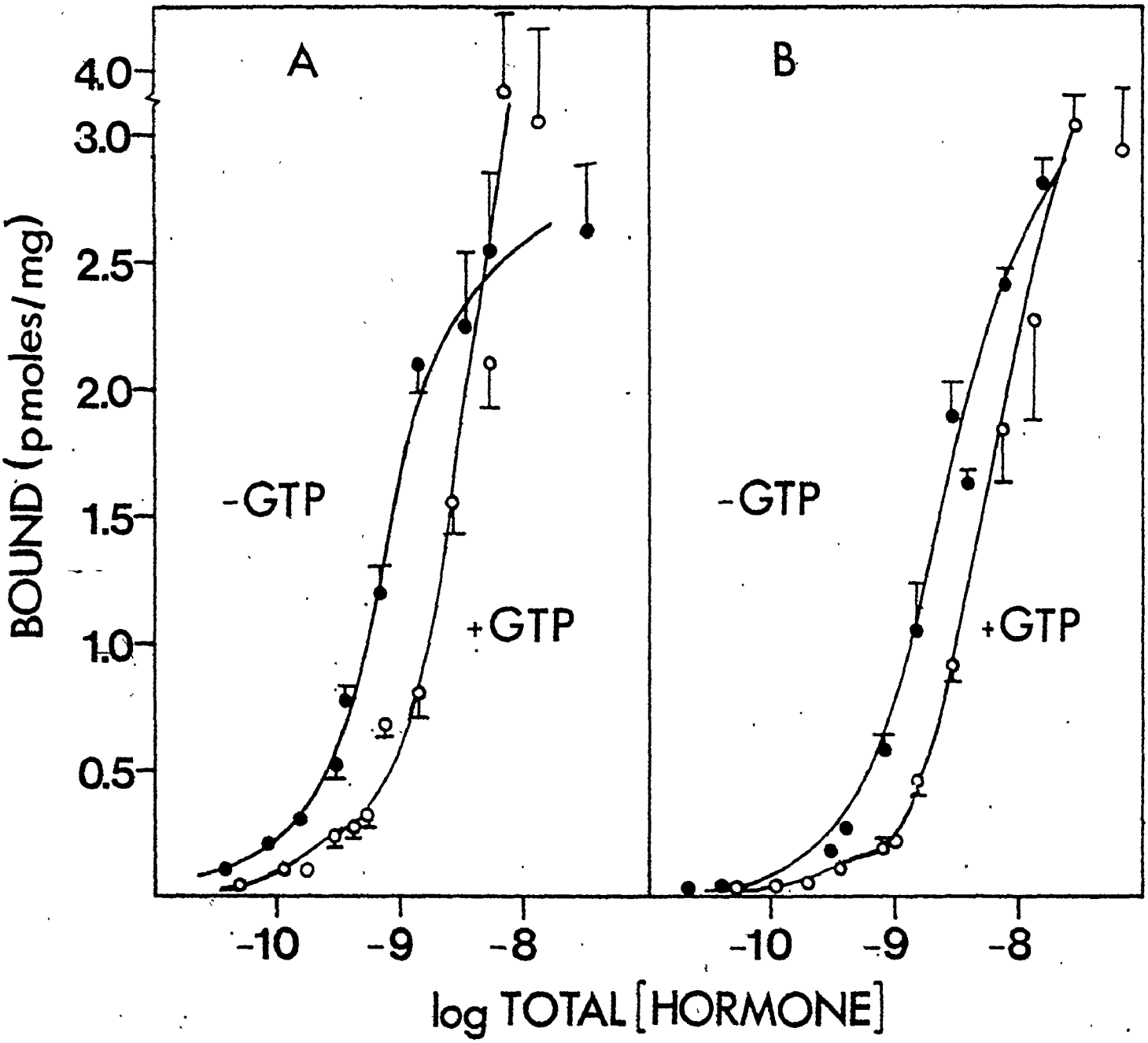

A
B
4.0
3.0
2.5
2.0
1.5
1.0
0.5
BOUND (p moles/mg)
-GTP
+GTP
-GTP
+GTP
-10
-9
-8
-10
-9
-8
log TOTAL [HORMONE]

Figure 24

enzyme. Therefore, in agreement with previous work (Rodbell et al. 1974) these studies suggest that all glucagon specific binding sites are coupled to adenylate cyclase and may represent functional receptor sites.

In the presence of GTP, saturation of the receptor sites was not observed at the concentrations of peptides tested. If the same number of saturable receptor sites is assumed in the presence and absence of the nucleotide, 50% occupation of receptor sites would occur at $\simeq 2.0 \times 10^{-9}$M for glucagon-NAPS and at $\simeq 5.0 \times 10^{-9}$M for glucagon (Fig. 24). The difference in the 50% values derived from the dose response and dose binding curves in the presence of GTP suggest that GTP may play the role of an amplifier in the stimulus-effect relationship of the hormone with the adenylate cyclase enzyme system.

The data from the displacement studies when treated as equilibrium data can be analyzed by the modified Scatchard method of Kahn et al. (1974). Scatchard plots of the above data were analyzed by the RIAGEN computer programme (Clinical Biochemistry Department, Health Sciences Centre, McMaster University, Hamilton, Ontario, Canada). Linear regression analyses were performed using the Hewlett Packard HP-65 programmable table calculator and programmes STAT 105A and STAT 1-22A. Treatment of the data assuming one set of binding sites for either glucagon or the derivative gave poor correlation coefficients (0.3-0.5), especially for the data obtained under "cyclase" conditions. The inflection points observed in the displacement and binding isotherm curves were also reflected in the Scatchard plots. Therefore, as a means of interpreting these results, two independent binding sites were hypothesized and the data analyzed in accordance with this model. The two sites were distinguished by the change of slope in

the Scatchard plots. The resultant regression lines (Figs. 25,26), irrespective of the ligand used, indicated two orders of specific receptor sites: a high affinity-low capacity site and a low affinity-high capacity site (Table XII). The differences in the affinities of glucagon and glucagon-NAPS for these sites were also reflected in the calculated equilibrium constants (Table XII).

Several possibilities were considered for the interpretation and significance of the two orders of binding sites observed in connection with the differences in the affinities displayed by the hormone peptides. As mentioned in the Introduction to this chapter, nonlinearity of Scatchard plots may reflect differences in the affinities between ligands for the receptor sites rather than multiplicity of sites, or site-site interactions.

Two factors were considered that may have contributed in altering the affinities of the ligands. One was the contribution of the iodotyrosyl residue in the stabilization of the radiolabeled ligand-receptor hydrophobic interactions; the other, which concerns the derivative only, was the contribution of the NAPS-reagent in further stabilization of these interactions. Both glucagon and glucagon-NAPS were iodinated and their monoiodoform purified by the same method. Therefore, it is more likely that the differences in the affinities of the ligands for the receptor sites are due to the presence of NAPS - rather than the presence of iodine on the peptides. Furthermore, the binding studies were conducted at pH 7.0 where contributions of the iodine should be minimal. Low radiolabeled ligand concentrations were used (10^{-11}M).

Both receptor sites are distinguishable in the ^{125}I-glucagon vs glucagon and ^{125}I-glucagon-NAPS vs glucagon-NAPS competition

Fig. 25. Scatchard plots of glucagon-NAPS binding to liver plasma membranes in the presence and absence of GTP

Mean of Scatchard plots of glucagon binding to liver cell membrane was calculated from binding studies of ^{125}I-glucagon-NAPS displacement by glucagon at 10^{-11} - 10^{-6}M after correcting for nonspecific binding and hormone degradation. The ratio of pmoles/mg bound and free hormone ($B/_F$) is plotted on the ordinate against the bound hormone (B) on the abscissa. The amounts of bound and free hormone in pmoles/mg for each of two independent experiments conducted in triplicate for each ligand concentration were calculated individually, but only the mean values ± SEM of ($B/_F$) and (B) are shown.
(●): absence of GTP; (o): presence of GTP.

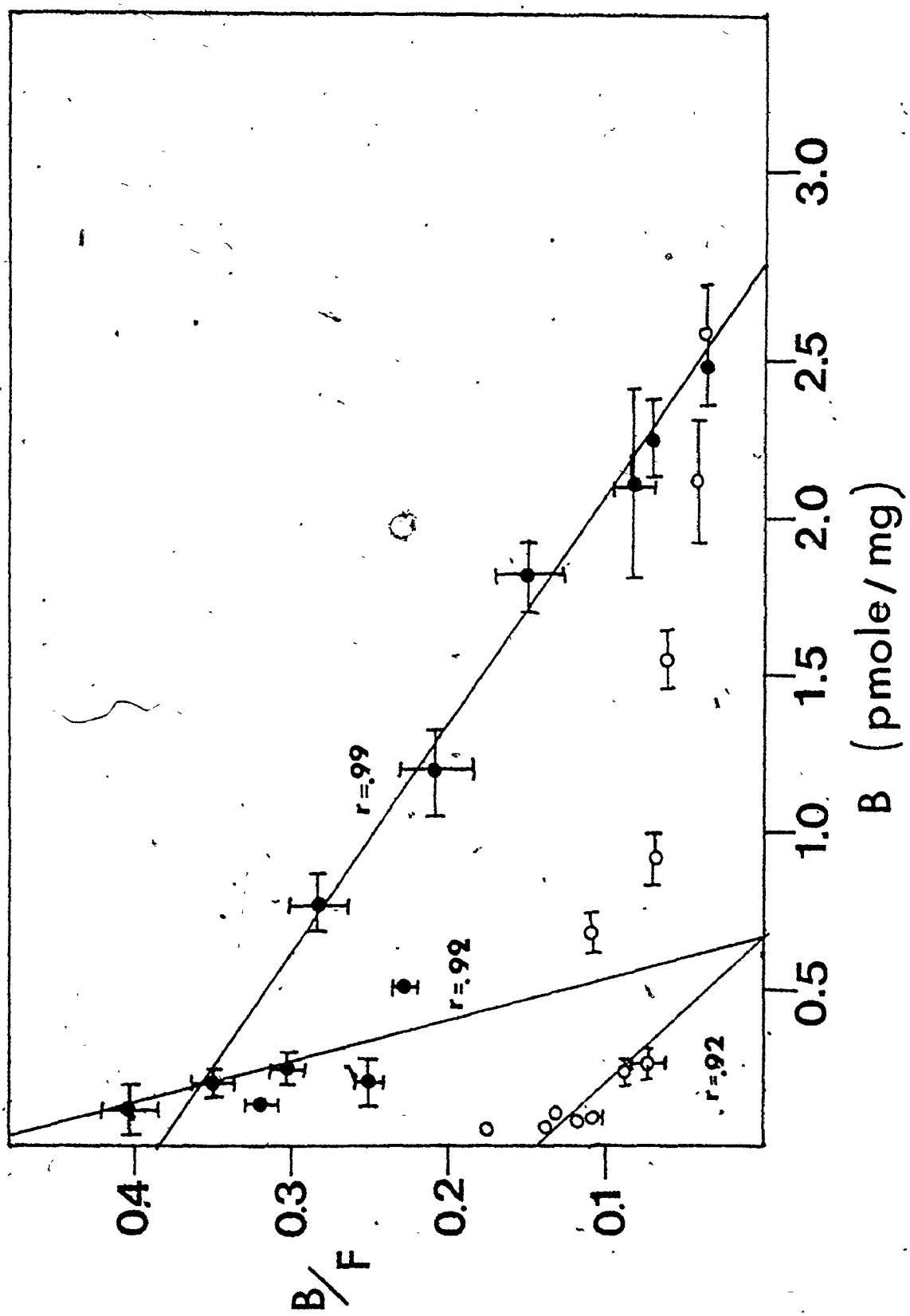


Figure 25

Fig. 26. Scatchard plots of glucagon binding to liver plasma membranes in the presence and absence of GTP

Mean of Scatchard plots of glucagon binding to liver cell membranes was calculated from binding studies of ^{125}I-glucagon displacement by glucagon (10^{-11} - 10^{-6}M) after correcting for nonspecific binding and for hormonal degradation. The ratio of pmoles bound/mg and free hormone ($B/_F$) is plotted on the ordinate against the bound hormone (B) on the abscissa. The amounts of bound and free hormone in pmoles/mg for each of two independent experiments conducted in triplicate for each ligand concentration were calculated individually, but only the mean values ± SEM of ($B/_F$) and (B) are shown.

(•): absence of GTP; (o): presence of GTP.

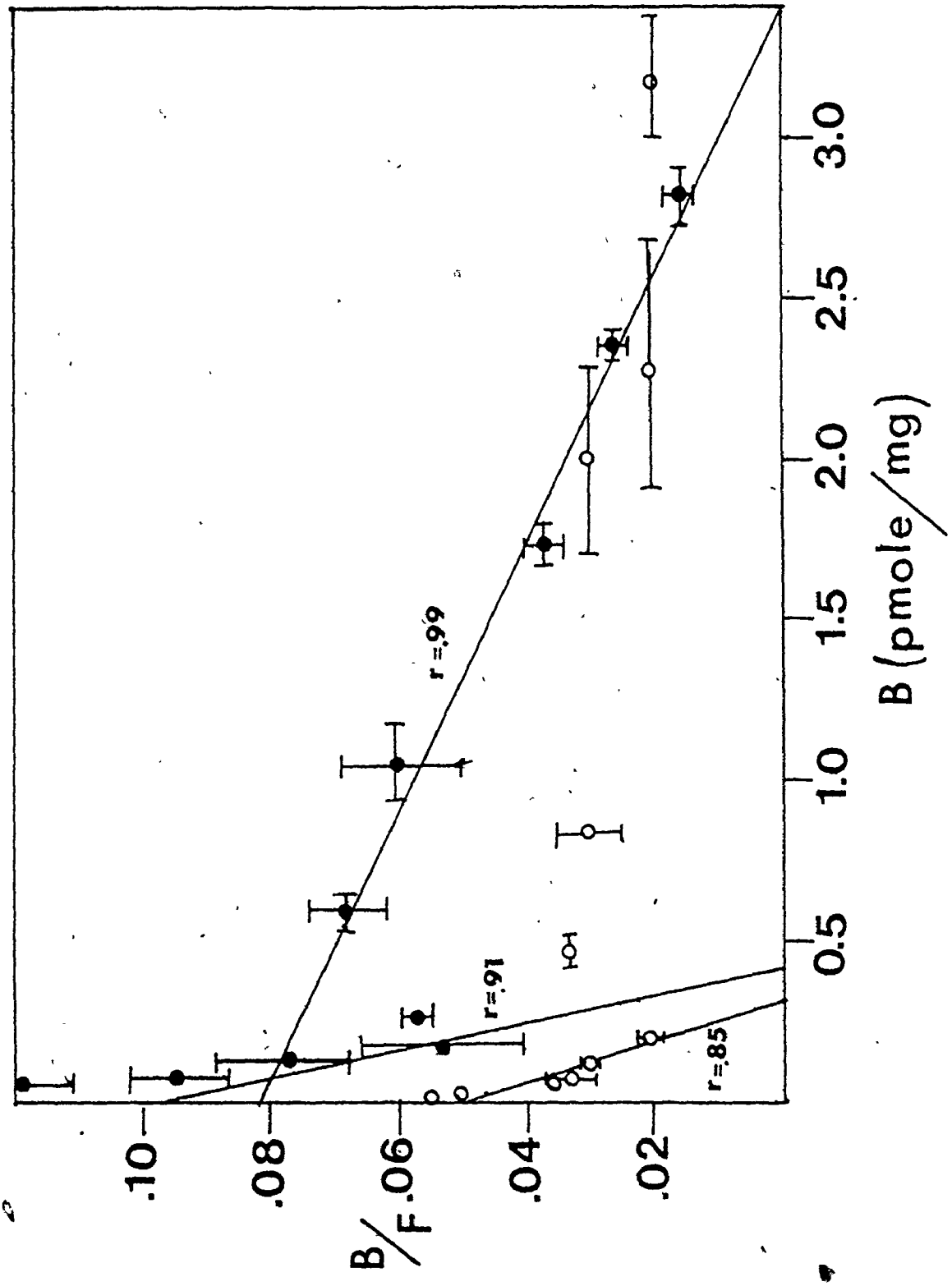


Figure 26

TABLE XII

Equilibrium dissociation constants of binding studies with glucagon and glucagon-NAPS in the presence and absence of 0.1 mM GTP

Ligand	GTP	Site	K_d (M)	Binding capacity (pmoles/mg)
Glucagon	-	High affinity	$3.52\ (\pm 0.72) \times 10^{-10}$	0.34 (±0.15)
		Low affinity	$4.17\ (\pm 1.05) \times 10^{-9}$	3.08 (±0.63)
	+	High affinity	$6.18\ (\pm 1.06) \times 10^{-10}$	0.31 (±0.11)
Glucagon-NAPS	-	High affinity	$1.31\ (\pm 0.10) \times 10^{-10}$	0.67 (±0.10)
		Low affinity	$7.28\ (\pm 1.11) \times 10^{-10}$	2.19 (±0.95)
	+	High affinity	$4.76\ (\pm 0.97) \times 10^{-10}$	0.67 (±0.21)

Equilibrium constants were calculated from Scatchard plots (Figs, 25,26) after treatment of the data according to Kahn et al. (1974). In brief, the K_d for the low affinity sites was obtained from the slope of a linear regression fit of data for concentrations of 40 nM-300 nM. The intercept of this line on the abscissa is the total number of binding sites. The contribution of the low affinity sites was subtracted from points obtained with hormone concentrations less than 40 nM. The resulting points were also fitted by a linear regression analysis. The high affinity constant was obtained from the slope of this fitted line. The intercept on the B axis is the concentration of high affinity sites. The concentration of low affinity sites was obtained by subtracting the high affinity sites from the total concentrations of binding sites. Low affinity sites and constants in the presence of GTP were not calculated since the systems did not reach saturation. Values represent the mean ± SEM of three independent determinations of two experiments.

studies as well as in the reciprocal studies (i.e. ^{125}I-glucagon vs glucagon-NAPS and vice versa) (Figs. 22.23), On the basis of these observations, it can be concluded that neither iodination nor the difference in the affinities of the ligands is responsible for the two receptor sites observed. Similar binding characteristics have been observed with ^{3}H-glucagon (Lin et al. 1977) in the presence and absence of GTP although the binding data were not analyzed by Scatchard plots. The observed changes in the relative affinities of the two ligands at concentrations larger than 10^{-10}M, may suggest two modes of binding interactions with the receptor(s).

