RAT LIVER NUCLEAR ENVELOPE INSULIN BINDING AND ITS EFFECTS ON ENDOGENOUS PROTEIN KINASES

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of the requirements for the
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Certification of Supervisor

In terms of paragraph eight of "General regulations for the degree of PhD." I as supervisor of the candidate, G.P. Sabbatini, certify that I approve of the incorporation into this thesis of the material that has already been published or submitted for publication.

Signed by candidate

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and UCT - FRD Research Centre for
Molecular Biology.

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I dedicate this thesis to Carol.

Scope of the Thesis

The postulated model for the insulin - stimulated induction of mRNA efflux (Purrello et al., 1983) is based on the demonstrated binding of insulin to intracellular membrane structures (see chapter 2, section 2.2.1), and the *in vitro* effect of insulin on nuclear envelope phosphorylation, NTPase activity, and mRNA efflux (see chapter 5, section 5.1). These independent observations have led to the development of a model for the direct induction by insulin, at the level of the nucleus, of mRNA efflux (figure I.1). However, the specific intracellular insulin binding has been inferred from kinetic or morphological studies which have not identified a discrete membrane - bound polypeptide(s) as an insulin docking molecule *in situ* (Goldfine, 1981). Also, the stimulation of NTPase activity has only been established by monitoring the level of general ATP hydrolysis of nuclear envelope fractions in the presence and absence of insulin (Purrello et al., 1983).

The scope of this thesis has been to further the understanding of this mechanism by attempting to a) unequivocally identify a specific nuclear envelope - associated insulin docking polypeptide *in situ* and b) to demonstrate that insulin directly affects the ATP - binding of nuclear envelope ATP - binding proteins. The latter would demonstrate a primary effect of insulin i.e. the modulation of the ATP - binding capacity of identified NTPases / protein kinases (or their release from some inactive storage form), and not a general phenomenon such as elevated ATP -

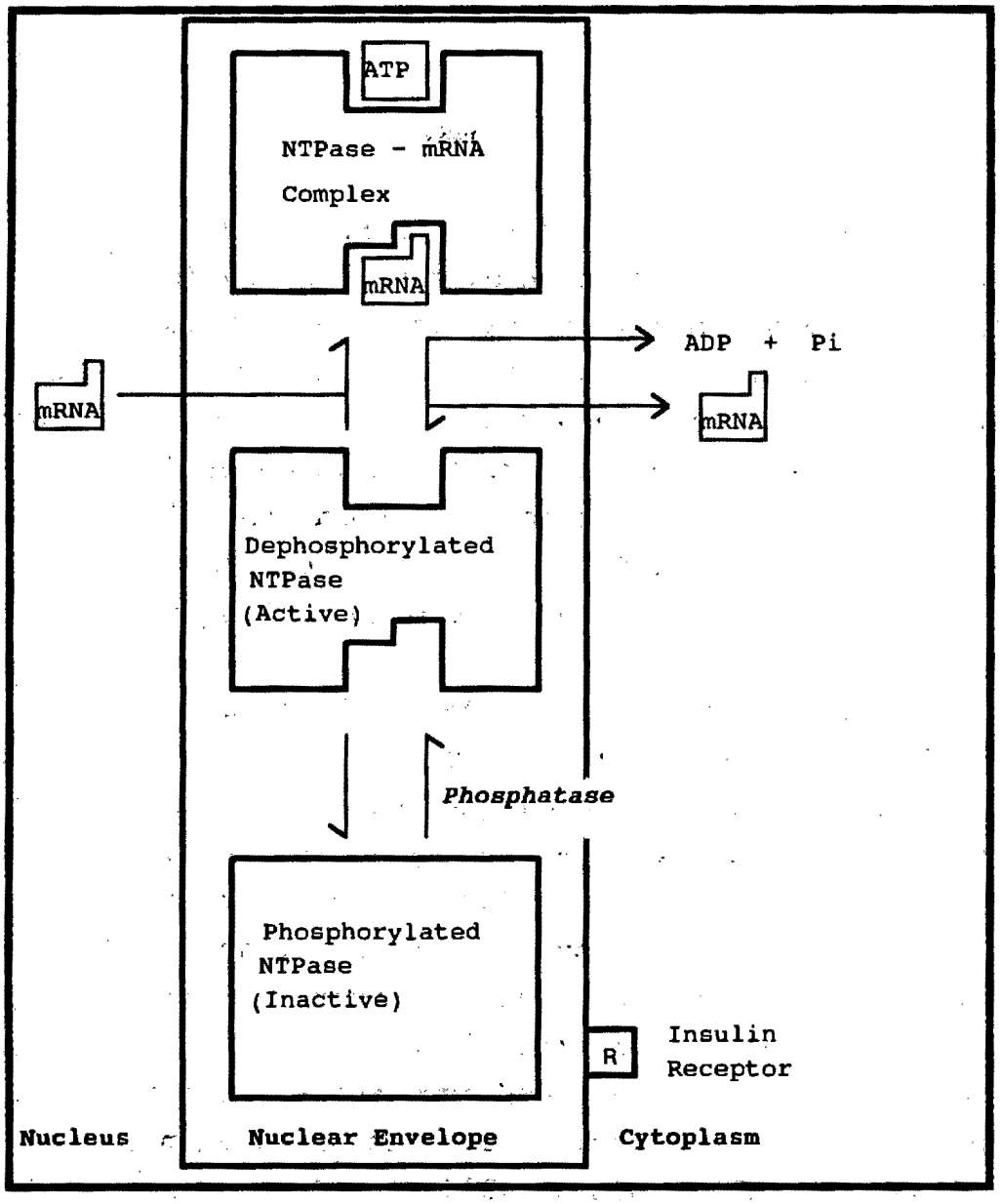

Figure I.1 The Proposed Mechanism for Insulin - Induced mRNA Efflux from the Nucleus (Purrello et al., 1983).

Insulin binding to a putative specific nuclear envelope receptor results in a general stimulation of phosphatase activity. One of the targets of this increased phosphatase activity is the inactive phosphorylated NTPase, which upon dephosphorylation, has an increased affinity for both ATP and mRNA. The consequence of this is the increase in both mRNA nucleocytoplasmic translocation and ATP hydrolysis.

Abbreviations

BOPA	2-tert-butoxy-carbonyloximino-2-phenylacetonitrile
BSA	Bovine serum albumin
cAMP	3',5' cyclic adenosine monophosphate
DMF	Dimethyl formamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase 1	Deoxyribonuclease 1
EDTA	Disodium ethylenediamine-tetraacetate
GHRC	Glucocorticoid hormone receptor complex
HPLC	High pressure liquid chromatography
IEF	Isoelectric focussing
mRNA	Messenger ribonucleic acid
N_3	Azido moiety
NEPHGE	Nonequilibrium pH gradient electrophoresis
PAGE	Polyacrylamide gel electrophoresis
PBS	Dulbeccos phosphate buffered saline
SASD	(Sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3-dithiopropionate
SDS	Sodium dodecyl sulfate
TA	Triamcinolone acetamide
TCA	Trichloroacetic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
U.V.	Ultra violet
WGA	Wheat germ agglutinin

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Chapter 1

The Synthesis of Carrier - Free [^{125}I]SASD - Derivatised Insulin and its Use in Identifying Putative Intracellular Insulin Receptors.

1.1 The Synthesis of Carrier - Free [^{125}I]ASD-Insulin

1.1.1 Introduction

Insulin has been reported to have direct effects at the level of the nucleus (see 4.1) and evidence has been published for specific insulin binding to isolated nuclei (see 1.2.1). These *in vitro* binding studies are corroborated by *in situ* results demonstrating the internalisation of insulin (see 1.2.1).
Wong et al. (1988) have isolated and characterised an insulin binding protein from nuclei but it would be of interest to demonstrate specific insulin binding to protein(s) in intact intracellular components. Such a demonstration would corroborate previous findings that insulin has a direct effect on nuclear activity. This chapter describes the strategy employed to probe for putative insulin receptors *in vitro* and *in situ*. Briefly, a carrier - free, radioactive, photoactivatable insulin derivative was synthesised and characterised for both integrity and biological activity (1.1.2). This derivative was then used to probe for insulin docking proteins (1.2.2).

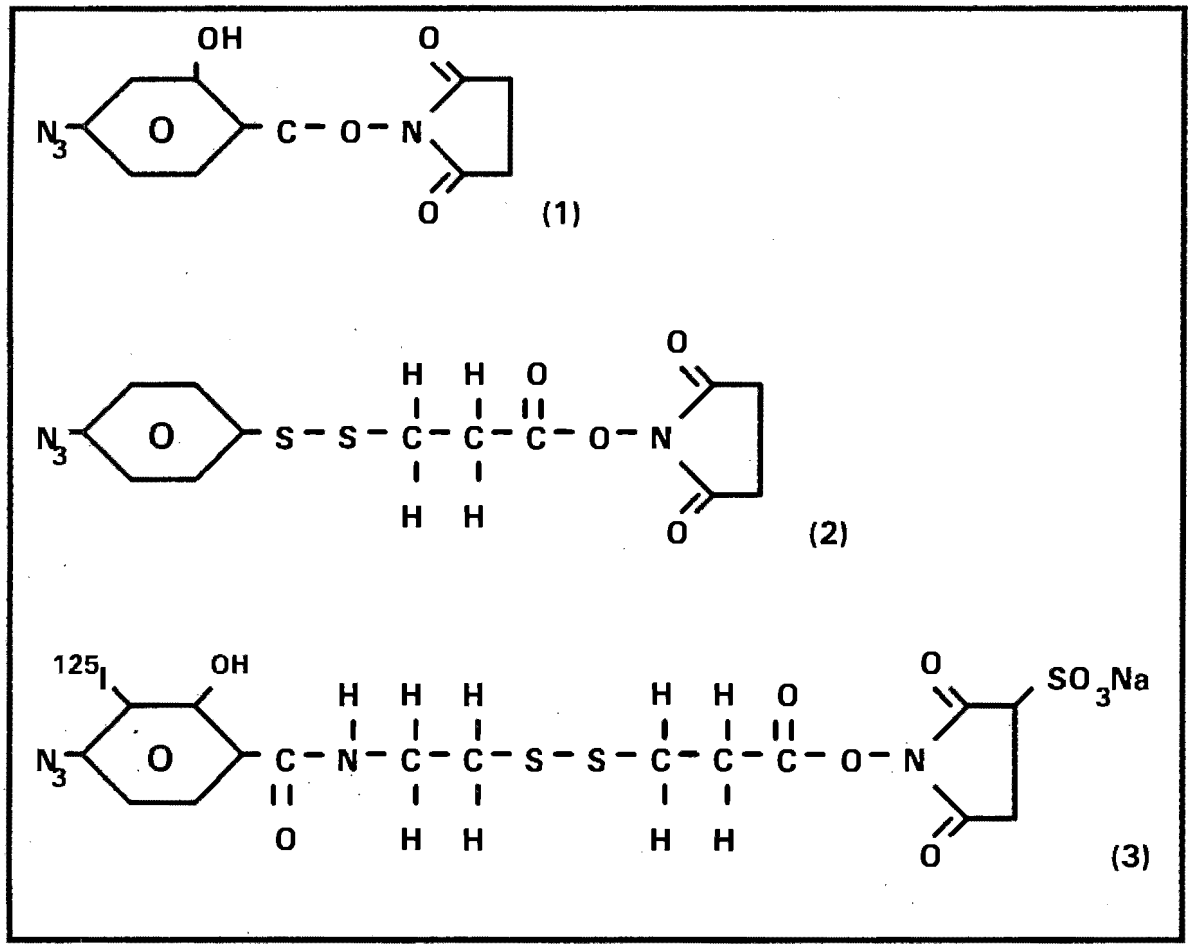


Figure 1.1.1 The Molecular Structure of Some Photoactivatable Heterobifunctional Reagents.

These examples illustrate the general structural characteristics of photoactivatable heterobifunctional reagents. Most consist of a photoactivatable phenyl azide moiety which is iodinatable at the meta position in the case of SASD (3) and a hydroxysuccinimide ester moiety for the coupling to an appropriate ligand. The length of the linker arm can be varied and may contain a cleavable disulfide bond as depicted in (2) and (3).
1) NHS-ASA (N-hydroxysuccinimide ester of 4-azidosalicylic acid.
2) NHS-APDP (N-hydroxysuccinimide ester of 3-((4-azidophenyl)dithio)propionimidate.
3)SASD, (sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3-dithiopropionate.