The high affinity-low capacity receptor sites observed in the absence of GTP have not been reported in earlier studies with ^{125}I-glucagon. Higher concentrations of radiolabeled hormone of lower specific activity and purity have been used by other investigators and may be the reason why the high affinity sites remained undetected. However, recently two sites have been reported in binding studies with intact rat hepatocytes, in the absence of GTP, as well as with liver plasma membranes using radiolabeled peptide concentrations in the range of 10^{-11}M (Sonne et al. 1978; Sperling et al. 1980).

The effects of GTP on the affinities and capacities of the hormone binding sites on liver plasma membranes have been reviewed in the first part of this chapter. The effects of GTP on the binding characteristics of glucagon and glucagon-NAPS (Figs. 22-26) were similar. The equilibrium constants calculated for the high affinity-low capacity receptor sites were within the same order of magnitude in the presence and absence of GTP (Table XII). It is possible therefore that the action of GTP may not be

related to these sites. However, GTP affected the low affinity-high capacity sites (observed in the absence of GTP) converting them into lower affinity sites which did not seem to be saturable (Figs. 24,25). If saturation was assumed to have been reached at the highest concentrations of the ligands tested, and the data from Scatchard plots were subjected to a linear regression analysis, a K_d value in the order of $10^{-8}M^{-1}$ was obtained. This value would be in agreement with that reported for the low affinity binding sites of ^{3}H-glucagon in the presence of GTP (Lin et al. 1977). The lack of saturation of the low affinity binding sites and the inflection points of the binding isotherm curves observed in the studies with ^{125}I-glucagon and ^{125}I-glucagon-NAPS under "cyclase" conditions were also observed with ^{3}H-glucagon (Lin et al. 1977).

The low affinity-high capacity equilibrium constants obtained in these studies in the absence of GTP are within the same order of magnitude as those measured by other investigators (Table XIII). Scatchard plots of ^{125}I-glucagon binding data with liver plasma membranes under "cyclase" and/or "noncyclase" conditions have not been reported.

Hill plot analysis of the data obtained under "cyclase" and "noncyclase" conditions are presented in Table XIV. Hill plots at ± 1.0 log free hormone, which corresponds to the hormone concentrations associated with the high affinity-low capacity sites, have a slope of ≈1.0 for both glucagon-NAPS and glucagon, indicative of noncooperativity. At higher concentrations of the hormone or the derivative, Hill plots were curvilinear with slopes >1.0 indicative of positive cooperativity. Previous studies have reported Hill plots with a slope of 1.5 for glucagon (Rodbell et al. 1974). The results from these studies correlate with the

TABLE XIII

Literature values of equilibrium constants

of glucagon binding

System	Equilibrium constant	Binding capacity	Reference
liver plasma membranes	4.0×10^{-9}M	2.6 pmole/mg	Rodbell et al. (1971a
fat cell "ghosts"	1.0×10^{-7}M		Birnbaumer (1973)
liver plasma membranes	4.0×10^{-9}M		
liver plasma membranes	$0.2\text{-}0.5 \times 10^{-9}$M (+GTP)	3.0 pmole/mg	Rodbell et al. (1974)
microsomal membranes	0.46×10^{-9}M		Desbuquois (1975)
liver plasma membranes	2.5×10^{-9}M		Lin et al. (1976)
liver plasma membranes	5.0×10^{-9}M		Srikant et al. (1977)
intact rat hepatocytes	0.7×10^{-9}M 1.3×10^{-8}M	2.0×10^{4} sites/cell 2.0×10^{5} sites/cell	Sonne et al. (1978)
liver plasma membranes	4.0×10^{-7}M $6.0\text{-}8.0 \times 10^{-6}$M	12.0-14.0 pmole/mg 30.0-40.0 pmole/mg	Lafuse and Edidin (19
liver plasma membranes	$1.2\text{-}1.6 \times 10^{-9}$M $3.4\text{-}5.3 \times 10^{-8}$M	82.3 pmole/mg	Sperling et al. (1980)

TABLE XIV

Hill plots of binding studies of glucagon and glucagon-NAPS to liver plasma membranes in the presence and absence of 0.1 mM GTP

(o)	glucagon (-GTP)	s = 1.15 ± 0.15	r = 0.97
(o)	glucagon (+GTP)	s = 1.27 ± 0.12	r = 0.99
(Δ)	glucagon-NAPS (-GTP)	s = 0.99 ± 0.10	r = 0.99
(Δ)	glucagon-NAPS (+GTP)	s = 1.01 ± 0.10	r = 0.97

Hill plots were calculated from the equilibrium binding studies with ^{125}I-glucagon and ^{125}I-glucagon-NAPS. Specifically bound hormone in pmole/mg of protein was calculated after correcting for nonspecific binding and hormone degradation. The results represent the mean ± SEM of three determinations of two experiments. (s) slope and (r) correlation coefficient were calculated from the data treated to a linear regression analysis.

cooperativity with the low affinity-high capacity receptor sites. The positive cooperativity observed with glucagon for the high affinity-low capacity binding sites has a larger error associated with it and is not statistically significant. However, it is possible that some cooperativity is present in the high affinity receptor-hormone interactions but may not be observable with glucagon-NAPS.

Considering the above results, it is possible that adenylate cyclase is activated by glucagon through receptor sites which have high and low affinities for the hormone and are distinguished within concentration ranges that reflect the number and affinity of these sites. Only the high affinity-low capacity sites may represent the glucagon receptor sites while the low affinity-high capacity sites may represent specific non-receptor sites. The specificity of the high affinity sites would be reflected in the interactions with specific peptide regions. Low affinity sites may involve hydrophobic interactions with membrane phospholipids, as discussed previously in the models proposed for glucagon receptor interactions (see this chapter). Interactions of the hormone with both sites may be required for a functional adenylate cyclase system. Taking into consideration that the hormone may associate in dilute solutions (see Chapter I), this association may be concentration dependent in the range used for hormone binding. It is also possible that the two binding sites observed may reflect two modes of hormone association with the membrane. The high affinity-low capacity sites may reflect a hormone-receptor adenylate cyclase associated state, while the low affinity-high capacity sites may reflect a state of the hormone receptor complex dissociated from the enzyme.

The above binding studies do not provide any information about the molecular nature of the binding sites observed, and the mechanisms of their interactions with the hormone. However, the higher affinity displayed by glucagon-NAPS for the low affinity-high capacity receptor sites in association with the higher hydrophobicity conferred to the peptide by the NAPS-reagent suggest that binding to these sites may indeed involve hydrophobic interactions.

The evidence that GTP modifies the states of receptor and adenylate cyclase through functionally distinct sites has been presented in the Introduction to this chapter. Although the present binding studies do not show whether the GTP effect on the low affinity-high capacity receptor sites is coupled to the adenylate cyclase enzyme system, they suggest that these sites may be the "receptor regulation" sites proposed by Yengar and Birnbaumer (1979).

Measurements of glucagon stimulation of adenylate cyclase in membranes pretreated with sulfhydryl reagents (Storm and Chase, 1975) have shown that the enzyme was inactivated. When glucagon binding to liver plasma membranes pretreated with sulfhydryl reagents was measured (Table XV), binding was reduced by ≈50% in the presence and absence of GTP. Binding in the presence of GTP was lower than that in the absence of the nucleotide, in both treated and untreated membranes. The reduction by GTP of hormone binding to membranes which should not respond to hormonal stimulation, suggests that modulation of glucagon binding by GTP may be independent of the adenylate cyclase stimulation by the nucleotide, in agreement with Yengar and Birnbaumer's (1979) studies.

The inhibition of NaF stimulation in membranes covalently

TABLE XV

Reduction of glucagon binding to sulfhydryl reagent pretreated liver plasma membranes

Treatment	% specific bound hormone	
	+ GTP (0.1 mM)	- GTP
Control	100.00 ± 2.66	100.00 ± 5.70
30 mM Tris-HCl pH 8.0	80.94 ± 6.50	74.49 ± 3.78
1 mM DTT	47.93 ± 2.90	54.22 ± 3.06
1 mM DTT + 1 mM iodoacetamide	44.12 ± 1.17	42.04 ± 2.0
1 mM DTT + 1 mM iodoacetic acid	45.11 ± 1.80	47.28 ± 2.35
1 mM iodoacetamide	69.96 ± 1.74	66.39 ± 2.99
1 mM iodoacetic acid	51.23 ± 3.74	62.40 ± 2.83

Membrane samples (≃ 4.0 mg/ml) pretreated with the sulfhydryl reagent as described in "Methods" were assayed for binding of 2.90×10^{-9} M glucagon at 0.2 mg/ml final concentration. ^{125}I-glucagon (2.25×10^{-11} M, specific activity 2.77×10^{6} cpm/pmole) was included as a tracer. Binding was assayed by the centrifugation method as previously described. Specific binding was calculated in pmole/mg protein, after correction for nonspecific binding. The amount of hormone specifically bound is expressed as percent of hormone bound to fresh untreated membranes used as controls. Specific binding to control membranes was 0.52 ± 0.02 and 0.84 ± .07 pmole/mg in the presence and absence of 0.1 mM GTP, respectively. Results are the mean ± SEM of three determinations in parallel.

labeled with glucagon-NAPS suggest that the common regulatory subunit involved in NaF and GTP stimulation of adenylate cyclase (Martin et al. 1979) may also be involved in glucagon binding.

Studies on the rate of dissociation of bound glucagon in the presence of GTP have demonstrated it to be a noncompetitive process (Rodbell et al. 1971a,b, 1974). The enhancement of activity by GTP observed after occupation of the receptor sites by glucagon (Rodbell et al. 1974) indicated that the action of GTP occurs subsequently to or upon the binding action of glucagon on the receptor system. Therefore, it has been suggested (Rodbell et al. 1974) that either glucagon increases the affinity of the nucleotide binding sites for GTP or induces the formation of states that have increased reactivity with the nucleotide. Whether the low affinity-high capacity receptor sites are related to this state can not be determined from these studies alone.

7. Dissociation studies using liver plasma membranes containing covalently bound ^{125}I-glucagon-NAPS

Measurements of the amount of ^{125}I-glucagon-NAPS remaining bound to liver plasma membranes after irradiation and treatment of membranes with GTP are presented in Table XVI. As indicated at concentrations 10^{-10} - 10^{-8}M, more than 80% of the radiolabeled peptide remained bound to the membrane after irradiation. The small decrease observed in the photolysed samples compared to nonphotolysed ones, ≈10-15% may be due to decomposition of iodine as a result of irradiation (see Chapter I). GPT displaced more than 90-95% of the hormone bound in both photolysed and unphotolysed

TABLE XVI

Dissociation of bound ^{125}I-glucagon-NAPS from liver plasma membranes after irradiation

^{125}I-glucagon-NAPS	Unphotolysed		Photolysed	
	+ GTP	- GTP	+ GTP	- GTP
^{125}I-glucagon-NAPS specific binding (pmole/mg) before dissociation				
1.7×10^{-8}M	4.6 ± 1.5	2.4 ± 1.2	3.1 ± 0.2	2.1 ± 0.7
1.7×10^{-9}M	1.3 ± 0.3	2.3 ± 0.7	1.4 ± 0.7	1.4 ± 0.2
8.5×10^{-10}M	0.8 ± 0.1	1.7 ± 0.2	1.3 ± 0.3	1.3 ± 0.1
^{125}I-glucagon-NAPS specific binding (pmole/mg) after dissociation with 0.1 mM GTP				
1.7×10^{-8}M	0.01 ± 0.03	0.05 ± 0.05	0.06 ± 0.01	0.12 ± 0.08
1.7×10^{-9}M	0.02 ± 0.02	0.04 ± 0.03	0.05 ± 0.03	0.10 ± 0.02
8.5×10^{-10}M	0.02 ± 0.02	0.04 ± 0.06	0.04 ± 0.02	0.10 ± 0.01

^{125}I-glucagon-NAPS (specific activity 3.0-2.5 x 10^6 cpm/pmole) was preincubated with liver plasma membranes (2.5 mg/ml) in the above concentrations under "cyclase" or "noncyclase" conditions previously described, for 10 min, and subsequently photolysed for 2 min or kept in the dark. The final volume was 0.5 ml. The amount of radiolabeled hormone specifically bound to membranes was measured in 50 µl samples (in triplicate) as described in the equilibrium binding studies. Bound hormone was dissociated from the remaining sample with 0.1 mM GTP as described in "Methods". Bound ^{125}I-glucagon-NAPS remaining after dissociation was measured as above. Bound hormone was calculated in pmole/mg protein after correcting for nonspecific binding and hormone degradation. Results are the mean ± SEM of triplicate determinations.

samples. Approximately 5% of peptide remained specifically bound to the membranes in irradiated samples in the absence of GTP and 2-3% in its presence after the dissociation step. The amount of covalent crosslinking, however, could not be evaluated because there were no significant differences between control and experimental values due to the residual radioactivity associated with control samples. A slow dissociating component of peptide may have contributed to this radioactivity. Incomplete dissociation may explain the higher basal stimulation observed in samples preincubated with hormone in the irradiation vs activity studies (see Chapter II). It is also possible that there may be a contribution of slow chemical reactions that may occur in the dark during the incubation similar to those observed in the studies with the glucagon specific antibody (see Chapter I). The yield of labeled photoproducts did not increase by prolonging the time of photolysis (data not shown). One would have to use another means, i.e. SDS-polyacrylamide gel electrophoresis, for the determination of specific covalent labeling upon irradiation (see Chapter III). Nonspecific labeling in the presence of excess cold glucagon (10^{-6}M) in irradiated samples after dissociation ranged between 9-30% depending on the concentration of radiolabeled peptide and the presence of GTP, indicative of hormone association with membrane at sites other than the receptor sites.

Assuming that the amount bound to membranes in the irradiated samples reflects covalent crosslinking of specific receptor sites, a relatively low yield is observed in comparison with the number of sites defined by the equilibrium binding studies. When degradation of the iodo-peptide was investigated by trichloroacetic acid precipitation, the low molecular weight degradation products did not exceed 5-10% after incubation

of the radiolabeled peptide with the membranes for an equivalent amount of time (data not shown). However, about 40% of ^{125}I-glucagon was shown to be unable to rebind to liver plasma membranes after preincubation with membranes (Table XVII).In the presence of GTP, 90% of the free hormone could rebind. It is possible, therefore, that the reduction in the amount of peptide hormone able to bind may be due to some other form of degradation associated with membrane bound hormone. Because GTP causes dissociation of the hormone from the membranes it may explain the larger amounts of hormone able to bind after preincubation with the membranes

Although a low yield of specific covalent crosslinking is observed, crosslinking upon irradiation may in fact be greater. Because of the electrophilic character of the nitro-azide and the hydrophobic properties of the derivative membrane phospholipids may be labeled; and intermolecular crosslinking may also take place. Nonspecific covalent labeling of phospholipids or other lipophilic membrane component may contribute to the higher nonspecific labeling (30-40%) observed in irradiated samples when compared with controls. The implications of nonspecific binding to these sites are presented in Chapter III.