1.1.1.1 Photoactivatable Heterobifunctional Reagents

Recently, considerable development of photoactivatable heterobifunctional derivatives to investigate protein - protein interactions has taken place (Vanin & Ji, 1981; Ji & Ji, 1982; Jaffe et al., 1980; Knutson, 1987; Lowndes et al., 1988).
The reagents consist of a hydroxysuccinimide ester moeity and an iodinatable phenyl azide group (figure 2.1.1) which is photoactivatable. The resultant nitrene reacts to form a covalent bond with a neighbouring protein. The great advantage of these reagents is that they are chemically inert until photoactivated, thus allowing considerable freedom in the design of experiments. Thus, by coupling the reagent to a ligand via the hydroxysuccinimide ester, it is possible to identify molecules with which the ligand is interacting by simply photoactivating the azido moeity. The resultant activated nitrene then reacts with the molecule, thus covalently crosslinking the interacting molecules.
Of special interest is the use of iodinatable, cleavable reagents such as SASD (sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate), which after crosslinking the ligand to its relevant counterpart, can be cleaved by the addition of a reducing agent. This results in the dissociation of the ligand and the radioactive tagging of the interacting molecule. This has proved to be invaluable in the study of subunit - subunit and ligand - receptor interactions.

Amongst other uses these reagents have been employed to label glycopeptides (Ji, 1977; Shephard et al., 1988); to study ribosomal subunit interactions (Maassen, 1979); to localise and identify prostaglandin and toxin receptors (Kattelman et al., 1987; Lima et al., 1988); and to study the identity, localisation, and internalisation of peptide

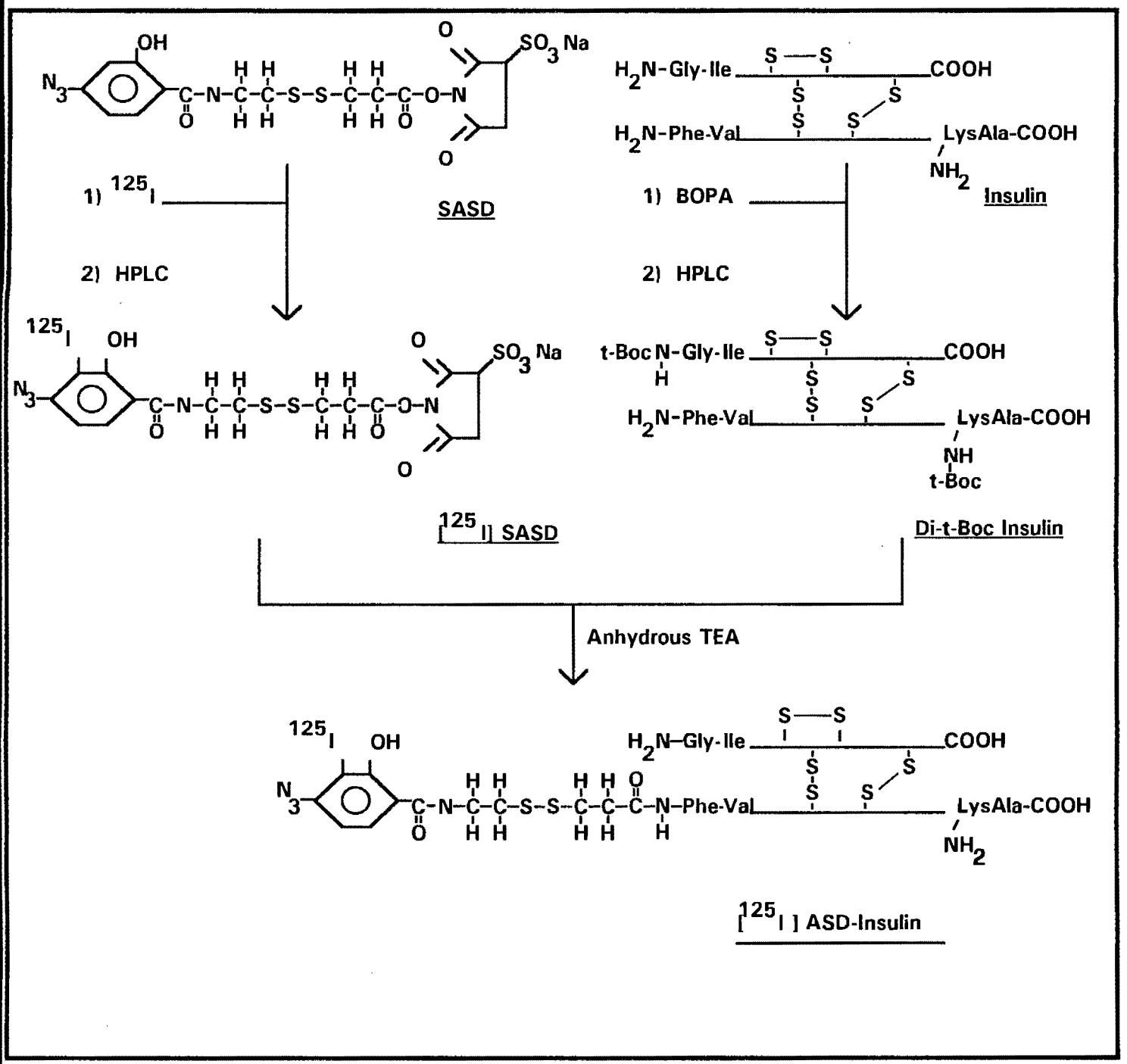


Figure 1.1.2 Protocol for the Synthesis of Carrier - Free [^{125}I]ASD-Insulin.

hormone and neuronal receptors (Horowitz et al., 1988; Kramer et al., 1988; Nikolics et al., 1988; Niznik et al., 1988; Ricard et al., 1988; Whiting & Lindstrom, 1987). Of special interest is the use of these reagents to investigate the identity and internalisation of the insulin receptor (Podlecki et al., 1987; Hofmann et al., 1981; Yip et al., 1980; Knutson, 1987).

1.1.1.2 The Derivatisation of Insulin with SASD

There are a number of important considerations when synthesising a photoactivatable insulin derivative:

1) The insulin should be derivatised in such a way as to limit the loss of biological activity either as a result of perturbing the tertiary structure of the hormone or by modifications of essential amino acid side chains during derivatisation.
2) Radiolabelling of the derivatised insulin should be on the reagent only and not the insulin molecule itself.
3) The derivatised insulin should be of the maximum specific activity i.e. preferably carrier - free so that even low abundance receptors may be detected.
4) The derivatised insulin preparation should be free of any uncoupled radioactive reagent which would indiscriminately label upon photolysis.

The protocol adopted for the synthesis of carrier - free [^{125}I]ASD-insulin is illustrated in figure 1.1.2 and was based on the above - mentioned considerations.
The photoreactive reagent SASD was chosen to derivatise insulin because it is iodinatable and cleavable, ideal

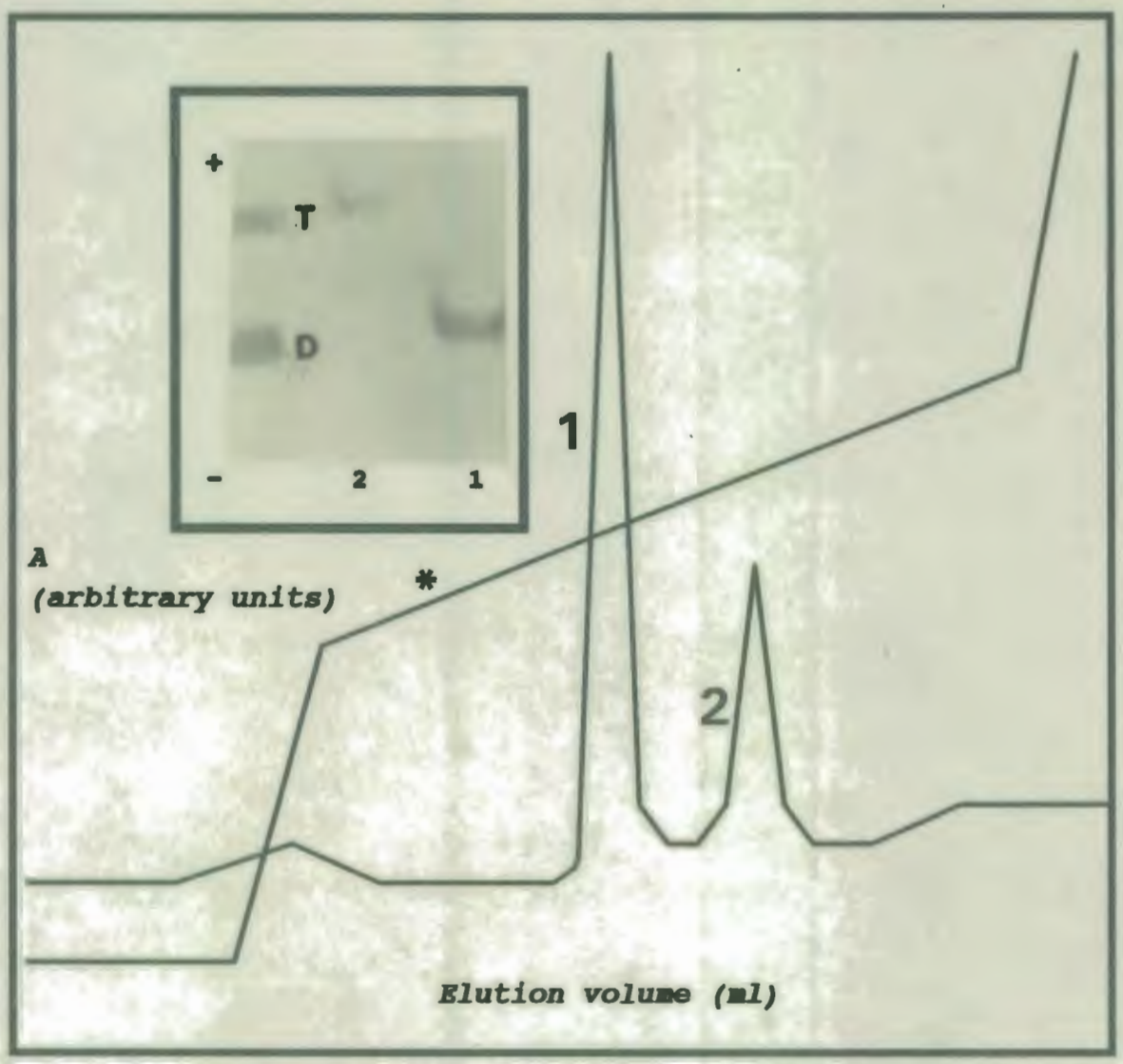


Figure 1.1.3 **HPLC Separation of Boced-Insulin Products**

Insulin was derivatised with BOPA as described in 6.2.2 and the products subjected to HPLC.
Peaks 1 and 2 were collected, lyophilised and subjected to acid-urea PAGE for positive identification (inset)
Inset: The lanes 1 and 2 represent peaks 1 and 2 respectively. The standards lane consists of diboc-insulin (D) and triboc-insulin (T).

* *denotes the position at which insulin elutes.*

properties to probe for insulin receptors.
It was considered essential that the photoaffinity moiety be iodinated prior to coupling to insulin. If done after coupling to insulin, as described by Knutson (1987), undesireable iodination of the insulin may occur. Indiscriminate radioiodination of insulin has been demonstrated to result in considerable loss of biological activity (Fraenkel - Conrat & Fraenkel - Conrat, 1950) and diiodination of tyrosine is thought to deactivate insulin (Linde & Hansen, 1974). Also, iodination of the affinity labelled insulin by using chloramine - T may result in various monoiodinated species of insulin, which have been shown to have different affinities for the insulin receptor (Podlecki et al., 1983). Thus, as shown in figure 1.1.2 only the SASD was iodinated, and after separation from noniodinated SASD, coupled to insulin.

The methodology to derivatise the β chain of insulin at its N - terminal was established previously in this laboratory by Grant and von Holt (1987) resulting in a biologically fully active derivative. Thus it was decided to couple carrier - free [^{125}I]SASD to B1 only. There are potentially 3 coupling sites for SASD: phe B1; gly A1; and lys B29. To ensure coupling to B1 only, the insulin was derivatised to (N-Boc-gly^{A1}, N^{6}-Boc-lys^{B29})insulin prior to coupling to the SASD reagent.