On the basis of the above observations, total crosslinking to membranes in irradiated samples ranged between 5-10% depending on the concentration of the photoreactive radiolabeled ligand. The contribution of intermolecular crosslinking could not be quantitated.

A low amount of covalent crosslinking upon irradiation of phenyl azides is generally attributed to the limitations of phenylnitrenes and their electrophilic character which may result in reaction with nucleophilic groups such as water or buffer components as discussed by

TABLE XVII

Inactivation of ^{125}I-glucagon with respect to binding to liver plasma membranes in the presence and absence of GTP (0.1 mM)

GTP	^{125}I-glucagon preincubated with plasma membranes		^{125}I-glucagon preincubated without plasma membranes		% amount of hormone able to rebind
	[^{125}I-glucagon]	Bound specific pmoles/mg	[^{125}I-glucagon]	Bound specific pmoles/mg	
-	9.25×10^{-11} M	.110 ± .008	10.19×10^{-11} M	.183 ± .015	60.37 ± 1.92
+	9.67×10^{-11} M	.047 ± .004	10.55×10^{-11} M	.052 ± .007	93.00 ± 3.64

1.56×10^{-10} M ^{125}I-glucagon was preincubated with or without liver plasma membranes (.12 mg/ml) at 30°C for 10 min. Free hormone was separated from bound and tested for binding to fresh plasma membranes as described in "Methods". The amount of hormone able to rebind was calculated as percent of ^{125}I-glucagon bound when preincubated in the absence of membranes, assuming the same specific activity (cpm/pmole) before and after incubation. Values are the mean ± SEM of three determinations in parallel. Specific binding was calculated after correcting for nonspecific binding and hormone degradation.

Bayley and Knowles (1978a). Treatment of the samples with 50 mM dithiothreitol before photolysis, which has been predicted to act as a scavenger of nitrenes and other reactive species derived from it (Staros, 1978), reduced specific labeling to less than 0.001 ± 0.002% (data not shown). The reduction was more than would be expected even after taking into account the reduction of hormone binding to liver plasma membranes in the presence of thiol reagents. This implies that the generated reactive photoproduct may have a half-life long enough that it can be scavenged efficiently. It may also suggest that although the -COOH terminus of glucagon-NAPS may contribute to the interaction of the molecule with the receptor sites, it may not be tightly bound to the membrane and therefore the site may easily be perturbed by the scavenger.

In summary, the binding studies with ^{125}I-glucagon and ^{125}I-glucagon-NAPS and the stimulation of adenylate cyclase by glucagon-NAPS indicated that the peptide derivative can interact with the specific receptor sites of glucagon. Specificity in covalent labeling of the receptor sites by glucagon-NAPS was not clearly shown, however, inhibition in adenylate cyclase stimulation by glucagon or glucagon-NAPS after labeling suggest that labeling may occur at glucagon-receptor sites involved in adenylate cyclase activation. Even though the % of covalent labeling of the membranes sites is low, it can be compensated for by the high specific activity of ^{125}I-glucagon-NAPS so that detection and characterization of the receptor sites may be possible.

CHAPTER III

Apparent Molecular Weights of Glucagon Binding Sites in Hepatic Plasma Membranes Photolabeled by the Radiolabeled Photoreactive Glucagon-NAPS Derivative

CONTENTS OF CHAPTER III

INTRODUCTION

Activation of membrane associated adenylate cyclase by peptide hormones and the characteristic response of the enzyme to glucagon upon binding to specific membrane receptor sites have been discussed in the previous chapters. Although several mechanisms for hormonal stimulation of adenylate cyclase have been proposed, these mechanisms are still ill-defined primarily because hormone enzyme complexes have not been solubilized and purified in a hormonally sensitive form. Even though recent findings have identified several components of the adenylate cyclase system like the GTP-binding protein (Pfeuffer, 1977), it is still not known how many protein subunits comprise the system, what the relationship between hormone-receptor and the catalytic subunit is, how the enzyme is integrated in the membrane and what is the role of the membrane lipid phase in the hormonal control of the activity and in the catalytic process of the adenylate cyclase enzyme complex is.

An understanding of the nature of hormone receptors can be aided by the proper description of the conformation of the hormone when bound to the receptor. Models concerning the interactions of glucagon with the receptor have been proposed on the assumption that at physiological concentrations ($\approx 10^{-11}$M) glucagon exists as a monomer and it is the only form involved in receptor binding. Because of the decreased stability of binding at low temperatures or upon addition of urea or detergents, the importance of hydrophobic interactions with the receptor have been emphasized (Birnbaumer et al. 1968; Sasaki et al. 1975). The

X-ray crystallographic data, as previously discussed, have shown that the helical conformation of glucagon is stabilized by non-polar residues clustered in two surface regions. A similar conformation stabilized by the interactions of two hydrophobic regions on the receptor was therefore proposed for glucagon, either as an oligomer or as a complex with the receptor (Rodbell et al. 1971; Sasaki et al. 1975). An alternative model based on the same type of interactions, would involve an initial binding of the -COOH terminal helical hydrophobic region to the receptor which in turn would induce a helix in the other hydrophobic region in a stepwise fashion so that the second region can bind. The length of 40Å of helical glucagon and the distance of 20Å between the hydrophobic regions of glucagon were considered to support the above models (Sasaki et al. 1975).

The occurrence of a nonpolar residue every third or fourth amino acid in the sequence of glucagon, the ability of the hormone to form amphipathic helices when interacting with phospholipids in a stable conformation similar to that found in the crystal state, and the requirements of phospholipids in hormonal adenylate cyclase activation (see Chapter II) have been considered in the model proposed by Epand (1980). According to this model glucagon may interact directly with membrane phospholipids largely by hydrophobic interactions without any rigid conformational requirements. Specificity in the interaction with the receptor would be rendered by polar groups which have been shown to have a functional role besides binding. This model was supported by the extensive studies with chemically modified glucagon derivatives at specific residues, with partial sequences of glucagon (Desbuquois, 1977;

Epand, 1980), and with glucagon-phospholipid complexes (Epand et al. 1977a,b; Jones et al. 1978). Proton NMR studies of glucagon self-association have demonstrated the absence of structure in the amino terminal region of glucagon suggestive of flexibility for specific receptor interactions (Wagman et al. 1979).

Proton NMR studies at 360 MHz (Boesch et al. 1978) have also shown that a local, rigid nonrandom spatial structure is contained in the conformation of monomeric glucagon in dilute solutions. On the basis of these findings, it was proposed that this structure may be preserved at physiological concentrations and serve as a recognition site for binding to the membrane. Secondary structural rearrangements of the bound hormone would provide a reactive receptor-glucagon complex.

The two-fold symmetry observed with glucagon is not uncommon. Symmetrical features in polypeptide hormones like luteinizing hormone releasing hormone (LH-RH), thyrotropin releasing hormone (T-RH), angiotensin II and bradykinin have been identified and it has been suggested that these symmetrical features are reflected in the conformation of the peptides at two symmetrically related similar or identical subunits in the receptor (Beddell et al. 1977). The importance of the amino acid sequence of these peptide hormones is reflected by the fact that they have been highly conserved through evolution.

Initial attempts to characterize the glucagon receptor in solubilized liver plasma membranes with the nonionic detergent Lubrol-PX identified a binding protein for both glucagon and insulin under non-denaturing conditions (Bletcher et al. 1974). Purification by 3,000-fold by a combination of protein precipitation, reduction of detergent concentration,

gel filtration and chromatography on hydroxylapatite gave a macromolecule with a 190,000 molecular weight. The binding of glucagon to this component displayed tissue specificity, rapid binding, saturability, reversibility and sensitivity to urea.

The myocardial adenylate cyclase glucagon complex solubilized by Lubrol-PX and chromatographed on Sephadex G-100 showed two components, one having a catalytic activity and a molecular weight greater than 100,000 and the other, a glucagon binding fraction with a molecular weight of 24,000-28,000 (Levey, 1975).

Labeling of rat liver plasma membranes with radioactively labeled iodoacetamide or iodoacetic acid in the presence and absence of glucagon, showed,on SDS-polyacrylamide gel electrophoresis, a component with an apparent molecular weight of 240,000 accounting for 1.3% of total membrane protein and a second component with a molecular weight of 10,000. Enhancement of labeling on the 240,000 daltons component in the presence of hormone, was suggestive of its being an integral component of the adenylate cyclase receptor complex (Storm and Chase, 1975).

Photoaffinity labeling of the glucagon receptor with the inactive derivative which is able to bind to liver plasma membrane, ^{125}I-N$^{\varepsilon}$-4-azido-2-nitrophenyl-glucagon, identified two components with a molecular weight range of 23,000-25,000 as being the receptor sites for glucagon (Bregman and Levy, 1977).

Although irradiation inactivation studies do not necessarily relate solely to the structural components of the hormone-receptor adenylate cyclase complex, they may give an idea about changes in the association of components involved in the presence of different ligands in situ.

Irradiation inactivation studies by Houslay et al. (1977) identified a molecular component of 160,000 daltons by F^- stimulation in liver plasma membranes, a 217,000 daltons component by ^{125}I-glucagon binding and a 389,000 component by glucagon stimulated activity. When the complex was irradiated in the presence of glucagon, the apparent target size for ^{125}I-glucagon binding had a value of 310,000, for F^- stimulation 380,000 and for glucagon stimulation 421,000 daltons. If glucagon was removed after irradiation and activity was assayed by readdition of glucagon, the apparent target size increased to 811,000 daltons. Similar studies by Schlegel et al.(1979) have identified a 150,000 daltons component using Mg/ATP as substrate. Pretreatment with NaF or guanine nucleotides showed a 230,000 component in the presence of Mg/ATP which increased to 350,000 daltons after preincubation with glucagon and GTP, and to 670,000 with glucagon alone. The basal state of the enzyme was found to be 1.3×10^6 daltons. Martin et al. (1979, 1980) showed the basal state to be 328,000 daltons, decreasing to 237,000 with Gpp(NH)p and F^- at 0°C and to 219,000 at 30°C. Glucagon and Gpp(NH)p increased the target to 460,000 at 0°C but decreased it to 223,000 at 30°C.

Although the picture of target analysis of the components involved in glucagon-receptor-adenylate cyclase interactions may look complicated, and even though the structure and composition of the targets are not defined, the size difference of targets preincubated or tested with a certain ligand may provide rough estimates of the molecular weights of the components involved. The differences between basal and F^- or GTP tested states indicate the involvement of a component(s) of ≈80,000 - 90,000 daltons; the differences between fluoride or GTP pretreated and glucagon

plus GTP pretreated states suggest a component(s) of ≈110,000 - 120,000. Similarly, the difference between the state tested for F^- stimulation after glucagon pretreatment and that tested for ^{125}I-glucagon binding is 80,000 daltons, whereas that measured with ^{125}I-glucagon binding from that measured by adenylate cyclase stimulation differs by ≈110,000 daltons. Furthermore, the state measured with ^{125}I-glucagon binding differs from that measured by adenylate cyclase stimulation by ≈172,000 daltons; F^- stimulated state differs from that of glucagon stimulated state by ≈67,000 daltons and glucagon adenylate cyclase activated state differs from that activated after preincubation of glucagon by ≈22,000 daltons.

Covalent labeling of the specific receptor sites of glucagon in plasma membranes with ^{125}I-glucagon-NAPS upon irradiation was expected to label at least two sites, as indicated from the binding studies with the iodohormone. Radiolabel was expected to be displaced from these sites in the presence of excess cold glucagon or glucagon-NAPS. For further confirmation of the specificity of photolabeling, the use of two chemically modified derivatives of glucagon were employed: N^{α}-trinitrophenyl-glucagon which behaves as an antagonist of glucagon possessing 0.1% of glucagon adenylate cyclase activity and 7% of glucagon binding activity; and the derivative $Asp^{9,15,21}$-tri-glycinamide-glucagonyl-[glycinamide] with 0.004% and 0.05% glucagon adenylate cyclase and binding activity, respectively (Epand, 1980).

SDS-polyacrylamide gel electrophoresis was selected as the means to determine the molecular weight of the components labeled. Gel filtration of Lubrol-PX solubilized covalently radiolabeled liver plasma membranes was used to further purify and characterize the receptor components.

METHODS

1) Photolabeling of glucagon receptor sites with ^{125}I-glucagon-NAPS

Liver plasma membranes (0.30-0.60 mg/ml) were preincubated with ^{125}I-glucagon-NAPS (7.2×10^{-10} - 1.0×10^{-8} M, specific activity $3.0\text{-}3.5 \times 10^{6}$ cpm/pmole) at 30°C for 10 min in the dark. The incubation medium was either 30 mM Tris-HCl, pH 7.0, 750 KIU/ml Trasylol, 0.25 mg/ml BSA, or the media used for binding studies (see "Methods" in Chapter II), in the absence and presence of 0.1 mM GTP. Volumes of samples ranged between 0.25-0.30 ml. After incubation, the samples were transferred to ice. Each sample was saturated with N_2 gas (water saturated) and subsequently photolysed for 2 min. under N_2 as previously described. Immediately after photolysis the sample was diluted with 10 ml of ice cold 30 mM Tris-HCl, pH 7.0, 0.1 mM GTP solution in a Corex tube (15 ml capacity) and incubated for 15 min at 30°C to dissociate noncovalently bound label. After incubation, the sample was centrifuged at (12000 x g) at 4°C for 30 min. After centrifugation, the supernatant was removed by gentle aspiration, the pellet was washed once with 0.5 ml 30 mM Tris-HCl, pH 7.0 and resuspended in 100-150 µl 6 M urea, 10% SDS for gel electrophoresis. Control samples included: membranes preincubated with 10^{-6} M cold glucagon, glucagon-NAPS or any of the glucagon derivatives tested; membranes preincubated with buffer and photolysed; membranes preincubated with prephotolysed ^{125}I-glucagon-NAPS and either photolysed or not; membranes preincubated with radiolabeled ^{125}I-glucagon (1.5×10^{-9} M, specific activity 2.0×10^{6} cpm/pmole) and photolysed; ^{125}I-glucagon-NAPS preincubated

in buffer and photolysed; membranes preincubated with ^{125}I-glucagon-NAPS, nonphotolysed; trypsin treated membranes preincubated with ^{125}I-glucagon-NAPS and photolysed.

2) Polyacrylamide gel electrophoresis

The discontinuous buffered SDS-polyacrylamide gel electrophoresis system described in "Methods" of Chapter I was used to analyze the plasma membrane components after photolysis. 7.5%, 10.0% and 12.5% acrylamide crosslinked cylindrical gels or slabs were used. Solubilized membranes were heated at 60°C for 30 min. and left overnight at room temperature. Samples were reduced with 10 mM dithiothreitol for half an hour at room temperature, under N_2 gas before application on gels. High and low molecular weight standards described in "Methods" in Chapter I were run in parallel on separate gels or on parallel slab wells. Slab gels were run for 1-2 hr. at 120 volts and then at 180 volts. After electrophoresis, gels were either fixed with 10% trichloroacetic acid or stained and destained as previously described in Chapter I. Cylindrical gels were scanned at 550 nm with a Gilford spectrophotometer with a linear transport and then frozen and cut into 1 or 2 mm slices for counting. Results were corrected for any discrepancies between the recorded lengths of gels and number of slices obtained. After staining and destaining, slab gels were dried on a Bio-Rad gel drier for 45 min. and then exposed to Kodak XR-2, X-Omat-R film for 10-15 days.

3) Membrane trypsinization

Liver plasma membranes 0.3 mg/ml were incubated in 30 mM Tris-HCl,

pH 7.0 with 0.10 mg/ml trypsin (10,000 units/mg) at 30°C for 30 min. At the end of this period the enzyme was inhibited by the addition of 0.12 mg/ml soybean trypsin inhibitor. Aliquots were taken for photoaffinity labeling of membrane receptor sites with ^{125}I-glucagon-NAPS.