1.1.2 Results

1.1.2.1 The Synthesis of (N-Boc-Gly^{A1}, N^{6}-Boc-Lys^{B29})Insulin

The diboc insulin was synthesised essentially as described by Geiger et al. (1971) except that the bocing agent was 2-tert-butoxy-carbonyloximino-2-phenylacetonitrile (BOPA).

The resultant product was analysed by reverse phase HPLC (figure 1.1.3) and the various fractions collected, lyophilised, and subjected to acid - urea PAGE. It is apparent from figure 1.1.3 that the predominant product (peak 1) is diboc-insulin.
Quantitation of the products revealed that the diboc-insulin constituted approximately 80% of the product, a figure very similar to that reported by Geiger et al. (1971). Of interest is the fact that the reaction ran to completion under these conditions, since no insulin remained (figure 1.1.3).

There are theoretically two forms of diboc-insulin, differing in the residues which are derivatised with BOPA. Irrespective of reaction conditions employed, the gly A1 is always derivatised so the two possible diboc-insulins are (N-boc-gly^{A1}, N^{6}-boc-lys^{B29})insulin or (N-boc-gly^{A1}, N-boc-phe^{B1})insulin. It was thus essential to confirm that the former derivative is the major product as shown by Geiger et al. (1971). The diboc-insulin was thus subjected to the first two steps of Edman degradation (figure 1.1.4). The first cleavage step (4a) produced a predominance of phenylalanine (1) and trace amounts of glycine (2) indicating that only phe B1 is unboced. The second cleavage step (4b) produced glycine A1 and valine B2 and some

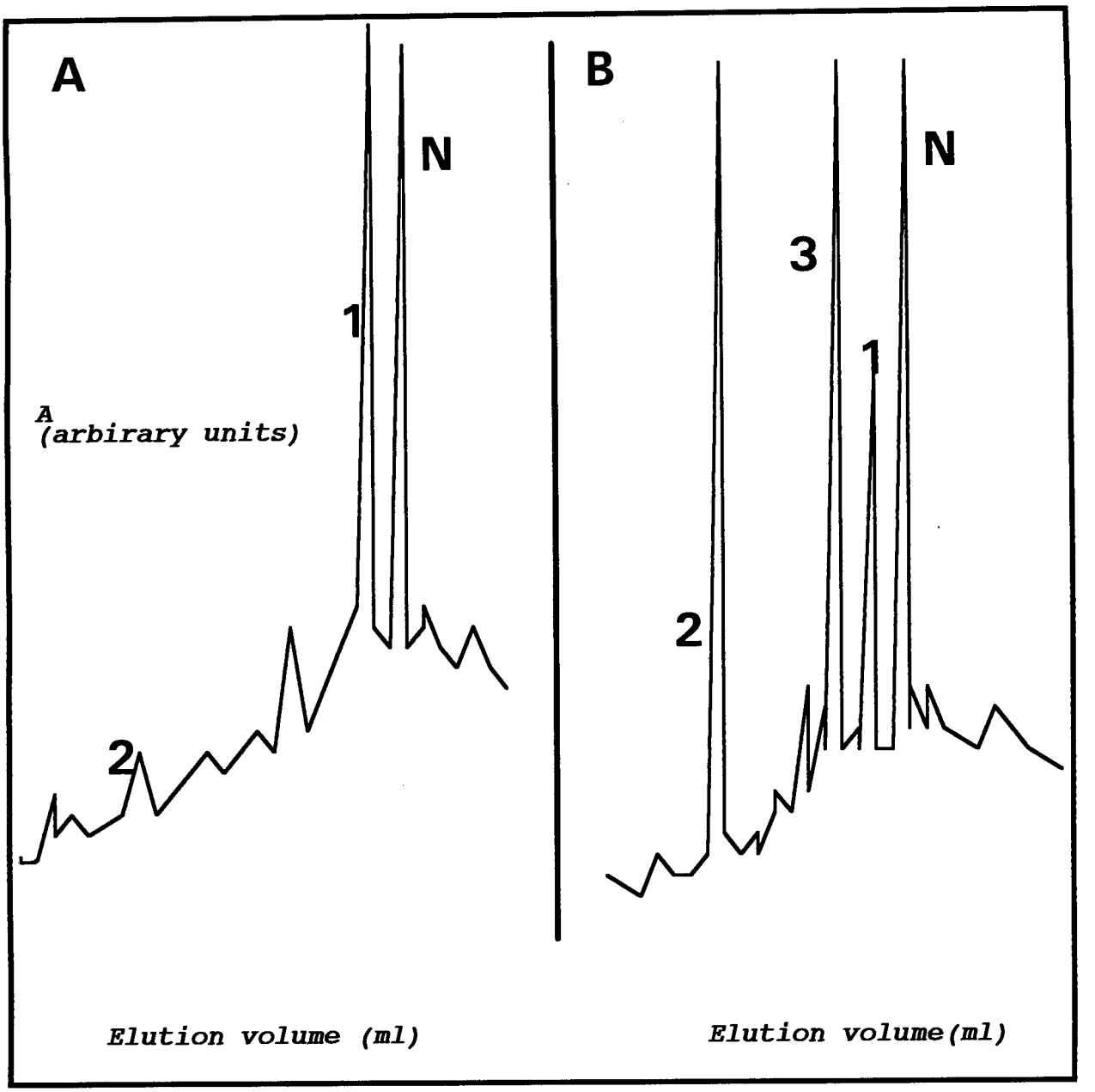


Figure 1.1.4 Edman Degradation of Diboc-Insulin to Establish the Identity of Peak 1 as (N-Boc-Gly^{A1}, N-Boc-Lys^{B29})Insulin.

A quantity of the lyophilised peak 1 fraction was subjected to the first (A) and second (B) steps of Edman degradation to ascertain whether phe-B1 was boced. The peaks are phenylalanine (1), glycine (2), and valine (3). The internal standard (N) is norleucine. This analysis was kindly undertaken by Prof. Brandt and Dr. Rodrigues.

phenylalanine carry - over due to insufficient coupling in the first step. The glycine cleavage results from the debocing of this residue during the first cleavage step. The results in figure 1.1.4 conclusively show that the synthesised diboc-insulin is (N-boc-gly^{A1}, N-boc-lys^{B29})insulin.

1.1.2.2 The Synthesis of Carrier - Free [^{125}I]SASD

1.1.2.2.1 The Iodination of SASD

The iodination of a cleavable, photoactivatable heterobifunctional reagent should be performed under conditions which do not alter the azido moiety, the disulphide bridge, or the hydroxysuccinimide ester moiety. The latter two functions are theoretically vulnerable to reducing agents such as mercaptoethanol, whereas the ester moiety may be susceptible to hydrolysis under aqueous conditions.
Despite reports that SASD has been iodinated in the presence of reducing agents (Knutson, 1987) and an aqueous enviroment (Shephard et al., 1988) it was deemed prudent to undertake the iodination in anhydrous conditions in the total absence of reducing agents. Thus SASD was dissolved in DMSO (10 mg / ml) and iodinated using iodogen (1,3,4,6 - tetrachloro-3α,6α - diphenylglycouril) instead of the commonly used chloramine - T method which requires the addition of reducing agent to terminate the reaction. Iodogen was coated onto the walls of the reaction vessels by the addition of 1 ml of a 1 mg / 50 ml chloroform solution to the vessels and the subsequent evaporation of the solvent.

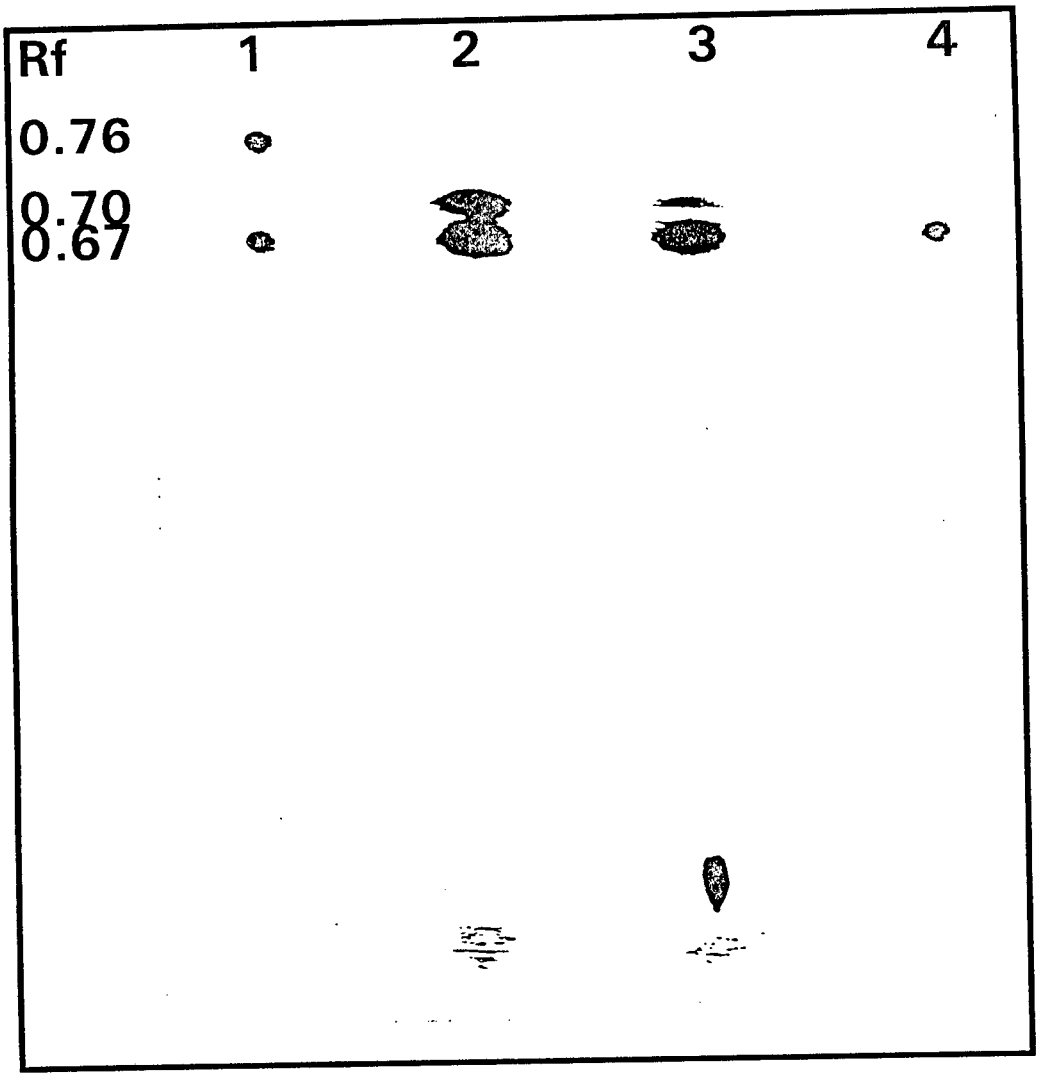


Figure 1.1.5. TLC Analysis of Iodinated SASD.

SASD (50 µg / 30 µl of DMSO) was iodinated with 5 µl of ^{125}I (5 mCi / 50 µl) for 1 minute in an iodogen tube as described in 6.2.5. After iodination a predetermined quantity was applied to a TLC plate and the remainder incubated with 100µl of 0.2 M glycine, pH 10.0 for 10 minutes. On completion of incubation a predetermined amount was applied to the TLC plate and run in a solvent of benzene:chloroform:ethyl acetate:acetic acid (1:1:1:0.1). The developed plate was then autoradiographed (typically for 20 minutes).
Lane 1: 3 µg of unlabelled SASD
Lane 2: iodinated SASD
Lane 3: iodinated SASD incubated with 0.2 M glycine, pH 10.0 for 10 minutes.
Lane 4: unlabelled SASD incubated with 0.2 M glycine, pH 10.0 for 10 minutes.