4. Solubilization of ^{125}I-glucagon and ^{125}I-glucagon-NAPS bound liver plasma membrane receptor

Liver plasma membranes (5-10 mg/ml) were incubated according to Welton et al. (1977) (except for some modifications), with ^{125}I-glucagon (1.7×10^{-9}M, specific activity 3.7×10^{6} cpm/pmole) or ^{125}I-glucagon-NAPS (1.23×10^{-9}M, specific activity 3.3×10^{6} cpm/pmole) in 0.6 ml of 30 mM Tris-HCl buffer, pH 7.0, containing 0.04% BSA, 0.1 mM App(NH)p (adenosine-5'-(β,γ-imido)triphosphate, 10 μM cAMP, 30 μM Gpp(NH)p and 5 mM $MgCl_2$. Incubation was at 30°C for 20 min in the dark. After incubation, the samples were transferred to ice. The cooled samples were subsequently purged with water saturated N_2 gas for one min and then photolysed for 2 min under N_2, as previously described (see "Methods", Chapter II). The photolysed mixtures were transferred in cellulose nitrate tubes (3/16" x $1^5/8$") and centrifuged at 50,000 x g for 20 min at 4°C, in a Beckman Model L2-65B ultracentrifuge using an SW 50.1 rotor. The supernatant was discarded and the pellet (96% of radiolabel) was resuspended in 1.0 ml 10 mM Tes buffer, pH 7.5, 1 mM dithiothreitol, 25% (w/v) sucrose, with or without 0.1 mM GTP. Lubrol-PX (10% w/w) in 10 mM Tes buffer, pH 7.5, was added to give a final concentration of 1.0% (v/v). The mixture was blended on a Vortex mixer for one min and sonicated for another min in a Branson 12 sonicator. The

solubilized membranes were subsequently centrifuged at 4°C for 30 min as described above. The supernatant fluid containing the solubilized adenylate cyclase glucagon receptor (≈40-50% of radiolabel) was treated as described below. Incubated samples which were not photolysed were also treated as above.

5) Gel Filtration using Ultrogel AcA22

The solubilized membrane fractions prepared as above were chromatographed on an Ultrogel AcA22 column (LKB, 1.5 x 90 cm, fraction range $1 \times 10^6 - 6 \times 10^4$ daltons), in the presence of 10 mM Tes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 25% sucrose and 0.01% (v/v) Lubrol PX. When GTP was added to the Lubrol-soluble extract of the membrane, 10 µM GTP was also included in the elution buffer. Fractions (1.0 ml) were collected at a rate of 10-15 ml/hr. Samples (150 µl) were removed from each fraction for counting and protein determination, according to the modified mini-Lowry procedure of Hess et al.(1978). The column was calibrated with Dextran blue, apoferritin, human γ-globulin, bovine serum albumin and dinitrophenyl-glycine. The molecular weight standards were eluted with the same buffer in the absence of detergent and dithiothreitol. From the elution profile, fractions of each radiolabeled peak were pooled into Corex tubes (30 ml capacity) and the protein was precipitated with saturated ammonium sulphate solution. Precipitated protein samples were centrifuged at (12000 x g) at 4°C for 1 hr. The supernatant was discarded and the precipitated protein was redissolved in 100-150 µl 6 M urea, containing 10% SDS for gel electrophoresis.

RESULTS

1) Glucagon receptor characterization

Binding of ^{125}I-glucagon-NAPS derivative to liver plasma membranes, followed by photolysis, resulted in the labeling of a number of proteins (Fig. 27). The most intensely labeled bands had apparent sizes of ≈27,500, 50,000, 63,000, 69,000 and possibly 70,000 daltons. A band with a molecular weight greater than 200,000 and another found in the interface between the stacking and the separating gels were also labeled. High molecular weight aggregates appeared at the top of the gels, and a number of other bands were labeled, although less intensely. A band with a molecular weight of 12,000-13,000 was also seen in 12.5% gels. A broad band ahead of the dye front appeared in all gels (data not shown).

Irradiation did not cause any alterations in the number and size of membrane proteins. The same Coomassie blue stained protein profile was observed in irradiated and nonirradiated electrophoresed samples (Figs. 28,29). Apparent changes in the molecular weights and number of radiolabeled bands were observed in unreduced samples (Fig. 27). The mobility of the 69,000-70,000 daltons protein band(s) was altered when reduction was omitted (Fig. 27, lanes F,H), as well as the intensities of the 50,000 and 27,500 bands. The mobility of the 27,500 was also altered. The amount of label found in the stacking gel was higher in unreduced samples. The mobility of BSA was similarly affected. The presence of guanyl nucleotides in the incubation medium and during photolysis did not alter the numbers and molecular sizes of bands labeled (Fig. 27, lanes G-K).

Fig. 27. Autoradiographic identification of glucagon-binding sites in liver plasma membranes fractionated on 10% acrylamide/SDS slab gels after irradiation in the presence of ^{125}I-glucagon-NAPS

Liver plasma membranes (0.13 mg/ml) were preincubated with ^{125}I-glucagon-NAPS (1.63×10^{-9}M, specific activity 2.53×10^{6} cpm/pmole) under the various conditions described below, in 0.3 ml binding assay media ± GTP. After preincubation at 30°C for 10 min, membranes were photolysed then subjected to the GTP dissociation step and pelleted by centrifugation as described in "Methods". After one wash with 30 mM Tris-HCl pH 7.0, the pelleted membranes were treated for gel electrophoresis in 10% acrylamide 0.1% SDS 0.5 M urea slab gels prepared as described in "Methods" of Chapter I. Approximately equivalent amounts of ^{125}I-cpm were applied on the gel (0.633-0.9 mg of protein). Gels were run, stained and distained, then dried and exposed for 18 days. The illustration represents two slab gels which were run in parallel. The Coomassie Blue stained protein profiles and the molecular weight distribution of standards are presented in Figs. 28,29. Numbers indicate the molecular weights of standards (right) and unknowns (left).

A : membranes preincubated with ^{125}I-glucagon-NAPS.

B : membranes preincubated with ^{125}I-glucagon-NAPS and 10^{-6}M cold glucagon.

C : membranes preincubated with ^{125}I-glucagon-NAPS and 10^{-6}M cold N^{α}-trinitrophenyl glucagon.

D : membranes preincubated with ^{125}I-glucagon-NAPS and 10^{-6}M cold $Asp^{9,15,21}$-tri-glycinamide-glucagonyl-[glycinamide].

E : trypsinized membranes preincubated as in A but under "cyclase" conditions.

F : membranes preincubated as in A, nonreduced.

G : membranes preincubated as in A under "cyclase" conditions.

H : same as G - membranes, nonreduced.

I : membranes preincubated as in B under "cyclase" conditions.

J : membranes preincubated as in A plus 30 µM Gpp(NH)p

K : membranes preincubated as in G plus 30 µM Gpp(NH)p

L : ^{125}I-glucagon-NAPS (10^{-9}M) in 30 mM Tris-HCl pH 7.0, 1 mg/ml BSA, 750 KIU/ml Trasylol, preincubated, unphotolysed.

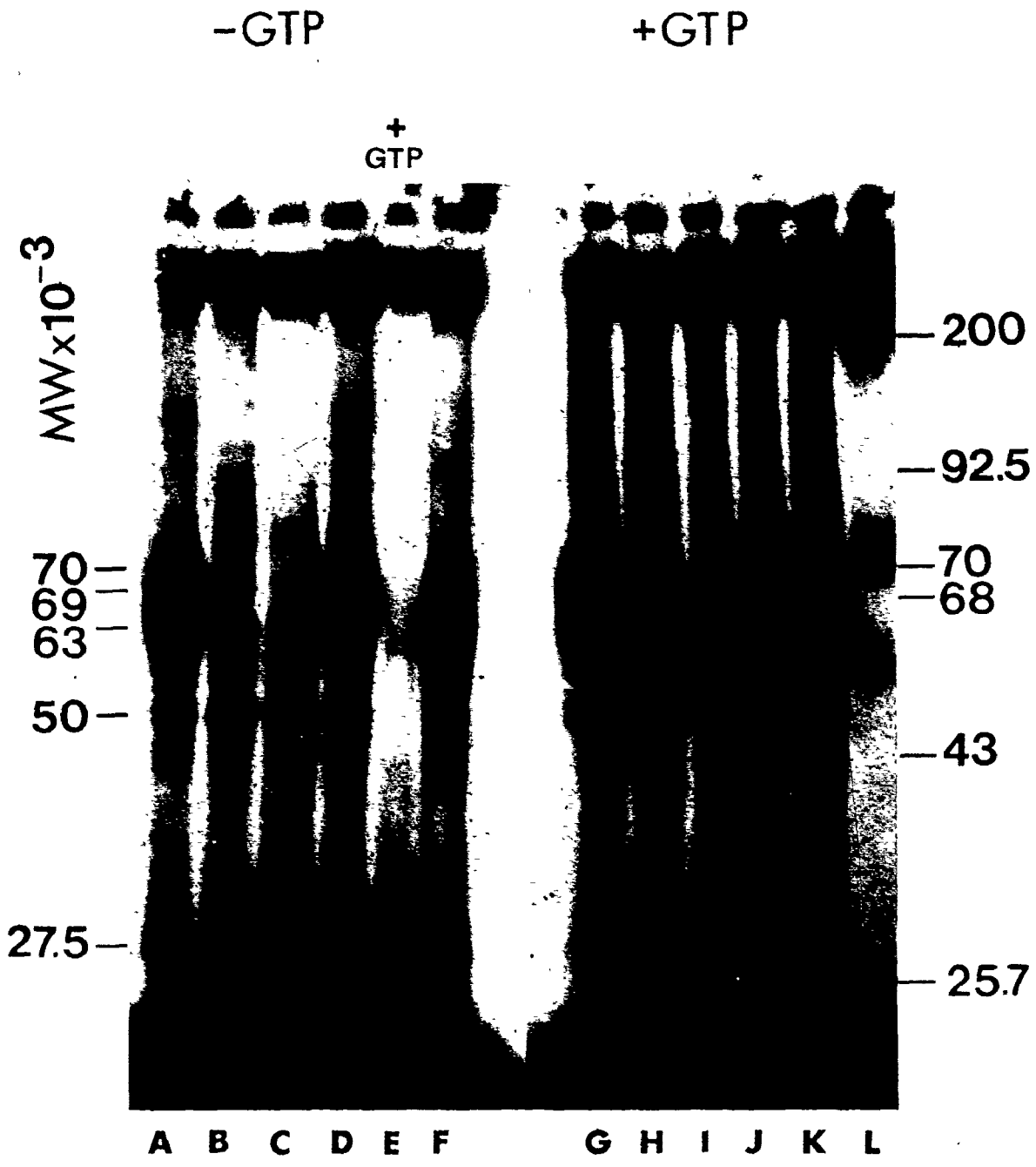

-GTP
+GTP
+
GTP
MW x10⁻³
200
92.5
70
68
43
25.7
70
69
63
50
27.5
A B C D E F
G H I J K L

Figure 27

Fig. 28. Coomassie Blue stain protein profiles of liver plasma membranes in the 10% acrylamide /SDS slab gels (Fig. 27).

(A-D, F):Liver plasma membranes preincubated with ^{125}I-glucagon-NAPS under "noncyclase" conditions before irradiation. E: trypsinized membrane preincubated under "cyclase" conditions. (A'):untreated liver plasma membranes. (B'):low molecular weight standards (ovalbumin 43K; α-chymotrypsinogen 25.7K; β-lactoglobulin 18.4K; lysozyme 14.3K; cytochrome c 12.3K; bovine trypsin inhibitor 6.2K; insulin 3.0K; C': myosin (H-chain) 200K; phosphorylase b 92.5K; bovine serum albumin 68K; ovalbumin 43K; α-chymotrypsinogen 25.7K; β-lactoglobulin 18.4K; cytochrome c 12.3K.

Samples (A-F) were photolysed under the conditions described in the legend to Fig. 27. Arrows indicate the position of the radiolabeled bands observed in autoradiograms (Fig. 27).

Figure 28

Fig. 29. Coomassie Blue stain protein profiles of liver plasma membranes in the 10% acrylamide/SDS slab gels (Fig. 27).

(G-K): Liver plasma membranes preincubated with ^{125}I-glucagon-NAPS under "cyclase" conditions before irradiation.

(L): ^{125}I-glucagon-NAPS unphotolysed, preincubated with 1 mg/ml BSA.

(A'): untreated liver plasma membranes.

(B'): low molecular weight standards.

(C'): high molecular weight standards.

Samples G-K photolysed under the conditions described in the legend to Fig. 27. Arrows indicate the position of labeled bands observed in the autoradiograms (Fig. 27).

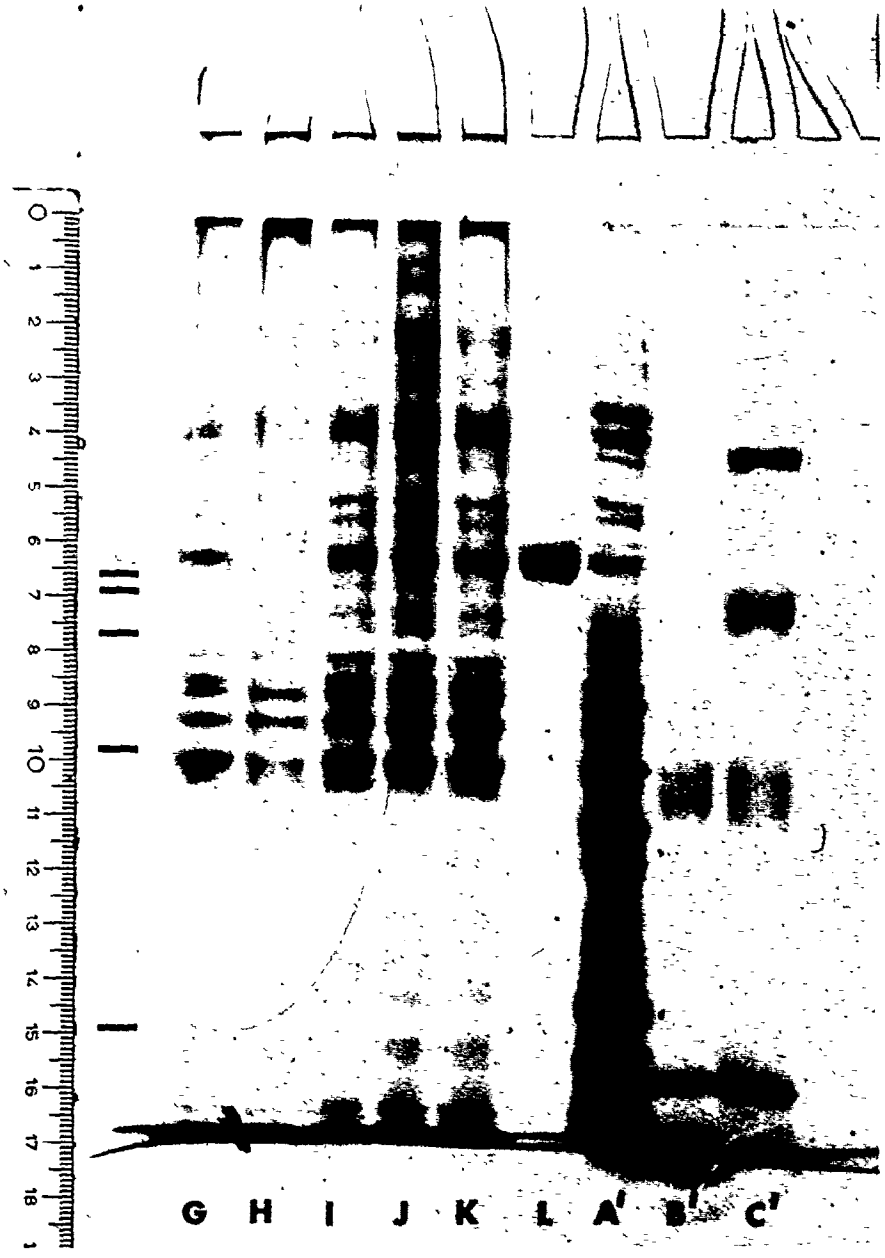


Figure 29

The band labeled significantly above background with an apparent molecular weight of 69,000-70,000 was the only band which was specifically labeled. The specificity of the labeling was ascertained from the fact that the intensity of the band was significantly reduced relative to the rest of the labeled bands when an excess of native unlabeled glucagon (10^{-5} - 10^{-6}M) was included in the binding medium and during irradiation of the sample. Because of the breadth of this band and the close proximity, it was not possible to clearly define its relative mobility. Therefore, the molecular weight may be over or underestimated by 5,000-10,000. The breadth may be due to proteolysis of a single protein component. The anomalous electrophoretic mobility observed with varying percentages of acrylamide suggest anomalous SDS binding (Fig. 30). The long autoradiographic exposure for visualization of bands containing minor amounts of label resulted in darkening of major bands. This, as well as the breadth of the bands, made correlation of relative densities difficult. When samples were run in cylindrical gels, sliced and counted directly, the almost complete displacement of radioactivity from the 69,000-70,000 daltons band in the presence of excess cold hormone was more evident (Fig. 31). Glucagon-NAPS was equipotent to native glucagon in displacing the radioactively labeled peptide (data not shown). The same specific receptor site was labeled in the presence or absence of GTP, although the amount of labeling was different (Fig. 27, A vs G; B vs I).