Typically, 50 μg of SASD was iodinated in an iodogen tube with 3 μl of ^{125}I (5 mCi / 50 μl, Amersham) for 1 minute. Analysis of the iodinated products by thin layer chromatography (figure 1.1.5) revealed the presence of two iodinated products (lane 2) with Rf values of 0.70 and 0.67. The unlabelled SASD also contains two products with Rf values of 0.76 and 0.66. These values differ quite markedly from those of Shephard et al. (1988) who reported Rf values of 0.48 and 0.36 for unlabelled SASD and 0.42 and 0.38 for iodinated SASD. This discrepancy may result from the fact that these authors did not use analytical grade solvents for the TLC solvent system (personal communication). Different amounts of water and / or acetic acid in the solvent system may affect the mobilities of the SASD components.

Typically, approximately 70% of input ^{125}I was incorporated into the SASD constituents and of this 60% was incorporated into the component with a Rf value of 0.70.

To determine which of components is the intact ester and not the hydrolysed ester i.e. 2-(p-azidosalicylamido)-1,3-dithiopropionate (Shephard et al., 1988), the iodinated SASD was reacted with 0.2 M glycine, pH 10.0. Any ester present would be subjected to nucleophilic attack by glycine at the hydroxysuccinimide ester. As indicated in figure 1.1.5 (lanes 3 & 4) there is a decrease in the components with Rf values 0.76 and 0.70 (mainly in the former) and a concomitant increase in a product with a very low mobility - this probably represents the glycine derivative. The radioactive component with Rf 0.67 also decreased but to a smaller, and variable extent (figure 1.1.6, lane 3). From this it was concluded that both iodinated components are reactive, but that the more mobile derivative is the principal intact ester component of SASD, an observation previously made by Shephard et al. (1988). This

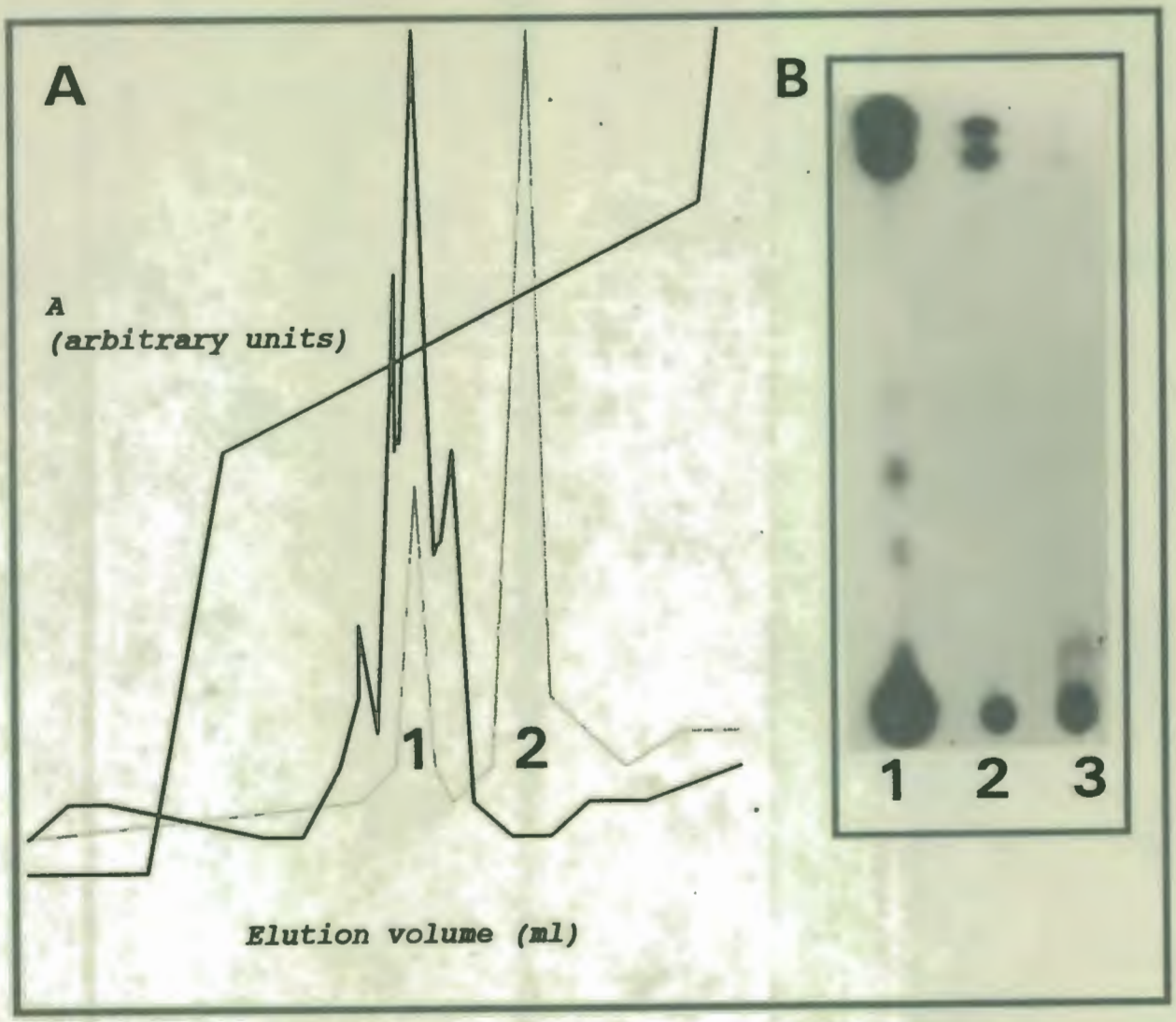


Figure 1.1.6. The Separation of [^{125}I]SASD from Unlabelled SASD by Reverse Phase HPLC.[4]

SASD was iodinated and subjected to reverse phase HPLC on a C18 column (figure 1.1.6 A) as described in 6.2.5. The elution of radioactivity was monitored by means of a Geiger counter and collected as 1 ml fractions (dotted line). After lyophilisation the fractions were resolubilised in a minimum volume of DMSO and pooled with the other fractions of the same peak. The pooled peaks were then subjected to rotary evaporation to remove the DMSO and the dried peaks stored at -20°C until required.
An aliquot of Peak 2 was subjected to TLC and autoradiography as described in figure 1.1.5. Figure 1.1.6 B is a typical result although for any given column it was

found that there was a gradual increase in origin - bound radioactivity throughout the entire elution range with sustained column use. This is most probably due to a gradual leaching of ^{125}I that had bound to the column.

Lane 1: unfractionated [^{125}I]SASD
Lane 2: peak 2
Lane 3: peak 2 incubated with 0.2 M glycine, pH 10.0.

experiment also provides evidence that the ester moiety survives the iodination procedure. It is apparent that the second component is a contaminant of commercially available SASD (figure 1.1.5, lane 1), possibly the free acid, and not a result of the iodination procedure.
Besides this contaminant there is evidence that the SASD consists of a mixture of differently charged species (section 1.1.2.4).

1.1.2.2.2 Preparing Carrier - Free [^{125}I]SASD

The iodinated SASD preparation consists of a mixture of mainly ^{125}I, [^{125}I]SASD, and underivatised SASD. In order to synthesise a carrier - free insulin derivative it is thus necessary to purify the [^{125}I]SASD.
Initial attempts at separating out the [^{125}I]SASD from the contaminants involved the use of LH20 column chromatography in absolute ethanol but this system proved not to have the required resolving power to separate ^{125}I from [^{125}I]SASD. Next, reverse - phase HPLC using a C18 column was attempted in order to separate out the unlabelled SASD from the [^{125}I]SASD. Using the conditions described in figure 1.1.6 it was possible to perform this separation. This procedure also removes any trace amounts of ^{125}I which eluted in the flow - through. The radioactive fractions were pooled and subjected to thin layer chromatography to ascertain their identities (figure 1.1.6 b). The radioactive fraction which coelutes with the unlabelled SASD does not contain any labelled SASD and its identity is unknown. However, peak 2 consists of [^{125}I]SASD , confirming that the [^{125}I]SASD is separable to a carrier - free state.

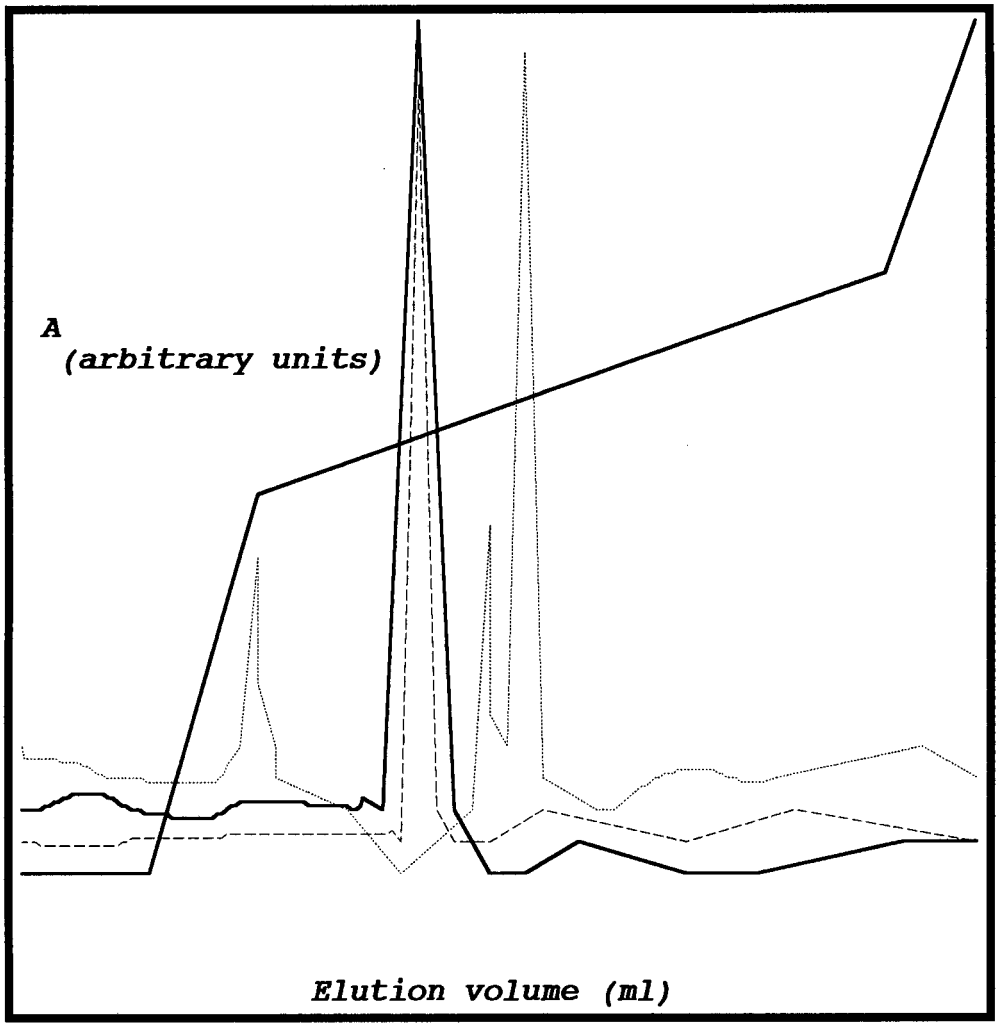


Figure 1.1.7. HPLC Analysis of Bovine Insulin Subjected To Coupling Conditions in Order to Ascertain Possible Disulfide Bond Exchange.

100 µg of bovine insulin was dissolved in 60 µl coupling medium (DMSO:TEA in a ratio of 300:0.16) and incubated for 16 hours at 37 ℃. The sample was then subjected to HPLC analysis as described in 6.2.3.
Shown on the same figure are native bovine insulin dissolved in DMSO and subjected to HPLC (solid line); bovine insulin subjected to the coupling conditions(dashed line); and bovine insulin dissolved in coupling medium containing 5 % ß-mercaptoethanol and boiled for 10 minutes, followed by cooling in the presence of air (dotted line).

To ascertain whether the ester moiety was intact after this purification procedure, an aliquot of peak 2 was reacted with 0.2 M glycine, pH 10.0 for 10 minutes. As is evident from figure 1.1.6 b, lane 3, the ester does react i.e. the ester is not destroyed in the separation process.
The penalty for obtaining carrier - free [^{125}I]SASD was the loss of approximately half of the [^{125}I]SASD due to irreversible adsorption by the column. Attempts to diminish losses at this step proved unsuccessful.
Routinely the relevant fractions were pooled, lyophilised, and stored at -20°C until required.