Specificity of labeling of this receptor site was also demonstrated when preincubation and irradiation were performed in the

Fig. 30. Relative mobilities of the radiolabeled components with varying percentages of acrylamide

Cylindrical gels of 7.5%, 10.0% and 12.5% acrylamide were prepared according to Laemmli (1970) with the modifications described in "Methods" of Chapter I. Membrane samples irradiated in the presence of ^{125}I-glucagon-NAPS prepared and treated as described in the legend to Fig. 27 were applied to the gels. Molecular weight standards (see legend to Fig. 28) were run in parallel gels. The relative mobilities were calculated with reference to the dye front. Molecular weights of unknowns were calculated from relative mobility vs log. Molecular weight plots obtained from molecular weight standards. (●): 27,500; (o): 50,000; (▲) 69,000-70,000 (as calculated from 10.0% acrylamide gels).

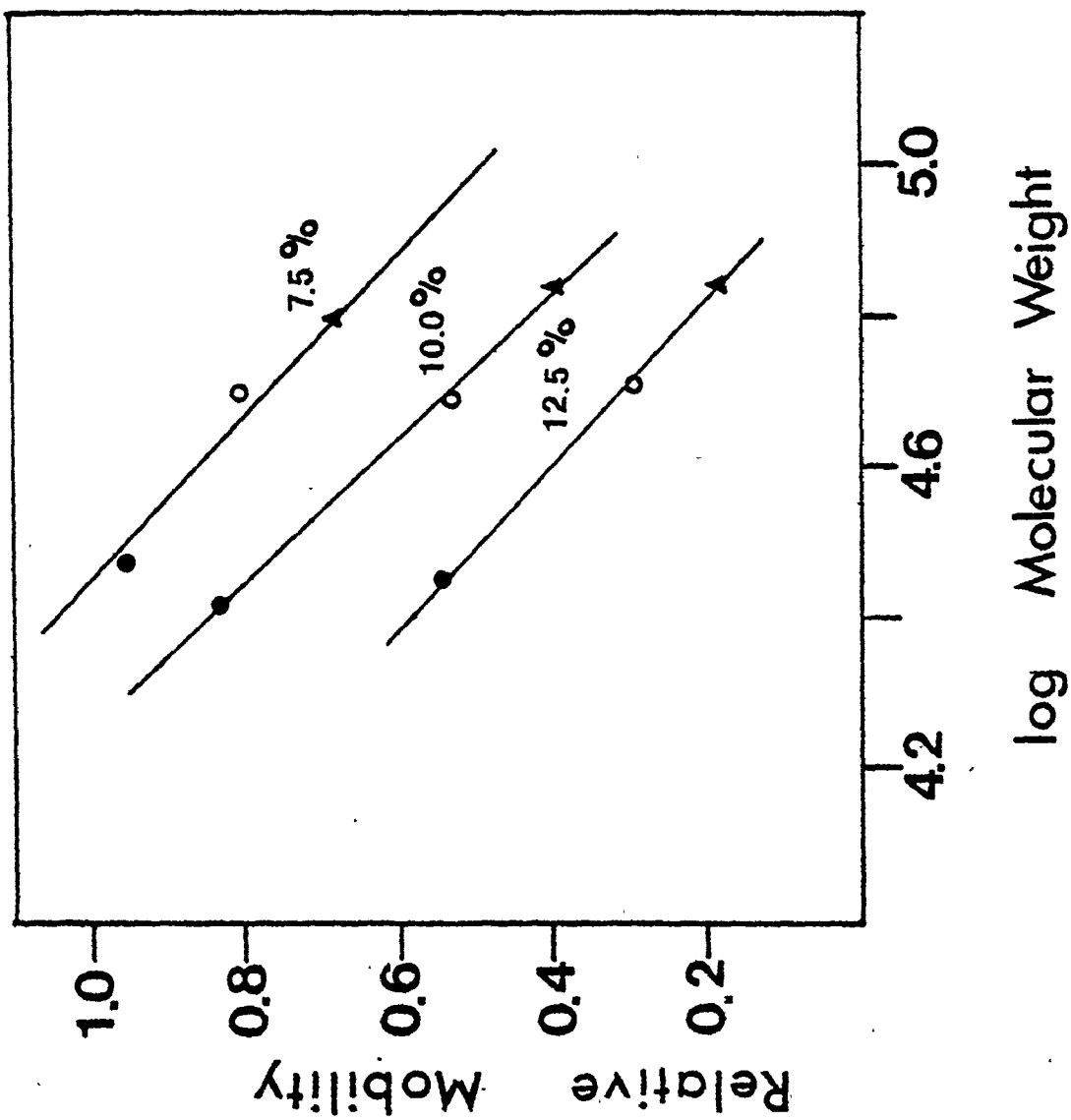
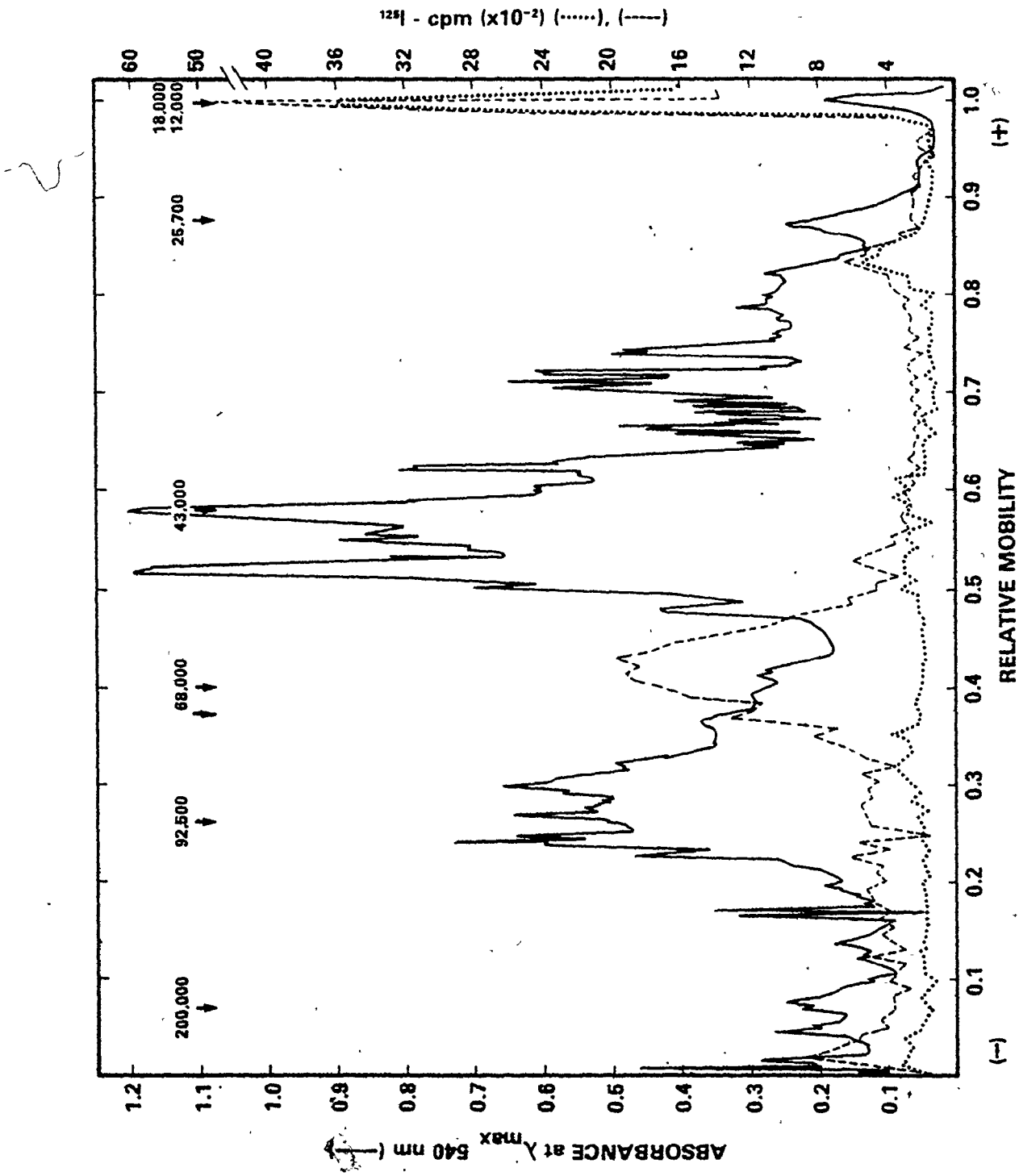

Relative Mobility
1.0
0.8
0.6
0.4
0.2
log Molecular Weight
4.2
4.6
5.0
7.5 %
10.0 %
12.5 %

Figure 30

Fig. 31. Densitometric scan and ^{125}I-cpm profile superimposed, of liver plasma membranes irradiated in the presence of ^{125}I-glucagon-NAPS and electrophoresed on 10% acrylamide/SDS cylindrical gels.

Liver plasma membranes (0.42 mg/ml) were preincubated with ^{125}I-glucagon-NAPS (7.2×10^{-10} M, specific activity 2.9×10^{6} cpm/pmole) in 0.21 ml 30 mM Tris-HCl, pH 7.0, 0.25 mg BSA/ml, 750 KIU/ml Trasylol at 30°C for 10 min. The samples were then photolysed under the conditions described in Chapter II, subjected to the GTP dissociation step and subsequently centrifuged as previously described. The pelleted membranes were solubilized with 0.15 ml 6 M urea, 10% SDS and treated for gel electrophoresis as described in Methods. The total sample was applied on (14.0 x 0.6 cm) cylindrical gels prepared as described in Chapter I. Molecular weight standards were run in parallel gels. After electrophoresis the gels were stained with Coomassie Blue and after distaining they were scanned at λ = 540 nm. The stained gels were frozen, sliced and counted for radioactivity. ^{125}I-cpm profiles were superimposed on densitometric scans after corrections for discrepancies between recorded lengths and number of slices obtained.

(——A 540_m); (- - -): ^{125}I-cpm of samples preincubated with ^{125}I-glucagon-NAPS, photolysed; (·····): ^{125}I-cpm of sample preincubated with ^{125}I-glucagon-NAPS and 10^{-6} M cold glucagon, photolysed.


^{125}I - cpm (x10^{-2}) (·····), (——)
60
50
40
36
32
28
24
20
16
12
8
4
18,000
12,000
25,700
43,000
68,000
92,500
200,000
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE at λ_{max} 540 nm (——)
(−)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
(+)
RELATIVE MOBILITY

presence of excess glucagon derivatives: N^{α}-trinitrophenyl glucagon was able to displace bound radioactivity from the receptor specific site (Fig. 27C). The total amount of ligand bound to membranes was equivalent to that in the presence of excess cold glucagon, after irradiation and centrifugation. A large amount of the bound radiolabeled ligand, 30-35% was associated with the 50,000 molecular weight band (calculated from cylindrical gels). $Asp^{9,15,21}$-triglycinamide glucagonyl-[glycinamide] did not compete for any of the specifically labeled receptor sites (Fig. 27D), however, 50% more label remained bound to membranes after dissociation with GTP than the amount bound in the presence of excess cold glucagon. The excess radioactivity was associated with the 27,500 molecular weight band as well as with the bands >200,000 (observed in cylindrical gels).

The high molecular weight bands (greater than 200,000) were considered to be molecular aggregates of the radiolabeled ligand, since they were observed when ^{125}I-glucagon-NAPS was electrophoresed under the same conditions but in the absence of membranes (Fig. 27, lane L). The intensity of these bands differed, depending on the total protein content and ligand in the incubation medium. A number of other bands with molecular weights of 50,000, 40,000-45,000, 27,500 and 20,000-25,000 displayed a similar behaviour. The 20,000-25,000 band may represent the equivalent molecular weight species observed in SDS gels of ^{125}I-glucagon-NAPS photolabeled BSA (see Chapter I), suggesting that it may be a glucagon aggregate. Irradiation of membranes in the presence of higher concentrations of unlabeled glucagon $>10^{-5}$ increased the radiolabeled ligand associated with the 40,000-45,000 band in SDS gels by more than 300% and a new band of molecular weight 110,000-115,000 appeared, suggesting that these bands may also be

Fig. 32. Gel electrophoresis profiles of labeled solubilized receptor and of plasma membranes irradiated in the presence of ^{125}I-glucagon-NAPS

Liver plasma membranes were preincubated and photolysed or kept in the dark under the conditions described in the legend to Fig. 27. After treatment of samples as previously described, the samples were run in 10% acrylamide cylindrical gels (see "Methods", Chapter I).

Liver plasma membranes solubilized with Lubrol were fractionated in Ultrogel AcA22. The fractions from the peak where radioactivity was displaced in the presence of GTP (Fig. 33) were pooled and the protein was precipitated as described in "Methods". The precipitated protein was solubilized in 6 M urea containing 10% (w/v) SDS and applied on the gels. After electrophoresis, the gels were treated as previously described, frozen and cut in 1 mm slices. The slices were counted in a γ-counter. Molecular weight standards (see Fig. 28) were run in parallel gels. A: (a) membranes were preincubated with ^{125}I-glucagon-NAPS and photolysed; (b): membranes were preincubated as in (a) and in the presence of 10^{-5}M unlabeled glucagon; (c): membranes were preincubated as in (a) but were not photolysed. B: (a) solubilized receptor obtained from Ultrogel AcA22 chromatography from fractions described above; (b) membranes preincubated with prephotolysed ^{125}I-glucagon-NAPS; (c) membranes preincubated as in A (a), reduced with 50 mM DTT before irradiation.

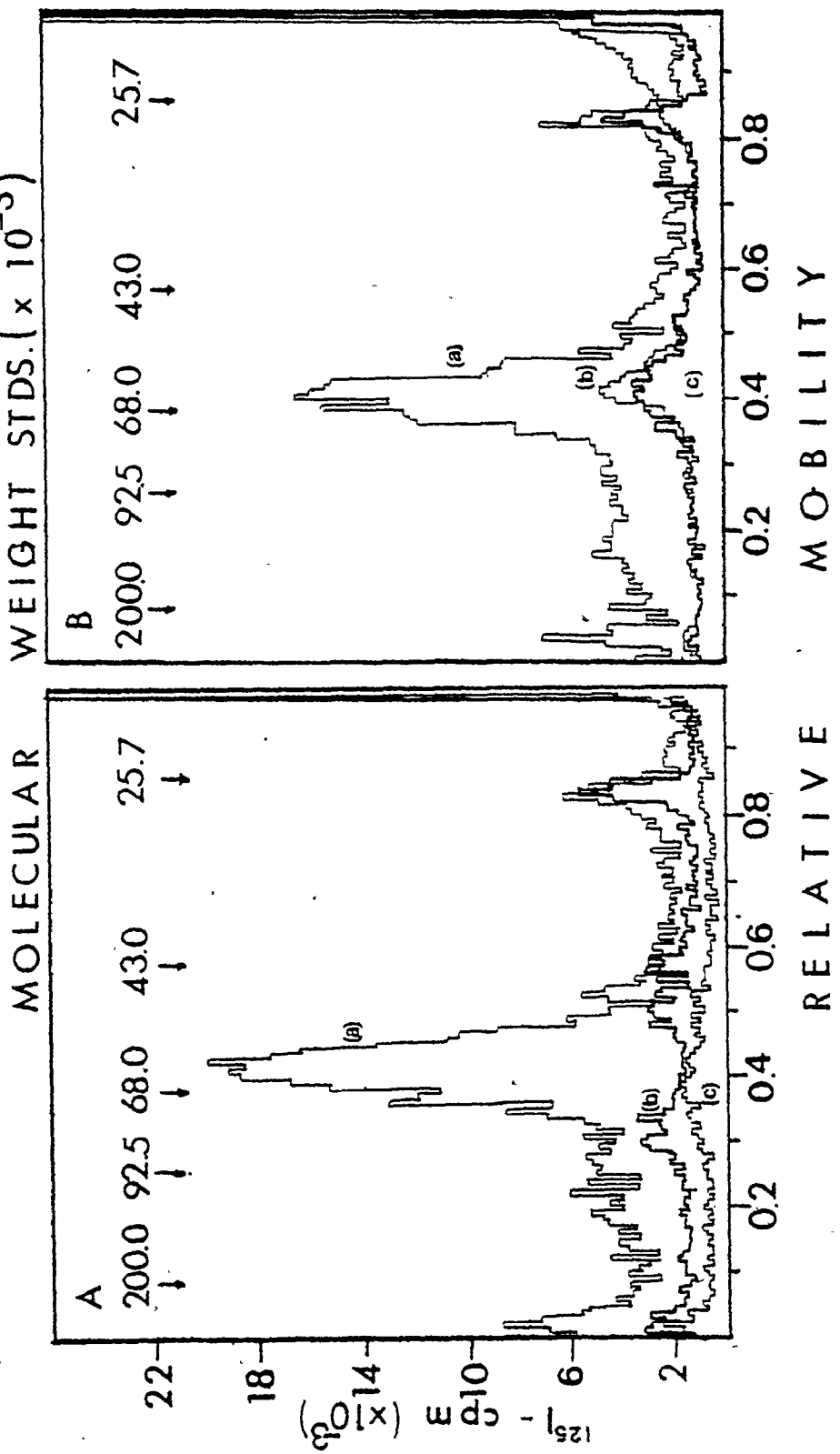

A
B
MOLECULAR WEIGHT STDS. (x 10^{-3})
200.0
92.5
68.0
43.0
25.7
(a)
(b)
(c)
^{125}I - cpm (x 10^{3})
22
18
14
10
6
2
0.2
0.4
0.6
0.8
RELATIVE MOBILITY

Figure 32

peptide aggregates associated with the membrane. It is possible that their aggregation is concentration dependent.