1.1.2.3 The Coupling of Carrier - Free [^{125}I]SASD to Diboc-Insulin

In order to preserve the integrity of the SASD ester moiety it was decided to undertake the coupling in DMSO, thereby eliminating the risk of hydrolysis which an aqueous medium would pose. The coupling was allowed to proceed overnight at 37°C for convenience, despite initial evidence indicating that maximum coupling was achieved within 4 hours.

Of importance in the DMSO coupling medium is the amount of base required to ensure that the amine on the insulin remains unprotonated for the reaction. At the same time the conditions should be such that disulphide bond exchange in the insulin molecule does not occur. To optimise the coupling yield, various ratios of DMSO to triethylamine (TEA) were tested. For 100 μg diboc-insulin as the TFA salt per 60 μl coupling medium, DMSO : TEA ratios of 300 : 0.10 and 300 : 0.16 (v/v) were found to be optimal.

Disulfide bond exchange within the diboc-insulin under these

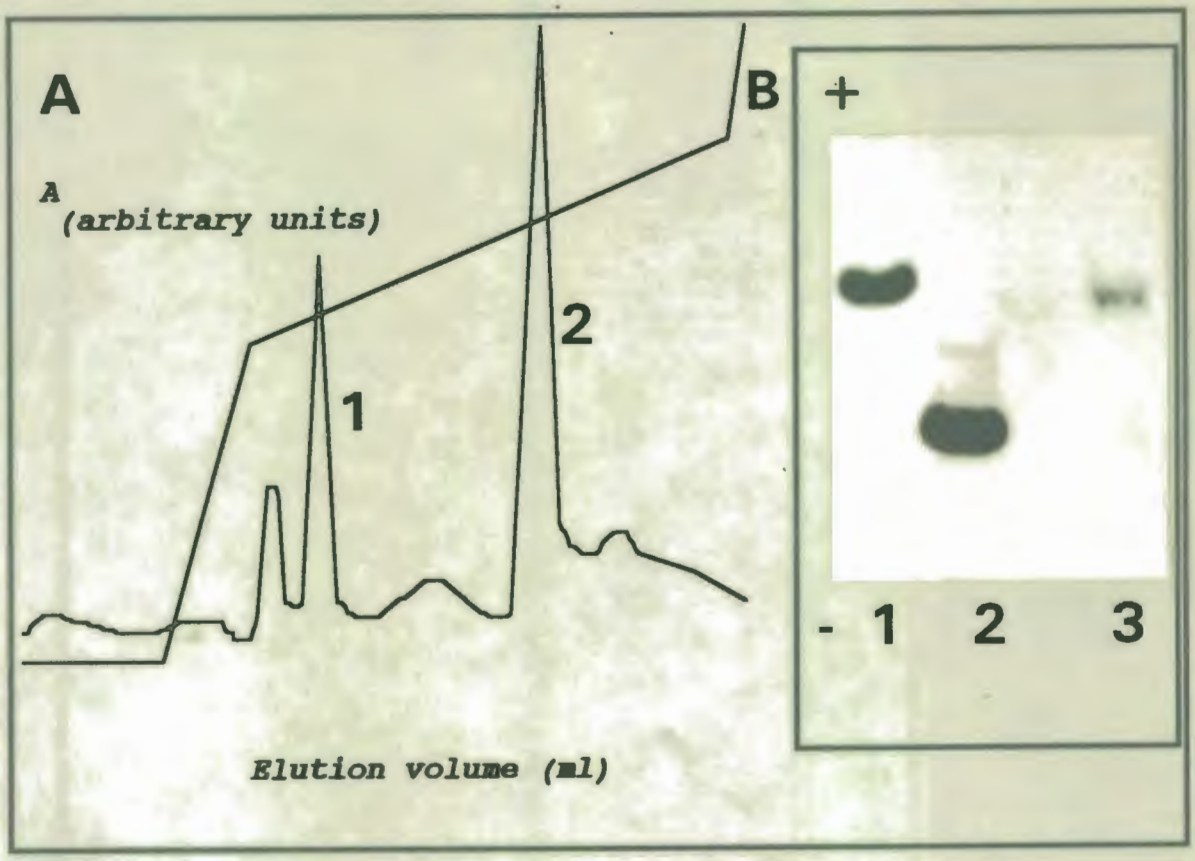


Figure 1.1.8. HPLC Analysis of Coupling Products.

200 µg diboc-insulin was incubated with 40 µg unlabelled SASD as described in 6.2.3. Upon completion of coupling, the reaction medium was evaporated by rotary evaporation and 50 µl of anhydrous TFA added to facilitate debocing. After 3 minutes, the TFA was evaporated and the residue redissolved in 40 µl DMSO and subjected to HPLC separation (figure 1.1.8 A).
1ml fractions of peak 2 were collected, lyophilised and identity confirmed by acid - urea PAGE (figure 1.1.8 B).
Lane 1: diboc-insulin
Lane 2: insulin
Lane 3: peak 2
Peak 1 is uncoupled insulin.

conditions was investigated by incubating diboc-insulin in these media for 16 hours at 37°C. Subsequent HPLC analysis revealed no change in retention time (figure 1.1.7). Intermolecular or intramolecular rearrangement would probably have resulted in the formation of heteropolymers with different elution profiles. It was thus concluded that the insulin molecules are not affected by exposure to these conditions.

On completion of coupling, the reaction cocktail would consist of coupled [^{125}I]ASD-diboc-insulin, [^{125}I]SASD, and uncoupled diboc-insulin. It was decided to deboc the products prior to purification of the photoactivatable product ([^{125}I]ASD-insulin) as this would minimise the number of manipulations.
Initial experiments to define optimal conditions for the synthesis of SASD derivatised insulin in the DMSO / TEA buffer system and to test the separation of the reaction products were undertaken using unlabelled SASD as described in figure 1.1.8. The decrease in the amount of insulin (peak 1) and concurrent appearance of peak 2 served to monitor the coupling yield. The presence of insulin (peak 1) and total absence of residual diboc-insulin indicates that the 3 minute debocing procedure is sufficient.
Confirmation that peak 2 is derivatised insulin was obtained by acid - urea PAGE (figure 1.1.8 b), where the derivative having the same number of charged groups as diboc insulin exhibits a similar mobility.
The determination of which DMSO : TEA ratio to use (300 : 0.10 or 300 : 0.16) was done by coupling carrier - free ^{125}I]SASD to diboc-insulin and determining the degree of incorporation by thin layer chromatography (figure 1.1.9). Derivatised insulin remains at the origin thus allowing direct determination by scraping and counting. This value was corrected to take into account the small amount of

protocol step	% radioactivity incorporated	% radioactivity recovered
SASD iodination(1)	70%	/
HPLC(2)	/	36%
Coupling(3)	75%	/
HPLC(4)	/	40%

Table 1.1.1. Summary of the Efficiency of Recovery of Radioactivity at Each Step in the Synthesis of Carrier - Free [^{125}I]SASD - Derivatised Insulin.

% incorporation refers to the percentage of input ^{125}I incorporated into SASD(1) and the percentage of input carrier - free [^{125}I]SASD coupled to insulin(3).

% recovery refers to the percentage of input [^{125}I]SASD recovered as carrier - free product after HPLC purification(2) and the percentage of input [^{125}I]ASD-insulin recovered as carrier - free product after HPLC purification(4).

radioactivity (presumably ^{125}I) at the origin in lane 3. It was found that the coupling efficiency was 66% and 75% for the DMSO : TEA ratio of 300 : 0.10 and 300 : 0.16 respectively. Thus subsequent coupling procedures were executed at the latter ratio.

Using the optimised conditions and the finalised protocol illustrated in figure 1.1.2, carrier - free [^{125}I]SASD was coupled to diboc-insulin and the deboced product purified by HPLC (figure 1.1.10 a). The identity of the radioactive peak as carrier - free [^{125}I]ASD-insulin was confirmed by acid - urea PAGE (figure 1.1.10 b), the radioactive band (lane 4) having very similar mobility to that of unlabelled derivatised insulin (cf. figure 1.1.8 b).
Table 1.1.1 summarises the efficiency of transfer of ^{125}I along the synthetic pathway. The data indicate that approximately 8% of input ^{125}I is recoverable as carrier - free [^{125}I]ASD-insulin.

The possibility that the SASD may couple to other amino acid residues such as histidine was investigated by incubating triboc-insulin with carrier - free [^{125}I]SASD under the established coupling conditions. If coupling occurs only at the 3 residues (phe B1; gly A1; and lys B29) then triboc-insulin should not couple. Any observed coupling would indicate that other residues are reactive toward SASD. Any such spurious coupling may have serious implications in terms of the conformation of the derivatised insulin.
The amount of coupling to triboc-insulin was found to be less than 4% (compared to 75% coupling to diboc insulin (table 1.1.1) and it is possible that this represents the coupling of SASD to trace amounts of contaminating diboc-insulin, since the diboc- and triboc-insulins elute closely on HPLC (figure 1.1.3). It was thus concluded that the coupling of SASD occurs only at phe-B1.

Figure 1.1.9. Determination of Optimal Coupling Conditions.

Carrier - free [^{125}I]SASD was reacted with diboc-insulin in coupling media of differing DMSO:TEA ratios: either 300 : 0.10 or 300 : 0.16. Equal amounts of the coupled mixtures were then subjected to TLC and subsequent autoradiography. The relevant spots were scraped and counted to determine the degree of coupling.
Lane 1: coupling products coupled in DMSO : TEA (300 : 0.10).
Lane 2: coupling products coupled in DMSO : TEA (300 : 0.16).
Lane 3: carrier - free [^{125}I]SASD (post HPLC purification).
Lane 4: unfractionated iodinated SASD.

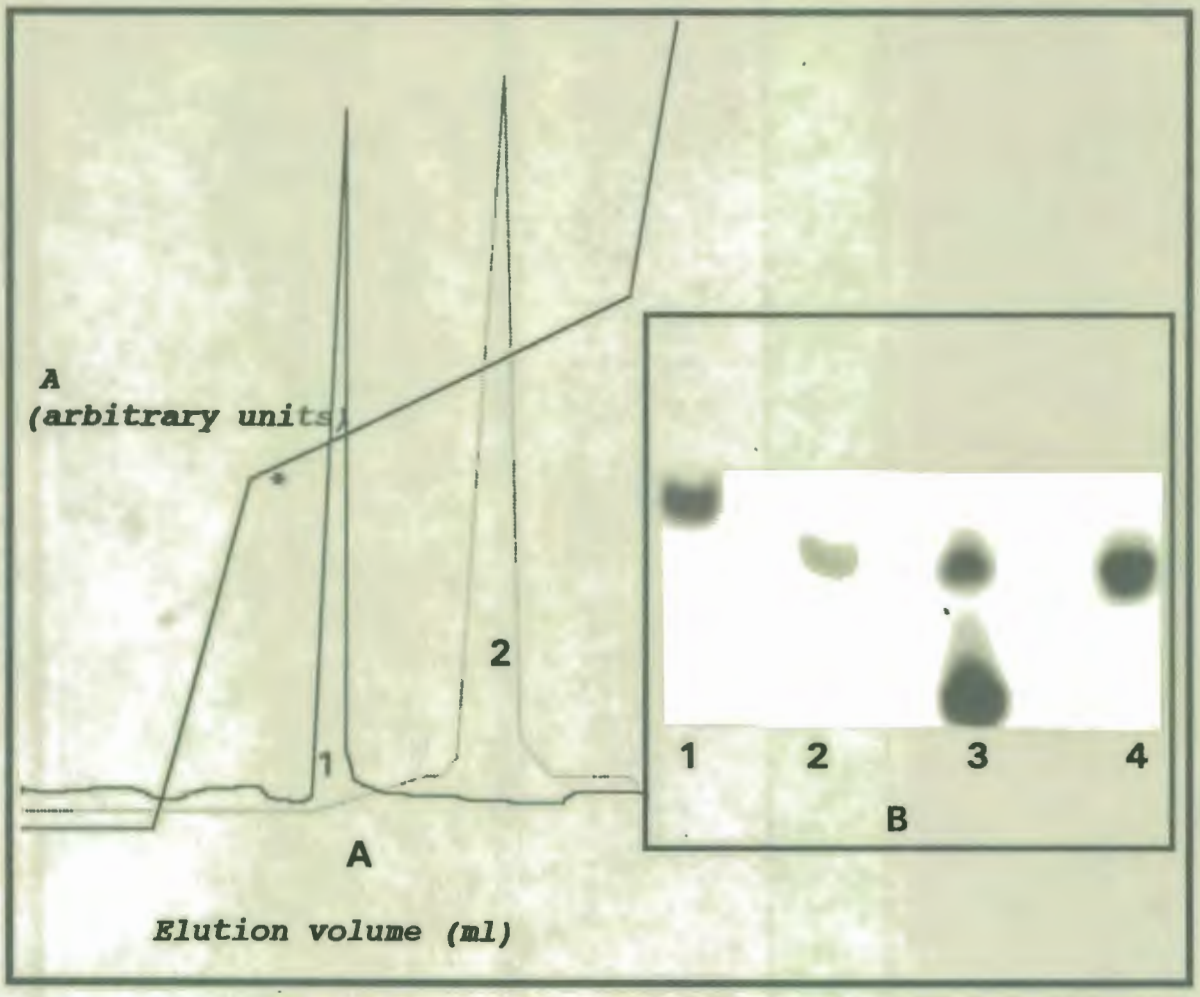


Figure 1.1.10. HPLC Separation of [^{125}I]SASD - Derivatised Insulin from Underivatised Insulin