Quantitation of specific covalent labeling by bound ^{125}I-glucagon-NAPS upon irradiation was calculated for the total amount of plasma membrane applied on the gels. Specific crosslinking, however, may be underestimated since both autoradiography and protein stain showed aggregates on the top of the gels. The percentages of specifically bound radioactive ligand were calculated from cylindrical gels which were sliced and counted. Under the irradiation conditions used, approximately 10-15% of bound radiolabeled glucagon-NAPS was associated with the specific glucagon receptor sites. This would correspond to 0.15 ± 0.02 pmole of bound ligand per mg membrane protein in the absence of GTP and in the presence of 7.2×10^{-10}M ligand. In the absence of photolysis and therefore covalent labeling, less than 0.10 ± .05% of the total bound ligand was associated with the specifically labeled receptor sites (Fig. 32). The total amount of bound ^{125}I-glucagon-NAPS which was prephotolysed and then incubated with plasma membranes was equivalent to that photolysed after incubation. Only a small percentage of total bound radioactivity from the prephotolysed sample (less than 0.1%) was associated with the glucagon specific binding sites after gel electrophoresis (Fig. 32). The extent of specific labeling of prephotolysed ligand was not greatly altered if the incubation medium was irradiated for a second time. A small increase (0.5% of total bound ligand) in the amount of labeling observed was attributed to migration of ^{125}I-ions during irradiation. In both cases, 2.3% of bound ligand was associated with the 12,000 - 13,000 band seen in 12.5% crosslinked gels. Preincubation with ^{125}I-glucagon followed by

irradiation resulted in approximately 0.6 - 1.0% of total bound radioactivity being associated with the specific receptor sites. Less than 0.1% of total hormone bound was associated with these sites in unphotolysed samples.

80-85% of ^{125}I-glucagon-NAPS bound to plasma membranes measured before electrophoresis, was associated with sites other than the specific receptor sites. This would correspond to 0.80 ± 0.10 pmole of nonspecific ligand bound per mg of membrane protein in the prescence of 10^{-10}M ligand.

Although there were no apparent changes in the number of bands labeled in the autoradiograms of samples preincubated and photolysed in the presence and absence of GTP (Fig. 27), there were differences in the amount of label incorporated under these conditions. Less radioactivity was associated with the specifically labeled band in the presence of GTP; Gpp(NH)p had an opposite effect (Fig. 27, lane J). An increase in the amount of label incorporated was observed for both the specifically and nonspecifically labeled sites. The Gpp(NH)p effect was not as pronounced when GTP was also included in the incubation medium (Fig. 27, lane K). The Gpp(NH)p effect, however, may not be significant because of the different amount of protein in the samples applied on the gels.

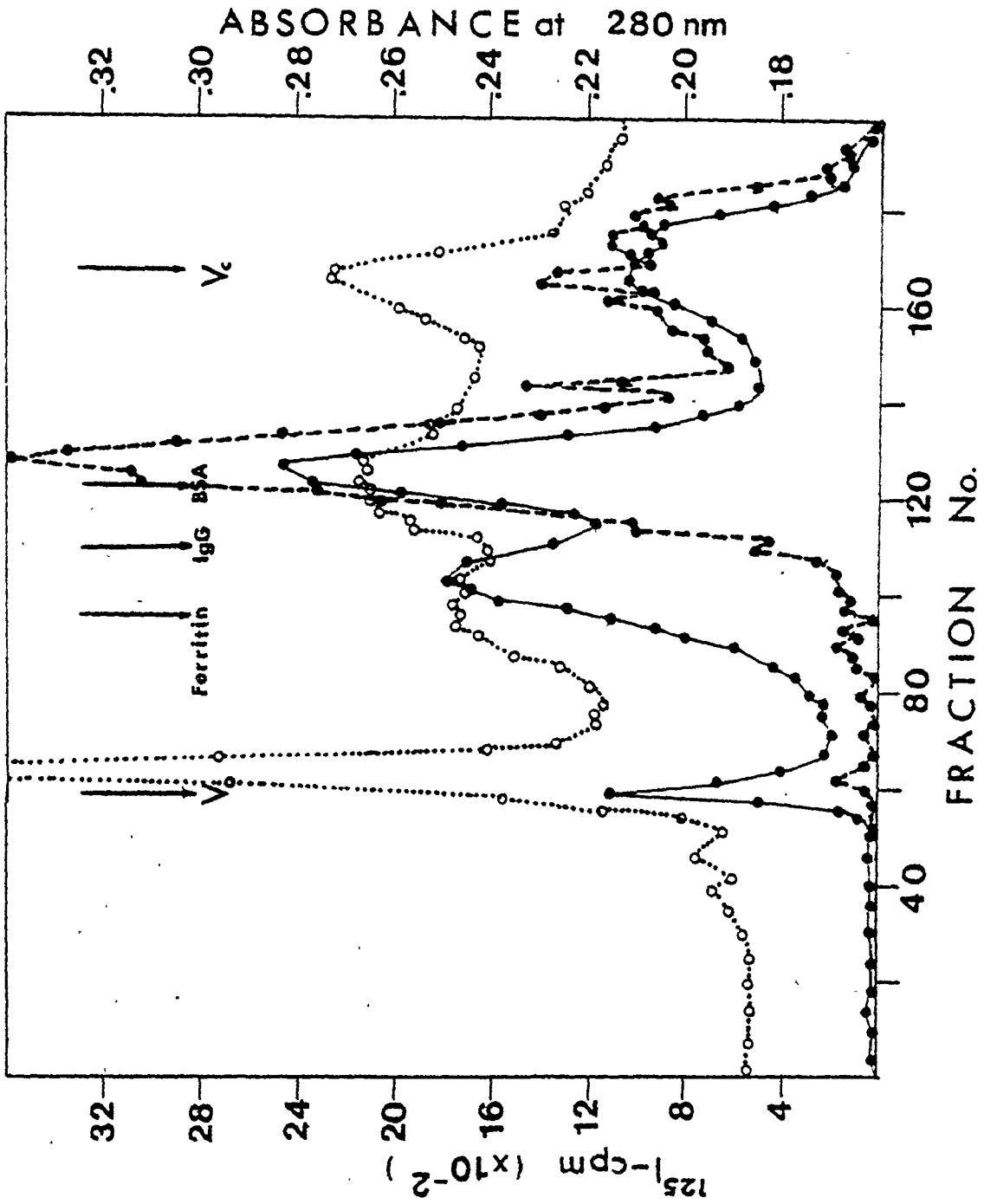
When trypsinized membranes were preincubated with ^{125}I-glucagon-NAPS and then photolysed, although 18% of the radiolabeled peptide remained bound on the membrane after the GTP dissociation step, there was no label incorporated in either the specifically or nonspecifically labeled bands. The high and low molecular weight aggregates of the radio ligand, however, were still present (Fig. 27E).

2) Receptor solubilization

The glucagon receptor, covalently labeled by ^{125}I-glucagon-NAPS, after preincubation and irradiation, was solubilized with 1% (w/v) Lubrol in the presence of 25% sucrose and subsequently subjected to chromatography on Ultrogel AcA22. The elution profile (Fig. 33) indicated that the glucagon receptor eluted from the gel in the included volume. The glucagon receptor peak was identified on the basis that radioactivity was completely dissociated when unphotolysed samples were solubilized and eluted in the presence of GTP. ^{125}I-glucagon was equally well displaced as ^{125}I-glucagon-NAPS in unphotolysed samples (data not shown). From the elution profile of the molecular weights used, the size of the receptor was estimated to be 200,000-250,000 daltons. Gel electrophoresis of the solubilized purified receptor covalently labeled with ^{125}I-glucagon-NAPS showed that the receptor was composed of the same subunits as those observed in unpurified samples (Fig. 32). Both the specifically labeled and nonspecifically labeled bands were observed. Bands identified as aggregates in the unpurified electrophoresed samples were not labeled significantly above background. After preincubation and irradiation and before solubilization, 90-95% of the radiolabeled ligand (10^{-9}M) remained bound to the membrane. Lubrol (1.0%) solubilized 15-20% of membrane; 35-40% radioactivity was associated with the soluble fraction. Approximately 30% of that was associated with the peak of the peptide-receptor complex in the elution profile of the Ultrogel.

Fig. 33. Elution profile on Ultrogel AcA22 of solubilized liver plasma membranes preincubated and photolysed in the presence of ^{125}I-glucagon-NAPS

Liver plasma membranes 6.0 mg/ml were incubated with ^{125}I-glucagon-NAPS (1.23×10^{-9} M, specific activity 3.3×10^{6} cpm/pmole) in 0.6 ml 30 mM Tris-HCl buffer, pH 7.0 containing 0.04% BSA, 0.1 mM App(NH)p, 10 µM cAMP, 30 µM Gpp(NH)p and 5 mM $MgCl_2$, at 30°C for 20 min in the dark. After incubation, the samples were photolysed, the unbound radiolabeled peptide was removed by centrifugation and the pelleted membranes were solubilized with Lubrol PX; recentrifuged again and chromatographed on Ultrogel AcA22 (1.5 x 90 cm) column (see Methods).
(●———●): Membranes preincubated with ^{125}I-glucagon-NAPS, photolysed, solubilized in the presence of 0.1 mM GTP, eluted with 0.01 mM GTP in 10 mM Tes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 25% sucrose and 0.01% (v/v) Lubrol PX.
(●----●): Membranes preincubated with ^{125}I-glucagon-NAPS, nonphotolysed, solubilized and eluted, as above.
(o·····o): Absorbance at λ = 280 nm of elution profile of solubilized liver plasma membranes. Samples 0.5 ml were counted. Fractions 1.0 ml were collected. The ^{125}I-glucagon profiles in the presence and absence of GTP are not shown for clarity.


ABSORBANCE at 280 nm
.32
.30
.28
.26
.24
.22
.20
.18
V_c
BSA
IgG
Ferritin
V_o
160
120
80
40
FRACTION No.
32
28
24
20
16
12
8
4
^{125}I-cpm (x10^{-2})

DISCUSSION

The aim of this project was to apply the techniques of photoaffinity labeling in the purification and characterization of the glucagon receptor sites in liver plasma membranes. The bifunctional photoaffinity reagent NAPSCl, specific for the modification of tryptophan containing peptides, was synthesized and used to modify glucagon. The photolabeling properties of the glucagon-NAPS derivative were tested and it was shown that it can covalently label macromolecules upon irradiation. The following lines of evidence suggested that the derivative could be used to photolabel the glucagon binding sites: a) glucagon-NAPS was able to activate the liver adenylate cyclase enzyme with a potency even higher than the native hormone; b) covalent crosslinking of glucagon-NAPS to membrane sites upon irradiation resulted in inhibiting stimulation of adenylate cyclase by glucagon; c) binding of the radiolabeled derivative was reduced specifically by native glucagon; d) the binding characteristics of the radiolabeled derivative were similar to those of the native hormone: two binding sites were identified for both the derivative and glucagon and their affinities for these sites were in the same order of magnitude; e) the binding characteristics and adenylate cyclase activity of glucagon-NAPS were affected by GTP in a similar fashion to that of the native glucagon; f) detergent solubilized ^{125}I-glucagon and ^{125}I-glucagon-NAPS receptor complexes displayed the same elution profile in gel fractionation of solubilized plasma membranes. Addition of GTP displaced ^{125}I-glucagon

NAPS from the solubilized ^{125}I-glucagon-NAPS complex as effectively as ^{125}I-glucagon in nonirradiated but not in irradiated samples.

SDS-polyacrylamide electrophoresis, under reducing conditions, of liver plasma membranes covalently crosslinked with ^{125}I-glucagon-NAPS, resolved a number of radiolabeled protein bands with molecular weights of 63,000-70,000, 50,000 and 27,500. Only the 69,000-70,000 daltons peptide were specifically labeled. A number of other bands with molecular weights 10,000-12,000, 20,000-25,000, 40,000-45,500, 100,000-110,000 and those >200,000 were identified as high molecular weight aggregates of the radiolabeled peptide since they were also observed in irradiated samples at high peptide concentrations in the absence of membrane. These components remained associated with the membrane even in the presence of excess cold peptide after dissociation with GTP, and removal of free ligand. Their association with the membrane suggests that glucagon may associate with the membranes before binding to the receptor. The sites of binding could be phospholipids. No changes in the protein profile of irradiated vs nonirradiated membranes were observed and prolonged irridation of membranes in the presence of ^{125}I-glucagon-NAPS for up to 10 min did not change the pattern of radiolabeled bands, suggesting that there is no perturbation of the labeled species by free radical crosslinking during irradiation. Reduction with DTT showed that the nitrene is accessible to the solvent and may react with neighbouring molecules, resulting in nonspecific binding.

The fact that the adenylate cyclase enzyme remains active in irradiated samples and can still bind glucagon under the experimental conditions used during irradiation (i.e. in the presence of protease

inhibitors, Trasylol and bacitracin) suggest that the specific radioactively labeled band(s) are less likely to be proteolytic products of a larger molecular weight receptor species. However, receptor related protease activity not inhibited by the protease inhibitors used, may account for the breadth of the band(s). The breadth of the bands observed may also be due to the glycoprotein nature of the labeled membrane peptides as suggested from the anomalous migration of the 69,000-70,000, as well as the 27,500 bands with different acrylamide concentrations. The apparent molecular weights of these components may differ from the actual size of the molecules as a result of their anomalous electrophoretic mobility. Changes in their electrophoretic mobility in unreduced vs reduced samples suggests the presence of intramolecular disulfide links. The extent of reduction therefore may also influence their mobility as suggested from studies with model protein containing cystine bonds (Griffith, 1972).

Specific displacement of label incorporated in the 69,000-70,000 band by cold glucagon suggested that this component may represent the glucagon receptor. The N^{α}-trinitrophenyl-glucagon derivative was also able to inhibit radiolabeling of this component. $Asp^{9,15,21}$-tri-glycinamide-glucagonyl-[glycinamide] did not displace any of the labeled components.

The apparent molecular weight of the solubilized glucagon-receptor complex was estimated by gel fractionation of detergent

solubilized membranes as 200,000-250,000. However, depending on the presence of detergent and its concentration, Stokes radii may alter and this value may be inaccurate. Phospholipids are possibly associated with this complex according to reports of similar studies with rat liver membrane solubilized adenylate cyclase (Welton et al. 1977, 1978), whose elution profile in Ultrogels overlaps with the glucagon-receptor complex. However, a component of 240,000 daltons has been identified as part of the adenylate cyclase-glucagon receptor complex (Storm and Chase, 1975).

SDS-gel electrophoresis of the purified ^{125}I-glucagon-NAPS receptor complex, covalently crosslinked by irradiation, identified a number of protein components with apparent molecular weights similar to those found in the unpurified samples. On the detection of the 27,500 and 50,000 components in the solubilized receptor complex, an important question arises as to whether these proteins represent true glucagon-receptor sites, since they were not significantly displaced in the presence of excess cold peptide. However, reduction of nitrenes immediately prior to photolysis resulted in inhibiting covalent crosslinking of the 69,000-70,000 components by more than ≃85%, the 50,000 component by ≃50% and the 27,500 component by ≃15%, suggesting that the 27,500 and 50,000 components may bind ^{125}I-glucagon-NAPS more tightly, and so the NAPS reagent may not be ascessible to solvent. Membrane protein components with molecular weight close to 27,500 daltons have been identified previously as glucagon-receptors (Levey, 1975; Bregman and Levy, 1977).

From the studies of Welton et al. (1977, 1978), the receptor sites associated with the solubilized glucagon-receptor complex have been identified as those of the high affinity-low capacity sites. This would

be in agreement with the low total amount 0.3-0.4 (±0.1) pmole/mg of protein, of ligand bound to these sites (the 27,500, 50,000 and 69,000-70,000 daltons components). The amount of label incorporated in the 69,000-70,000 daltons component in the gel electrophoresis pattern of irradiated membranes with ^{125}I-glucagon-NAPS is affected by GTP presence in the incubation medium. Similarly, the amount of bound radiolabeled ligand to detergent solubilized receptor of nonirradiated membranes and membranes preincubated with ^{125}I-glucagon is affected by the presence of GTP, suggesting that the 69,000-70,000 component may represent the specific glucagon receptor site in agreement with being displaced by unlabeled glucagon. It is possible that the 50,000 and 27,500 components are nonspecifically labeled peptides that may copurify with the receptor.

CONCLUSIONS

The low extent of covalent crosslinking of specific receptor sites to ^{125}I-glucagon-NAPS upon irradiation, as measured from the dissociation studies, the SDS-gel electrophoresis results, the insensitivity of adenylate cyclase to glucagon stimulation in irradiated membranes in the presence of glucagon-NAPS and the binding studies provided strong evidence that low capacity-high affinity sites of the receptor are involved in the adenylate cyclase enzyme activation by glucagon. Photoaffinity labeling and SDS-gel electrophoresis identified the glucagon receptor sites as having an apparent molecular weight of 69,000-70,000. The glucagon specific receptor copurified in a detergent soluble form with two components, 50,000 and 27,500 daltons. The radiolabeling of these components was not significantly displaced by cold peptide and may not be called specific receptor sites. The inhibition of covalent crosslinking with ^{125}I-glucagon-

NAPS to any of these sites in trypsinized membranes demonstrated the protein nature of these membrane receptor sites. The anomalous electrophoretic mobilities with different acrylamide concentrations suggests that the glucagon specific receptor site(s) may be a glycoprotein, in agreement with previous conclusions by other investigators.

If the low capacity-high affinity sites are the receptor sites responsible for adenylate cyclase stimulation, a question arises as to what is the role and nature of the low affinity-high capacity receptor sites observed in the binding studies. These sites could be phospholipids involving hydrophobic interactions with the peptide, as suggested in the studies related to the role of phospholipids in hormone receptor binding and adenylate cyclase activation. Membrane fluidization and collision coupling of the hormone-receptor with the adenylate cyclase enzyme could be facilitated through such types of interactions. It is also possible that association with the membrane may be required for recognition by the receptor. The above is in agreement with the models proposed for the glucagon receptor interactions and with the fact that all binding sites of glucagon are related to the adenylate cyclase enzyme activation.

REFERENCES

Alford, F.P. and Chisholm, D.J. (1979) Aust. N.Z. J. Med. 9, 733.

Ansorge, S., Bohley, P., Kirschke, H., Langer, J. and Hanson, H. (1971) J. Biochem. 19, 283.

Ariens, E.J. (1979) TIBS inaugural issue, p. 11.

Ariens, E.J., Beld, A.J., Rodrigues de Miranda, J.F. and Simonis, A.M. (1978) The Receptors, A Comprehensive Treatise, (R.D. O'Brien, ed). Plenum Press, New York and London, Vol. 1, p. 33.

Assan, R. and Slusher, N. (1972) Diabetes 21, 843

Bayley, H. and Knowles, J.R. (1977) Methods in Enzymol. 46, 69.

Bayley, H. and Knowles, J.R. (1978a) Biochem. 17, 2414.

Bayley, H. and Knowles, J.R. (1978b) Biochem. 17, 2420.

Beaven, G.H., Fratzer, W.B. and Davies, H.G. (1969) Eur. J. Biochem. 11, 37.

Beddell, C.R., Sheppey, G.C., Blundell, T.L., Sasaki, K., Dockerill, S. and Goodford, P.J. (1977) Int. J. Peptide Protein Res. 9, 161.

Berson, S. and Yalow, R. (1966) Science 152, 205.

Bhathena, J.J., Voules, N.R., Smith, S. and Recant, L. (1978) J. Clin. Invest. 6, 1488.

Biltonen, R. (1977) Fed. Proc. 36, 2112.

Birnbaumer, L. and Pohl, S.L. (1973) J. Biol. Chem. 248, 2056.

Birnbaumer, L., Beaver, C.F. and Iyengar, R. (1980b) J. Biol. Chem. 255, 3552.

Birnbaumer, L., Pohl, S.L. and Rodbell, M. (1968) J. Biol. Chem. 244, 3468.

Birnbaumer, L., Pohl, S.L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1857.

Birnbaumer, L., Pohl, S.L., Rodbell, M. and Sundby, F. (1972) J. Biol. Chem. 247, 2038.

Birnbaumer, L., Swartz, T.L., Abramowitz, J., Mintz, P.W. and Iyengar, R. (1980a) J. Biol. Chem. 255, 3542.

Blanchard, M.H. and King, M.V. (1966) Biochem. Biophys. Res. Comm. 25, 298.

Bletcher, M., Giorgio, N.A. and Johnson, C.B. (1974) Adv. in Enzym. Regul. (G. Weber, ed.), Pergamon Press, Oxford, Vol. 12, p. 289.

Blundell, T.L., Hodgkin, D.C., Dodson, G.G. and Mercola, D.A. (1972) Adv. Protein. Chem. 26, 279.

Blundell, T.L. and Wood, S.P. (1975) Nature 257, 197.

Boesch, C., Bundi, A., Oppliger, M. and Wüthrich, K. (1978) Eur. J. Biochem. 91, 209.

Boeynaems, J.M. and Dumont, J.E. (1975) J. Cyclic Nucleotide Res. 1, 123.

Bogert, M. T. and Stull, A. (1941) "Organic Synthesis", J. Wiley and Sons, Inc., New York, N.Y. Coll. Vol. I, p. 220.

Bornet, H. and Edelhoch, H. (1971) J. Biol. Chem. 246, 1785.

Bregman, M.D., Cheng,S, and Levy, D. (1978) Biochim. Biophys. Acta 539, 489.

Bregman, M.D. and Levy, D. (1977) Biochem. Biophys. Res. Comm. 78, 584.

Bretscher, M.S. (1971) Nature, London New Biol. 231, 229.

Bromer, W.W., Boucher, M.E. and Koffenberger, J.E. (1971) J. Biol. Chem. 246, 2822.

Bromer, W.W., Boucher, M.E. and Patterson, J.M. (1973) Biochem. Biophys. Res. Comm. 53, 134.

Bromer, W.W., Sinn, L.G., Staub, A. and Behrens, O.K. (1956) J. Am. Chem. Soc. 78, 3858.

Buers, C.M. and Khorash, N. (1950) J. Am. Chem. Soc. 72, 3599.

Canova-Davis, E. and Ramachandran, J. (1980) Biochem. 19, 3275.

Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA, 75, 2669.

Cassel, D., Levkowitz, H. and Selinger, Z. (1977) J. Cycl. Nucleotide Res. 3, 393.

Cassel, D. and Selinger, Z. (1977) Proc. Natl. Acad. Sci. USA, 74, 3307.

Cassim, J.Y. and Yang, J.T. (1969) Biochem. 8, 1947.

Catt, K.J. and Dufau, M.L. (1977) Ann. Rev. Physiol. 39, 529.

Catt, K.J., Dufau, M.L., Tsuruhara, T. (1972) Biochim. Biophys. Acta. 279, 194.

Chou, P.Y. and Fasman, G.D.((L975) Biochem. 14, 2536.

Chowdhry, V. and Westheimen, F.H. (1979) Ann. Rev. Biochem. 48, 293.

Chothia, C. and Janin, J. (1975) Nature 256, 705.

Contaxis, C.C. and Epand, R.M. (1974) Can. J. Biochem. 52, 456.

Cuatrecasas, P.and Anfinsen, C.D. (1971) Ann. Rev. Biochem. 40, 259.

Cuatrecasas, P.,Hollenberg, M.D., Chang, K.-J. and Bennett, V. (1975) Recent Progr. Hormone Res. 31, 37.

Das, M. and Fox, C.F. (1979) Ann. Rev. Biophys. Bioeng, 8, 165.

Davis, B.J. (1964) Annals N.Y. Acad. Sci. 121, 404.

De Meyts, P., Bianco, A.R. and Roth, J. (1976) J. Biol. Chem. 251, 1877.

Desbuquois, B. (1975) Eur. J. Biochem. 53, 569.

Desbuquois, B. (1977) First International Symposium on Hormonal Receptors in Digestive Tract Physiology, INSERM Symposium No. 3, Editors: Bonfils et al. p. 127.

Desbuquois, B., Krug, F. and Cuatrecasas, P. (1974) Biochim. Biophys. Acta 343, 101.

Diaconescu, C., Thamm, P., Saunders, D.J. and Brandenburg, D. (1980) in "Insulin, Chemistry, Structure and Function of Insulin and Related Hormones". Pub. Walter de Gruyter & Co., Berlin, New York, p. 353.

Di Nunno, L., Modena, G. and Scorrano, C. (1966) Ricerca Sci. 36, 825.

Dipple, I. and Houslay, M.D. (1978) Biochem. J. 174, 179.

Earp, H.S. and Steiner, A.L. (1978) Ann. Rev. Pharmacol. Toxicol. 18, 431.

Edelhoch, H. and Lippolt, R.E. (1969) J. Biol. Chem. 244, 3876.

Eigler, N., Sacca, L. and Sherwin, R.S. (1979) J. Clin. Invest. 63, 114.

Eisentraut, A.M., Olmeda, A., Aquilar-Parad and Unger, R.H. (1968) Diabetes (Suppl. I), 17, 320.

Eisentraut, A.M., Whissen, N. and Unger, R.H. (1968) Am. J. Med. Sci. 255, 137.

Emmelot, P., Bos, C.J., Benedetti, E.L. and Rumke, P.H. (1964) Biochim. Biophys. Acta 90, 126.

Engelhard, V.H.,Esko, J.D., Storm, D.R. and Glaser, M. (1976) Proc. Natl. Acad. Sci. USA, 73, 4482.

Ensinck, J.W., Shepard, C., Doull, R.J. and Williams, R.H. (1972) J. Clin. Endocrinol. Metab. 35, 463.

Epand, R.M. (1971) Can. J. Biochem. 49, 166.

Epand, R.M. (1972) Arch. Biochem. Biophys. 148, 325.

Epand, R.M. (1980) Insulin, Chemistry, Structure and Function of Insulin and Related Hormones (D. Brandenburg and A. Wollmer, eds.) Walter de Gruyter & Co., Berlin, New York, p. 363.

Epand, R.M. and Cote, T.E. (1976) Biochim. Biophys. Acta 453, 365.

Epand, R.M., Jones, A.J.S. and Sayer, B. (1977) Biochem. 16, 4360.

Epand, R.M., Jones, A.J.S. and Schreier, S. (1977) Biochim. Biophys. Acta 491, 296.

Erecinska, M. (1977) Biochem. Biophys. Res. Comm. 76, 495.

Escher, E.H.F. and Guillemete, G. (1979) J. Med. Chem. 22, 1047.

Exton, J.H., Lewis, S.B., Ho, R.-J., Robinson, G.A. and Park, C.R. (1971) Ann. N.Y. Acad. Sci. 185, 85.

Falk, J.E. and Cole, P.W. (1965) J. Biol. Chem. 240, 193.

Fiske, C.H. and Subba Row, Y. (1925) J. Biol. Chem. 66, 375.

Fleet, G.W., Knowles, J.R. and Porter, R.R. (1972) Biochem. J. 128, 499.

Fleet, G.W.J., Porter, R.R. and Knowles, J.R. (1969) Nature 224, 511.

Foa, P.P., Bajaj, J.S. and Foa, W.L. (eds.) (1978) Glucagon, its role in physiology and clinical medicine, Springer Verlag, Berlin.

Fontana, A., Marchiori, F., Moroder, L. and Scoffone, E. (1966a) Tetrahedron Letters 26, 2985.

Fontana, A., Marchiori, F., Rocchi, R. and Pazetta, P. (1966b) Gazz. Chim. Ital. 96, 1301.

Fontana, A., Scoffone, E. and Banassi, C.A. (1968a) Biochem. 7, 980.

Fontana, A., Veronese, F.M. and Scoffone, E. (1968b) Biochem. 7, 3901.

Formisano, S., Johnson, M.L. and Edelhoch, H. (1977) Proc. Natl. Acad. Sci. USA, 74, 3340.

Fouchereau-Person, M., Rancon, F., Freychet, P. and Rosselin, G. (1976) Endocrinol. 98, 755.

Freychet, P. (1976) Diabetologia 12, 83.

Freychet, P., Kahn, C.R., Roth, J. and Neville, D.M. (1972) J. Biol. Chem. 247, 3953.

Galardy, R.E. and Jaimieson, J.D. (1977) Mol. Pharmacol. 13, 852.

Gebhardt, R. and Mecke, D. (1979) Eur. J. Bilchem. 97, 29.

Goding, J.W. (1978) J. Immunol. Methods 18, 183.

Goldstein, S. and Blecher, M. (1976) Methods in Receptor Research (Blecher, M. ed.) Marcel Dekker, Inc., New York and Basel.

Gratzer, W.B. and Beaven, G.H. (1969) J. Biol. Chem. 244, 6675.

Gratzer, W.B., Creeth, J.M. and Beaven, G.H. (1972) Eur. J. Biochem. 31, 505

Gratzer, W.B., Beaven, G.H., Rattle, H.W.E. and Bradbury, E.M. (1968) Eur. J. Biochem. 3, 276.

Grieves, M.F. (1977) Nature 265, 681.

Griffith, I.P. (1972) Biochem. J. 126, 553.

Hanoune, J., Lacombe, M.L. and Pecker, F. (1975) J. Biol. Chem. 250, 4569.

Hanstein, W.G. (1979) Methods in Enzymol. 48, 653.

Hanstein, W.G., Hatefi, Y. and Kiefer, H. (1979) Biochem. 18, 1019.

Hartman, F.C. and Wold, F. (1967) Biochem. 6, 2439.

Hazum, E., Chang, K.J., Schechter, Y., Wilkinson, S. and Cuatrecasas, P. (1979) Biochem. Biophys. Res. Commun. 88, 841

Heding, L.G. (1971) Diabetologia 7, 10.

Henkin, J. (1977) J. Biol. Chem. 252, 4293.

Hess, H.H., Lees, M.B. and Derr, J.E. (1978) Anal. Biochem. 85, 295.

Hew, C.L., Lifter, J., Yoshika, M., Richards, F.F. and Konigsberg, W.H. (1973) Biochem. 12, 4685.

Hill, A.V. (1910) J. Physiol. (London) 40, 190.

Hollenberg, M.D. and Cuatrecasas, P. (1979) The Receptors, a Comprehensive Treatise, Vol. 1, p. 193.

Holohan, K.N., Murphy, R.F., Buchanan, K.D. and Elmore, D.T. (1973) Clin. Chim. Acta 45, 153.

Holst, J.J. (1978) Digestion 17, 168.

Holst, J.J. (1980) Biochem. J. 187, 337.

Holst, J.J. and Aasted, B. (1974) Acta Endocrinol. 77, 715.

Houslay, M.D., Ellory, J.C., Smith, G.A., Hesketh, T.R., Warren, G.B. and Metcalfe, J.L. (1977) Biochim. Biophys. Acta 467, 208.

Houslay, M.D., Dipple, I. and Elliott, K.R.F. (1980) Biochem.J. 186, 649.