Diboc-insulin was reacted with carrier - free [^{125}I]SASD, deboced, and subjected to HPLC. Figure 1.1.10 A represents a typical separation profile (the dotted trace representing the radioactive profile) and is consistent with that obtained for non - radioactive SASD - derivatised insulin (see figure 1.1.8.). The asterisks (*) indicates the position at which unlabelled SASD - derivatised insulin elutes. 1 ml fractions of the major radioactive peak (peak 2) were collected, pooled and after lyophilisation, analysed by acid - urea PAGE (figure 1.1.10 B).
Lane 1: iodinated triboc - insulin standard.
Lane 2: iodinated diboc - insulin standard.
Lane 3: iodinated mixture of insulin, mono -, di -, and tri - boc insulins.
Lane 4: radioactive peak 2.

1.1.2.4 SASD Heterogeneity

Occasional heterogeneity of the synthesised ASD-insulin was observed, a typical example of which is illustrated in figure 1.1.11. Analysis by acid - urea PAGE revealed that these fractions differ in charge (inset). This phenomenon does not appear to be a function of the coupling conditions since coupling undertaken at DMSO : TEA ratios of 300 : 0.10 and 300 : 0.16 gave the same result. That this was not due to rearrangement of insulin under the coupling conditions is demonstrated in figure 1.1.7. Also discounted is the possible modification of insulin (eg deamination) during any one of the subsequent synthesis steps. As can be seen in figure 1.1.12, the charge of the insulin molecule is unaffected by any reagent with which it comes into contact during the modification reactions.
However, the use of a new C18 reverse phase column allowed the separation of SASD into various components (figure 1.1.13 a). The ratio of 1 : 2 : 3 : 4 was found to be 1 : 6.25 : 25 : 2.5 based on the absorbance at 260 nm. This heterogeneity was found to be identical for different batches purchased over a period of 24 months. Coupling of diboc-insulin with these separated components and subsequent acid - urea PAGE provided evidence that it is indeed the heterogeneity of the SASD which is responsible for the heterogeneity of the final insulin derivative (figure 1.1.13 b). Of interest is the observation that peaks 1 and 3 produce derivatives of the same charge. It is only peak 2 which differs. The lower bands in lanes 1, 3, and 4 represent uncoupled insulin which is still present owing to the fact that after coupling, the products were not subjected to HPLC. The very low coupling yield of fraction 4 may be the result of the comparatively small amount of ester input.
Iodination of the various SASD components reveals that all

contain the desired ester in different amounts, although peak 2 contains two readily iodinatable species with Rf values between 0.43 and 0.48 (figure 1.1.13 c). This is of interest since it is the insulin derivatised with peak 2 which differs in charge. Iodination of SASD "out of the bottle" also results in species with Rf values 0.40 - 0.50, indicating that this is a contaminant and does not result from any modification during use. The precise nature of these species is unknown and in the interest of minimising manipulations it was decided not to attempt to separate them out prior to derivatising insulin. This decision was based on TLC evidence that these species do not couple to diboc-insulin since there is no decrease in the species after coupling (figure 1.1.9, lanes 1 & 2). The possibility that these species are generated from the ester during TLC is excluded, since there is no concomitant increase of these species with increase in the amount of ester (figure 1.1.9, lanes 3 & 4). Spectroscopic analysis of the fractionated peaks in figure 1.1.13 a reveal that the various components of SASD have different absorption maxima (figure 1.1.14 a). However, exposure to ultraviolet light reduced the absorption of all fractions, indicating that all components are photoactivatable (figure 1.1.14 b - e). The absorption maxima and level of photoactivation are consistent with the findings of Wollenweber & Morrison (1985) for unfractionated SASD.

In conclusion, charge heterogeneity of the SASD - derivatised insulin is not a result of modifications during synthesis but is rather a reflection of the inherent heterogeneity of the SASD itself. Fractionation of the SASD prior to coupling to insulin was deemed to be unnecessary since all the components can couple to insulin, are iodinatable, and are photoactivatable.

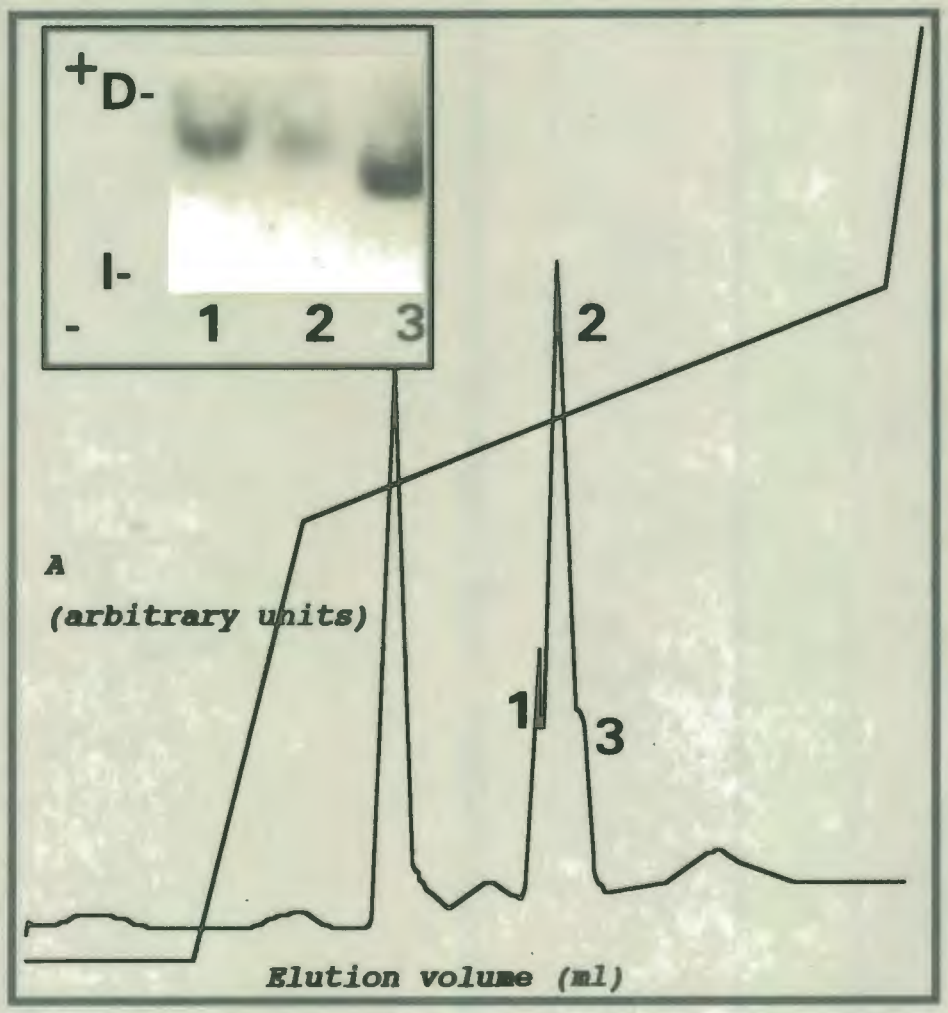


Figure 1.1.11. HPLC Demonstration of the Heterogeneity Of SASD - Derivatised Insulin.

Unlabelled SASD - derivatised insulin was synthesised (6.2.3) and subjected to HPLC. 1 ml fractions of the product peak were collected and the fractions corresponding to the sections 1, 2, and 3 of the peak pooled separately. After lyophilisation the pooled fractions were subjected to acid - urea PAGE (inset). The PAGE lane designations correspond to peak sections. Standards were diboc-insulin (D) and insulin (I).

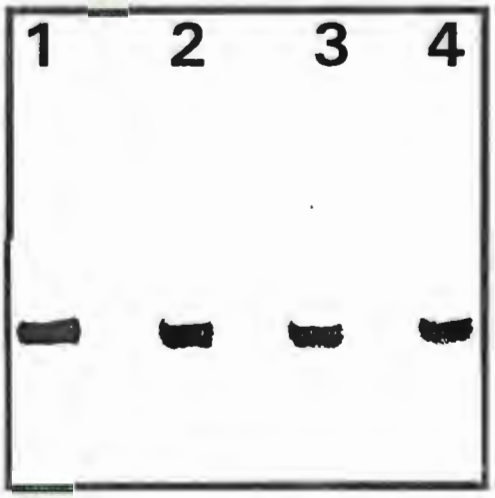


Figure 1.1.12. Acid Urea PAGE of Bovine Insulin Exposed To all the Solvents Used in the Synthesis of SASD - Derivatised Insulin.

100 µg of bovine insulin was subjected to various solvents under the identical conditions that are encountered during the synthesis, and then subjected to acid - urea PAGE to determine whether any charge changes had occurred.
Lane 1: insulin + DMSO
Lane 2: insulin + DMSO : TEA (300 : 0.16)
Lane 3: insulin + ether
Lane 4: insulin + TFA

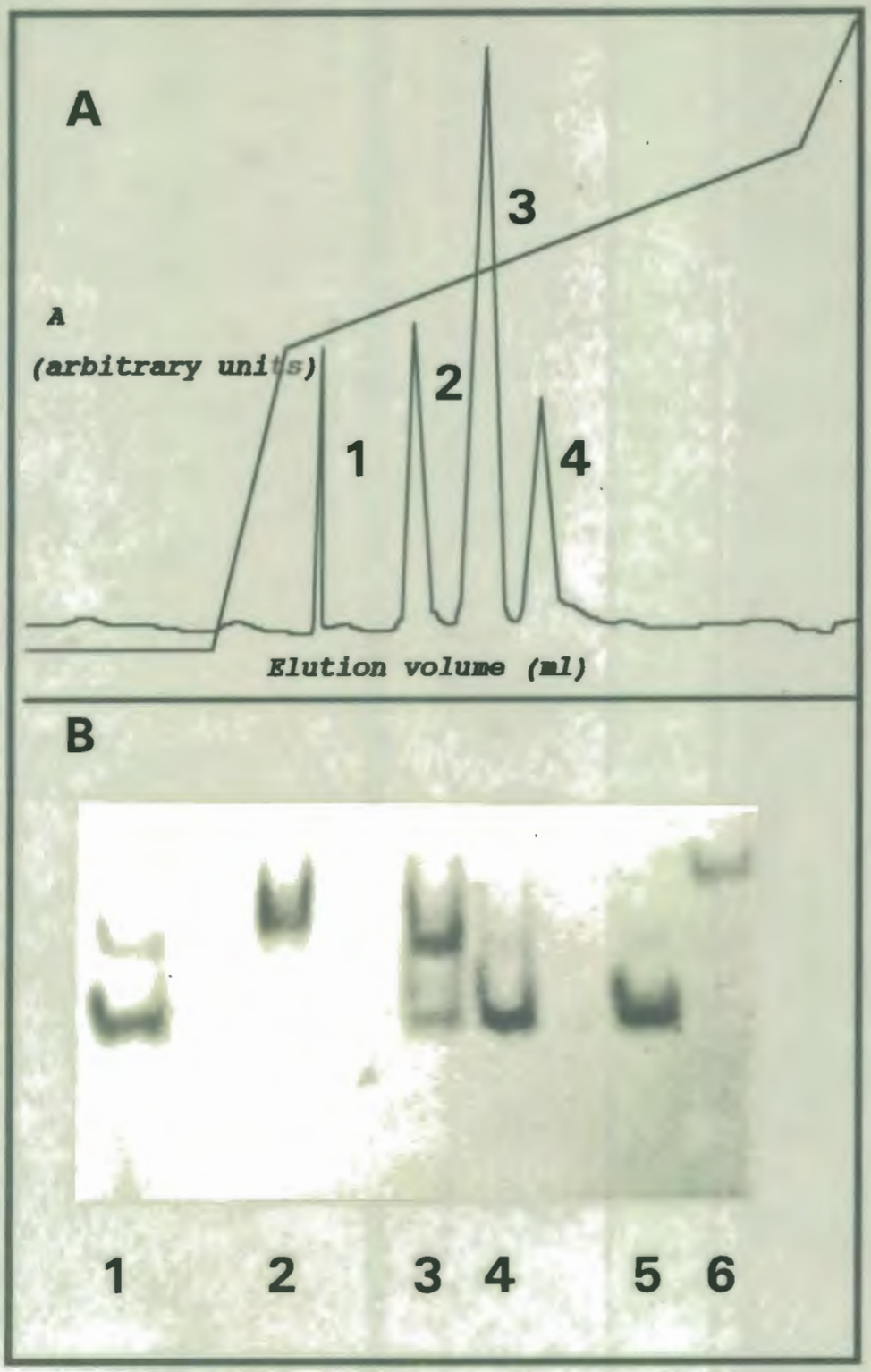

A
3
A
(arbitrary units)
2
1
4
Elution volume (ml)
B
1
2
3
4
5
6

Figure 1.1.13. Separation of SASD Into its Various Components and Their Subsequent Coupling to Diboc-Insulin.