Houslay, M.D., Hesketh, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C. (1976b) Biochim. Biophys. Acta 436, 495.

Houslay, M.D., Metcalfe, J.C., Warren, G.B., Hesketh, T.R. and Smith, G.A. (1976a) Biochim. Biophys. Acta 436, 489

Hunter, W.M. (1971) in Radioimmunoassay Methods (Kirkham, K.E. and Hunter W.M. eds.) p. 3, Churchill Livingstone, Edinburgh.

Insel, P.A. (1978) Int. Rev. Biochem. 20, 1.

Iyengar, R. and Birnbaumer, L. (1979) Proc. Natl. Acad. Sci. USA, 76, 3189.

Iyengar, R., Swartz, T.L. and Birnbaumer, L. (1979) J. Biol. Chem. 254, 1119.

Jacobs, S. and Cuatrecasas, P. (1976) Biochim. Biophys. Acta 433, 482.

Jacobs, S. and Cuatrecasas, P. (1977) New Eng. J. Med. 297, 1383.

Jacobs, S., Hazum, E., Schechter, Y. and Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. USA, 76, 4918.

Ji, T.H. (1977) J. Biol. Chem. 252, 1566.

Ji, T.H. (1979) Biochim. Biophys. Acta 559, 39.

Jirousek, L. (1979) in Radioiodination Notes, New England NUclear, p. 6.

Jörgensen, K.H. and Larsen, V.D. (1972) Horm. Metab. Res. 4, 223.

Jurcovicova, S. and Klimes, I. (1978) Radiochem. Radional. Letters 34, 187.

Kahn, R.C., Freychet, P. and Roth, J. (1974) J. Biol. Chem. 249, 2249.

Katzenellenbogen, J.A. and Hsiung, H.M. (1975) Biochem. 14, 1736.

Kessler, S.W. (1975) J. Immunol. 115, 1617.

Kessler, S.W. (1976) J. Immunol. 117, 1482.

Kimura, N. and Nagata, N. (1977) J. Biol. Chem. 252, 3829.

King, T.E. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E. eds.), Acad. Press, New York, Vol. 10, 216.

Kiss, Z. (1978) FEBS Lett. 92, 29.

Klausner, Y.S., McCormic, W.M. and Chaiken, I.M. (1978) Int. J. Peptide Protein Res. 11, 82.

Knowles, J.R. (1972) Acc. Chem. Res. 5, 155.

Krishna, G., Weiss, B. and Brodie,B.B. (1968) J. Pharmacol. Exp. Ther. 163, 379.

Kuehn, L., Meyer, H., Rutschmann, M. and Thamm, P. (1980) FEBS Lett. 113, 189.

Kühle, E. (1973) "The Chemistry of Sulfenic Acids", G. Thiebe Publs., Stuttgart, p. 7.

Kwok, Y.C. and Moore, G.J. (1980) Proc. West. Pharmacol. Soc. 23, 345.

Lad, P.M., Welton, A.E. and Rodbell, M. (1977) J. Biol. Chem. 252, 5942.

Laemmli, U.K. (1970) Nature 227, 680.

Lafuse, W. and Ediddin, M. (1980) Biochem. 19, 49.

Lee, T.T., Williams, R.E. and Fox, C.F. (1979) J.Biol. Chem. 254, 11787.

Lefebvre, P.J. and Unger, R.H. (eds.) (1972) "Glucagon), Pergamon Press, Oxford, p. 61.

Lefkowitz, R.J. (1974) J. Biol. Chem. 249, 6119.

Lefkowitz, R.J., Roth, J. and Pastan, I. (1971) Science 170, 633.

Lesniak, M.A., Roth, J., Gorden, P. and Gavin, J.R.(1973) Nature New Biol. 241, 20.

Levey, G.S. (1975) Metabolism 24, 301.

Levey, G.S., Fletcher, M.A. and Klein, I. (1975) Adv. Cycl. Nucleotide Res. 5, 53.

Levitzki, A. and Helmreich, J.M. (1979) FEBS Lett. 101, 213.

Liljenguist, J.A. and Rabin, D. (1979) J. Clin. Endocrinol. Metab. 49, 937.

Lin, M.C., Nicosia, S. and Rodbell, ,. (1976) Biochem. 15, 4537.

Lin, M.C., Nicosia, S., Lad, P.M. and Rodbell, M. (1977) J. Biol. Chem. 252, 2790.

Lockwood, D.H. and East, L.E. (1979) Diabetes 27, 580.

Londos, C., Salomon, Y., Lin, M.C., Harwood, J.P., Schramm, M., Wolff, J. and Rodbell, M. (1974) Proc. Natl. Acad.Sci. USA, 71, 3087.

Luby, P. and Shinitzky, M. (1979) Biochem. 18, 445.

Martin, R.B., Stein, J.M., Kennedy, E.L. and Doberska, C.A. (1980) Biochem. J. 188, 137.

Martin, R.B., Stein, J.M., Kennedy, E.L., Doberska, C.A. and Metcalff, S.C. (1979) Biochem. J. 184, 253.

Maturo, J.M. and Hollenberg, M.D. (1978) Proc. Natl. Acad. Sci. USA, 75, 3070.

McBride-Warren, P.A. and Epand, R.M. (1972) Biochem. 11, 3571.

Moran, E.C., Chou, P.Y. and Fasman, G.D. (1977) Biochem. Biophys. Res. Comm. 4, 1300.

Munger, B.L. (1972) "Glucagon", (Lefebvre, P.J. and Unger, R.H., eds.) Pergamon Press,, p. 7.

Muramoto, K. and Ramachandran, J. (1980) Biochem. 19, 3280.

Murlin, J.R., Glough, H.D., Gibbs, C.B.F. and Stokes, A.M. (1923) J. Hel. Chem. 56, 253.

Murov, S.L. (1973) Handbook of Photochemistry, Dekker, New York.

Nakayama, H., Nazawa, M. and Kanaoka, Y. (1979) Chem. Pharm. Bull. 27, 2775.

Neville, D.M. (1968) Biochim. Biophys. Acta 154, 540.

O'Keefe, E. and Bennett, V. (1980) J. Biol. Chem., 255, 561.

Orr, W.L. and Kharash, N. (1953) J. Am. Chem. Soc. 75, 60[redacted]

Ottensmeyer, F.P. and Korn, A.P. (1979) XIth International [redacted]ngress of Biochemistry, Toronto, Abstracts, p. 189.

Panijpan, B. and Gratzer, W.B. (1974) Eur. J. Biochem. 45, 547.

Parker, A.J. and Kharash, N. (1960) J. Am. Chem. Soc. 82, 3071.

Patel, D.S. (1970) Macromolecules 3, 448.

Pavazzola, M., Siperstein, A., Moody, A.J., Sundby, F., Jacobsen, H. and Orci, L. (1979) Endocrinol. 105, 499.

Pfeuffer, T. (1977) J. Biol. Chem. 252, 7224.

Pimental, E. (1978) Acta Cient. Venez. 29, 147.

Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1970) Fed. Proc. 29, 602.

Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971a) J. Biol. Chem. 246, 1849.

Pohl, S.L., Kraus, H.M.J., Birnbaumer, L. and Rodbell, M. (1971b) J. Biol. Chem. 246, 1861.

Pollock, H.G. and Kimmel, J.R. (1975) J. Biol. Chem. 250, 9377.

Pullen, R.A., Lindsay, D.G., Wood, S.P., Tickle, I.J., Blundell, T.L., Wollmer, A., Kraie, G., Brandenburg, D., Zahn, H., Glemann, J. and Gammestoft , S. (1976) Nature 259, 369.

Rall, T.W. and Sutherland, E.W. (1962) J. Biol. Chem. 237, 1228.

Ramachandran, J., Canova-Davis, E. and Behrens, C. (1979) Proc. Int. Cong. Biochem., 11th, Abstracts, 606.

Ramachandran, J., Muramoto, K., Kenez-Keri, M., Keri, G. and Buckley, D.I. (1980) Proc. Natl. Acad. Sci. USA, 77, 3967.

Rodbell, M. (1972) "Glucagon", (Lefebvre, P.J. and Unger, R.H, eds.), p. 5.

Rodbell, M. (1973) Fed. Proc. 32, 1854.

Rodbell, M. (1980) Nature 284, 17.

Rodbell, M., Lin, M.C. and Salomon, Y. (1974) J. Biol. Chem. 249, 59.

Rodbell, M., Birnbaumer, L., Pohl, S.L. and Sundby, F. (1971a) Proc. Natl. Acad. Sci. USA, 68, 909.

Rodbell, M., Kraus, H.M.J., Pohl, S.L. and Birnbaumer, L. (1971b) J. Biol. Chem. 246, 1872.

Rodbell, M., Lin, M.C., Salomon, Y., Londos, C., Harwood, J.P., Martin, B.R., Rendell, M. and Berman, M. (1975) Adv. Cycl. Nucleotide Res. 5, 3.

Ross, E.M., Haga, T., Howelett, A.C., Schwarzmein, J., Schleifer, L.S. and Gilman, A.G. (1978) Adv. Cycl. Nucleotide Res. 9, 53.

Roth, J. (1973) Metabolism 22, 1059.

Rubalcava, B. and Rodbell, M. (1973) J. Biol. Chem. 248, 3831.

Rubalcava, B. and Rodbell, M. (1973) J. Biol. Chem. 248, 6239.

Rupley, J.A. (1969) "Biological Macromolecules", Timasheff, S.N., Fasman, G.D, Marcel Dekker, Vol. 2, p. 291.

Salomon, Y. and Rodbell, M. (1975) J. Biol. Chem. 250, 7245.

Salomon, Y., Lin, M.C., Londos, C., Rendell, M. and Rodbell, M. (1975) J. Biol. Chem. 250, 4239.

Sands, H. and Richenberg, H.V. (1978) Int. Rev. Biochem. 20, 45.

Sasaki, K., Dockerill, S., Adamiak, D.A., Tickle, I. and Blundell, T.- (1975) Nature 257, 751.

Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660.

Schaffhausen, R.S., Silver, J.E. and Benjamin, T.L. (1978) Proc. Natl. Acad. Sci. USA, 75, 79-83.

Schlegel, W., Kempner, E.S. and Rodbell, M. (1979) J. Biol. Chem. 254, 5168.

Schneider, A.B. and Edelhoch, H. (1972) J. Biol. Chem. 247, 4986.

Scoffone, E., Fontana, A. and Rocchi, R. (1968) Biochem. 7, 971.

Schwyzer,R. (1963) Pure Appl. Chem. 6, 268.

Segrest, J.P., Jackson, K.L., Andrews, E.P. and Marchesi, V.T. (1971) Biochem. Biophys. Res. Comm. 44, 390.

Senyk, G., Nitecki, D. and Goodman, J.W. (1971) Science 171, 407.

Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133.

Soman, V. and Felig, P. (1978) J. Clin. Invest. 61, 552.

Sonne, P., Berg, T. and Christoffersen, T. (1978) J. Biol. Chem. 253, 3203.

Sonne, O. and Gliemann, J. (1977) Biochim. Biophys. Acta 499, 269.

Sperling, M.A., Ganguli, S., Voina, S., Kaptein, E. and Nicoloff, J.T. (1980) Endocrin. 107, 684.

Srere, P. and Brooks, G.X. (1969) Arch. Biochem. Biophys. 129, 708.

Srikant, C.B., Freeman, D., McCorkle, K. and Unger, R.H. (1977) J. Biol. Chem. 252, 7434.

Stadel, J.M., Goodman, D.B. P., Galardy, R.E. and Rasmussen, H. (1978) Biochem. 17, 1403.

Staros, J.V., Bayley, H., Standring, D.N. and Knowles, J.R. (1978) Biochem. Biophys. Res. Comm. 80, 568.

Staub, A., Sinn, L. and Behrens, O.K. (1953) Science 117, 628.

Staub, A., Sinn, L. and Behrens, O.K. (1955) J. Biol. Chem. 214, 619.

Stephenson, R.P. (1956) Brit. J. Pharmacol. 11, 379.

Storm, D.R. and Chase, R.A. (1975) J. Biol. Chem. 250, 2539.

Sundby, F. and Markussen, J. (1971) Horm. Metab. Res. 3, 184.

Sundby, F. and Markussen, J. (1972) Horm. Metab. Res. 4, 56.

Sundby, F., Frandsen, E.K., Thomsen, J., Kristiansen, K., and Brundpeedt, K. (1972) Horm. Metab. Res. 26, 289.

Sutherland, E.W., Rall, T.W. and Menon, T. (1962) J. Biol. Chem. 237, 1220.

Sutherland, E.W., Robinson, G.A. and Butcher, R.W. (1968) Circulation 37, 279.

Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462.

Swann, J.C. and Hammes, G.G. (1969) Biochem. 8, 1.

Tae, H.J.I. (1977) J. Biol. Chem. 252, 1566.

Tager, H.S. and Markese, J. (1979) J. Biol. Chem. 254, 2229.

Tager, H.S., Hohenboken, M. and Markese, J. (1977) Endocrinol. 100, 367.

Tell, G.P., Haour, F. and Saez, J. (1978) Metabolism 27, 1566.

Tolkovsky, A. and Levitzki, A. (1978) Biochem. 17, 3795.

Unger, R.H., Eisentraut, A.M., McCall, M.S., Keller, S., Lanz, H.C. and

Madison, L.L. (1959) Proc. Soc. Exp. Biol. Med. 102, 621.

Unger, R.H., Ketterer, H. and Eisentraut, A.M. (1966) Metabolism 15, 865.

Unger, R.H., Srere, P., Bromer, W.W. and Felig, P. (eds.) (1976) Glucagon Symposium, Metabolism 25 (Supple. 1), Grune and Stratton, New York.

Unger, R.H. and Orci, L. (1976) Physiol. Rev. 56, 778.

Veronese, F.M., Boccu, E. and Fontana, A. (1968) Ann. Chim. (Rome) 58, 1309.

Veronese, F.M., Boccu, E. and Fontana, A. (1970) Int. J. Protein Res. 11, 67.

Vinick, A.I. and Hardcastle, A. (1974) Horm. Metab. Res. 6, 396.

Vogel, A.I. (1956a) Practical Organic Chemistry, Third Ed., Wiley, New York, N.Y. p. 577.

Vogel, A.I. (1956b) Practical Organic Chemistry, Third Ed., Wiley, New York, N.Y. p. 581.

Von Schenck, H. (1977) Clin. Chim. Acta 80, 455.

Von Schenck, H. and Jeppson, J.-O. (1977) Biochim. Biophys Acta 491, 503.

Von Schenck, H., Larsson, I. and Thorell, J.I. (1976) Clin. Chim. Acta 69, 225.

Wagman, M.E., Dobson, L.M. and Karplus, M. (1979) Abstract. Am. Chem. Soc. 178th Natl. Meet., Washington, D.C.

Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1977) J. Biol. Chem. 252, 5947.

Welton, A.F., Lad, P.M., Newby, A.C., Yamamura, H., Nicosia, S. and Rodbell, M. (1978) Biochim. Biophys. Acta 522, 625.

White, A.A. and Karr, D.B. (1978) Anal. Biochem. 85, 451.

Wieland, T. and Sarges, R. (1962) Ann. 685, 181.

Wisher, M., Baron, M.D., Jones, R.H. and Sönksen, P.H. (1979) Biochem. Biophys. Res. Comm. 92, 492.

Wold, F. (1972) Methods in Enzymol. 25B, 623.

Wood, S.P., Blundell, T.L., Wollmer, A., Lazarus, M.R. and Neville, R.W.J. (1975) Eur. J. Biochem. 55, 531.

Wu, C.-S.C. and Yang, J.T. (1980) Biochem. 19, 2117.

Wünsch, E. and Weinges, K.F. (1972) "Glucagon", (Lefebvre, P.J. and Unger, R.H., eds.), Pergamon Press, p. 31.

Yeung, C.W.T., Moule, M.L. and Yip, C.C. (1980) Biochem. 19, 2196.

Yip, C.C., Yeung, C.W.T. and Moule, M.L. (1978) J. Biol. Chem. 253, 1743.

Yip, C.C., Yeung, C.W.T. and Moule, M.L. (1980) Biochem. 19, 70.

Yoshika, M., Lifter, J., Hew, C.L., Convese, C.A., Armstrong, M.Y.K., Konigsberg, W.H. and Richards, F.F. (1973) Biochem. 12, 4679.

Yu, N.T. and Liu, C.S. (1972) J. Am. Chem. Soc. 94, 127,