SASD was subjected to HPLC using a C18 column with larger plate number and a modified gradient profile (figure 1.1.13 A). The peaks were collected and pooled with corresponding peaks from successive runs and lyophilised.
The various fractions were then used to derivatise diboc-insulin in the usual manner. After debocing, the samples were subjected to acid - urea PAGE (figure 1.1.13 B). The lane numberings correspond to peak designations in figure 1.1.13 A.

Lane 5: insulin standard.
Lane 6: diboc - insulin standard.
Prior to coupling an aliquot of each peak was iodinated and the products analysed by TLC (figure 1.1.13 C). Lane designations are as in figure 1.1.13 A.

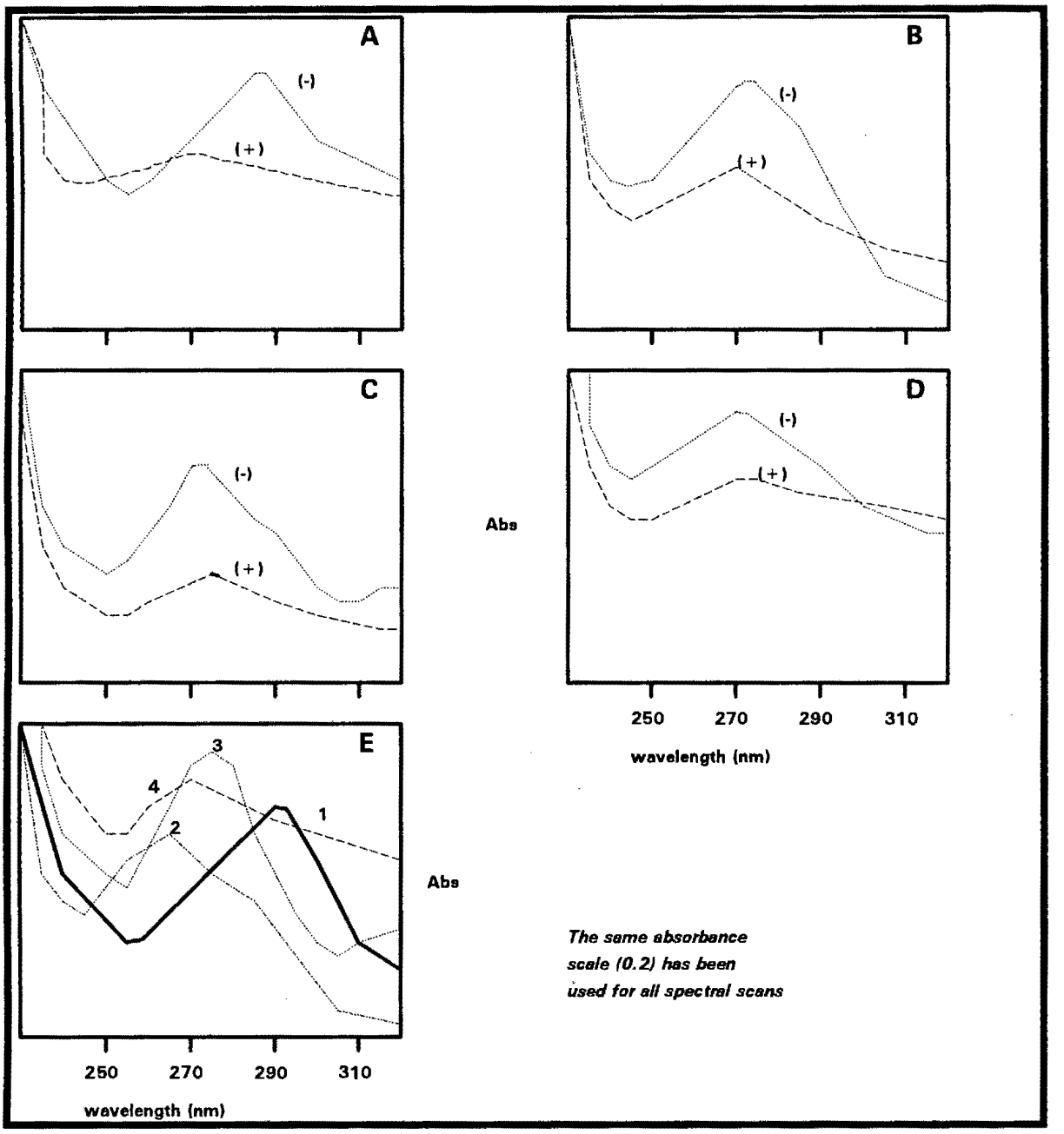


Figure 1.1.14 The Photolability of the Components of SASD.

SASD was separated out into its various components as described for figure 1.1.13 A. The separated fractions were lyophilised and then solubilised in a minimum of DMSO and then made to 1 ml with phosphate buffered saline (PBS), pH 7.4. The various peaks were scanned from 230 nm - 360 nm. The peaks were scanned before (-) and after (+) photoflashing. The results for peaks 1 - 4 (figure 1.1.13 A) are represented in A - D respectively. A direct comparison of the absorption spectra of the various components prior to photoflashing is shown in E. The peak designations correspond to the separated peaks in figure 1.1.13 A.

1.1.2.5 The Biological Activity of the Synthesised Carrier-Free [^{125}I]ASD-insulin

Before using the synthesised insulin derivative to probe for intracellular insulin binding sites it was important to establish its biological activity with respect to native insulin. This was done by determining the derivative's ability to bind plasma membrane - bound receptor *in vivo*; to induce known insulin target genes *in situ*; and to induce hypoglycaemia in mice (a reflection of the overall insulin effect).

The autoradiogram in figure 1.1.15 is typical of the binding pattern of the plasma membrane fraction achieved when the derivatised insulin was incubated with hepatoma cells. The major labelled band (approximately 130 kD) corresponds to the insulin - binding α subunit of the insulin receptor. The labelling of this band is insulin - specific (figure 1.1.15, lane 2) indicating that the insulin derivative does indeed bind to the insulin receptor in a specific manner. There is some minor labelling of a band in the 95 kD range, possibly the ß subunit of the insulin receptor (see 1.1.3). This may reflect some non - specific labelling of the subunit when the ASD-insulin is bound to the α subunit.

The ability of the derivatised insulin to induce a response "*in vivo*" was ascertained by testing its ability to induce a hypoglycemic state in mice. The results summarised in figure 1.1.16 demonstrate that the ASD - insulin readily induced a hypoglycemic coma at a level comparable to that of native insulin.

The mouse drop test represents a general *in vivo* response to insulin. It was therefore deemed desirable to investigate more specific effects i.e. at the level of the gene. A

Figure 1.1.15 The *In Situ* Labelling of the Hepatoma Plasma Membrane - Bound Insulin Receptor by Carrier - Free [^{125}I]ASD-Insulin.

The labelling studies were performed as described in 6.3.4. Hepatoma cells were grown to confluency (3 x 10^7 cells / flask) and starved of serum overnight prior to the labelling studies. The medium was removed and 4ml of binding medium (MEM, 0.1% BSA, 20 mM HEPES, pH 7.4) added. [^{125}I]ASD-insulin (2 µCi) was added to each flask and the cells were incubated for 2 hours at 4 ℃ to prevent receptor internalisation (lane 1). For competition studies 100 µM cold insulin was added to the medium (lane 2)
After incubation, the medium was decanted and the cells were extensively washed and fresh medium added. The flasks were then subjected to ultraviolet radiation for 3 minutes and subsequently detached. The cells were homogenised in 0.32 M sucrose, TKM and subjected to a 10 minute spin at 800 g. The resultant supernatant was centrifuged at 38000 g for 30 minutes and this plasma membrane fraction subjected to SDS PAGE.

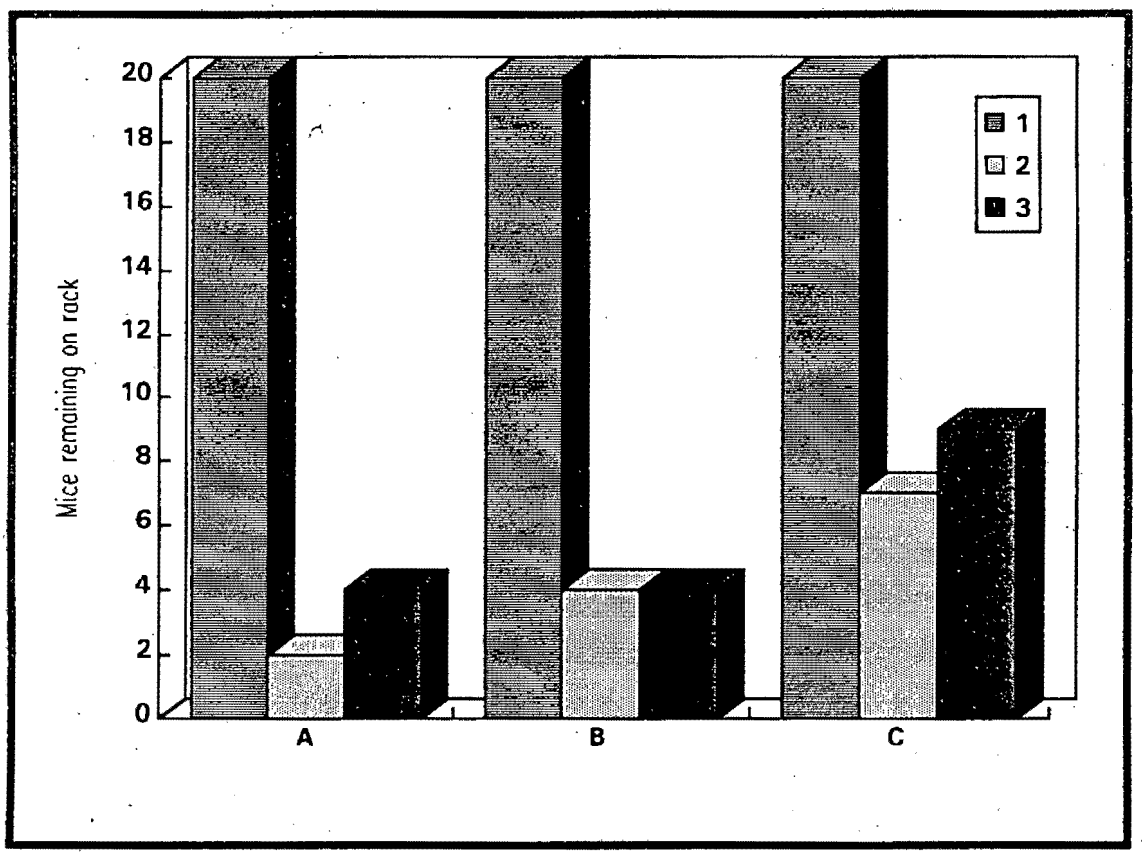


Figure 1.1.16 *In Vivo* **Response to ASD-Insulin: the Induction of Hypoglycemia in Balb C Mice.**

Groups of 20 mice each (average weight 30 g) were created by random selection and maintained in the same groupings for the duration of the experiments. These were designated insulin high, insulin low, ASD-insulin high, and ASD-insulin low.
The mice were starved for 6 hours and acclimatised at 37 °C for 1 hour prior to the experiments. The mice were injected intraperitoneally with either insulin (low dose 18 mU; high dose 36 mU) or ASD-insulin (low dose 0.8 µg; high dose 1.6 µg) and placed on mesh racks inclined at 60 °. Hypoglycemia resulted in the mice slipping off the racks (early warning system) and convulsing. The mice were resuscitated with an intraperitoneal injection of 0.5 ml of 15 % glucose (zero mortality rate was experienced). These mice were deemed to be hypoglycemic. The mice remaining on the racks after 1 hour were counted.
The experiment was repeated using different permutations of groups and dosages (see 6.3.7.). This figure represents an average value for the experiments.
Experiments were also performed using intermediate dosages (insulin 29 mU; and ASD-insulin 1.2 µg).
A, B, and C are high, intermediate, and low dosages respectively.
1, 2, and 3 are placebo, insulin, and ASD-insulin respectively.

hepatoma cell line (H4IIE) was incubated with ASD-insulin and the induction of known insulin - responsive genes was ascertained by the use of appropriate mRNA probes (pyruvate kinase and tyrosine amino transferase) as described in section 6.3.6. ASD-insulin increased pyruvate kinase mRNA levels 1,93 - fold and TAT mRNA levels 1,42 - fold above that of the control (figure 1.1.17). These increases are similar to those for native insulin (2,4 - fold and 1,67 - fold for pyruvate kinase and tyrosine amino transferase, respectively). I am indebted to Dr. Brenda Stickells, who optimised and carried out these gene - induction studies.

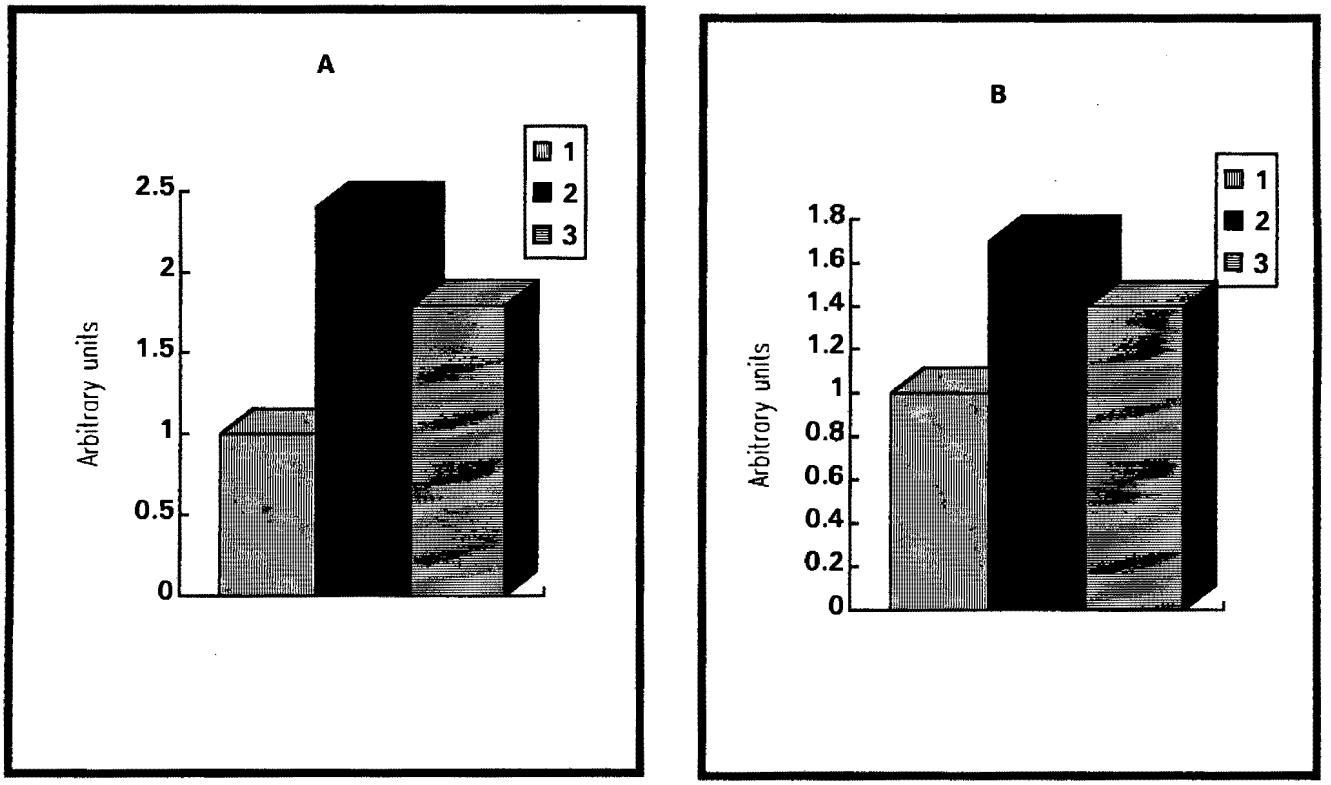


Figure 1.1.17. Determination of ASD-Insulin Stimulated Gene Induction in Hepatoma Cells

Pyruvate kinase and tyrosine amino transferase gene induction studies were undertaken as described in 6.3.6. Briefly, hepatoma H4IIE were incubated with either 10 mM ASD-insulin or natural insulin for 180 minutes and the total RNA isolated using CsCl gradient fractionation. After crosslinking to Hybond N, the RNA was probed with pyruvate kinase and tyrosine amino transferase probes. The blots were then autoradiographed and the resultant autoradiograms subjected to densitometric scans in order to quantitate the levels of mRNA present. The average SEM for two separate experiments was 0.2.

A: Pyruvate kinase gene induction.
1) control; 2) natural insulin; 3) ASD - insulin.
B: Tyrosine amino transferase gene induction.
1) control; 2) natural insulin; 3) ASD - insulin.

1.1.3 Discussion

This section details the synthesis and characterisation of carrier - free [^{125}I]ASD-insulin. The [^{125}I]ASD-insulin has been shown to be fully biologically active in that it specifically binds the plasma membrane insulin receptor (figure 1.1.15); it induces hypoglycemia in mice to a level comparable to native insulin (figure 1.1.16); and it induces the transcription of insulin - specific genes in hepatoma cells (figure 1.1.17).

The labelling pattern for plasma membrane receptor (figure 1.1.15) is similar to previous reports (Knutson, 1987; Hofmann et al., 1981; and Heidenreich et al., 1985) in that the major labelled protein is the 130 kD subunit. Knutson (1987) reports labelling of 130 kD exclusively, whereas the latter authors do show minor labelling of the 90 kD subunit. Podlecki et al. (1987) have also demonstrated the labelling of the 90 kD subunit. Of interest are the labelled high molecular weight bands (figure 1.1.15) which may represent unreduced receptor complex as demonstrated by Heidenreich et al. (1985). The labelling of the 130 kD subunit is in agreement with its role as the insulin binding subunit.

There are numerous reports of ligands derivatised with iodinated photoaffinity reagents but most are either not carrier - free or are iodinated after derivatisation i.e. both ligand and photoaffinity reagent are labelled (Knutson, 1987; Wollenweber & Morrison, 1985; Podlecki et al., 1987; Baenziger & Fiete, 1982; Heidenreich et al., 1985). However, a carrier - free cardiac glycoside photoaffinity derivative has been reported (Lowndes et al., 1988).
The derivatisation of insulin with photoaffinity labels has been reported (Knutson, 1987; Heidenreich et al., 1985; Yip et al., 1980; Yip et al., 1978; Podlecki et al., 1987) even

using SASD (Knutson, 1987) but either the insulin was iodinated (Knutson, 1987; Heidenreich et al., 1985; Yip et al., 1980; Yip et al., 1978) or the final product was not carrier - free (Hofmann et al., 1981; Yip et al., 1978). To my knowledge this is the first report of a carrier - free photoactivatable insulin derivative that is not iodinated on the insulin molecule.

The importance of a carrier - free insulin derivative is highlighted in the following consideration:
Assuming 1×10^4 insulin receptors per cell, an *in situ* experiment involving 1×10^7 cells, exhibiting, say, 5% unoccupied receptors, would be expected to result in 5×10^9 bound insulin molecules. Assuming that photoactivated cross - linking operates at approximately 2% efficiency, the final number of insulin molecules covalently cross - linked to their receptors is 1×10^8. As 1 Ci ^{125}I corresponds to 2.74×10^{17} atoms of iodine, the cross - linked insulin molecules correspond to $1 \times 10^8 / 2.74 \times 10^{17} = 3.65 \times 10^{-10}$ Ci or 810 d.p.m. on the plasma membrane. Thus the need for *carrier - free*, photoactivatable insulin derivative as a probe for intracellular receptors, where the receptor concentration may be much lower than on the plasma membrane, is obvious.

1.2. The Use of Carrier - Free [^{125}I]ASD-Insulin to Probe for Nuclear Envelope Insulin Docking Proteins

1.2.1 Introduction

In vitro studies using [^{125}I]insulin have been interpreted to demonstrate insulin binding sites at the level of the nucleus (Horvat et al, 1973; Goidl, 1979; Vigneri et al., 1978 a & b; Goldfine & Smith, 1976), the endoplasmic reticulum and the Golgi complex (Bergeron et al., 1973). Current opinion is that the major nuclear - binding site is the nuclear envelope (Vigneri et al., 1978 a) although it has been reported that binding at the level of chromatin does exist (Smith & Jarrett, 1987). These authors demonstrated insulin binding at the periphery of condensed chromatin near the nuclear pore complex. The evidence for insulin intracellular binding sites has been comprehensively reviewed (Goldfine, 1987; Goldfine et al., 1985; Goldfine et al., 1982 a & b).

Biochemical and immunological studies of the putative nuclear envelope - associated insulin receptor have shown it to be distinct from that of the plasma membrane receptor (Goldfine et al., 1977b; Vigneri et al., 1978 a) although a recent report (Wong et al., 1988) has shown the receptor isolated from nuclei to be virtually identical, by gel electrophoretic criteria, to that of the isolated plasma membrane receptor. These authors attribute this discrepancy to the fact that all previous studies were done with membrane - bound receptor and that differences in the receptor properties may actually reflect differences in membrane enviroments.

This *in vitro* evidence is corroborated by *in vivo* studies, demonstrating the internalisation and subsequent binding of label from radioactive insulin to intracellular organelles. Using insulin labelled by either ^{125}I, fluorescent dyes such as rhodamine, or electron dense moieties such as ferritin, the internalisation and eventual accumulation of label in the nucleus has been demonstrated in hepatocytes, rat diaphragm, lymphocytes, and adipocytes (for an extensive review see Goldfine, 1981). Briefly, these studies involved the administration of labelled insulin *in vivo* or *in vitro* and subsequent subfractionation. Determination of label localisation was by autoradiography, electron microscopy, or light microscopy (Carpentier et al., 1978; Carpentier et al., 1979; Goldfine et al., 1977a & b; Goldfine, 1981; Smith & Jarrett, 1987; Schlesinger et al., 1978), depending on the derivatisation of the insulin molecule. Label binding has been demonstrated to occur within 15 - 30 minutes (Goldfine et al., 1977a; Smith and Jarrett, 1987). The latter authors found 3% of internalised insulin to be accumulated at the nucleus within 60 minutes. These findings appear to be significant because excess cold insulin almost completely inhibited the association of ferritin - insulin with nuclei (Smith & Jarett, 1987).

The above findings strongly suggest that insulin interacts with the nuclear envelope and complement the findings that insulin directly influences nuclear envelope functioning (see 4.1). In an attempt to expand on the above evidence, the photoactivatable insulin derivative (synthesised as described in 1.1.2) was used to probe for putative intracellular insulin binding proteins. This section summarises the findings of this investigation.

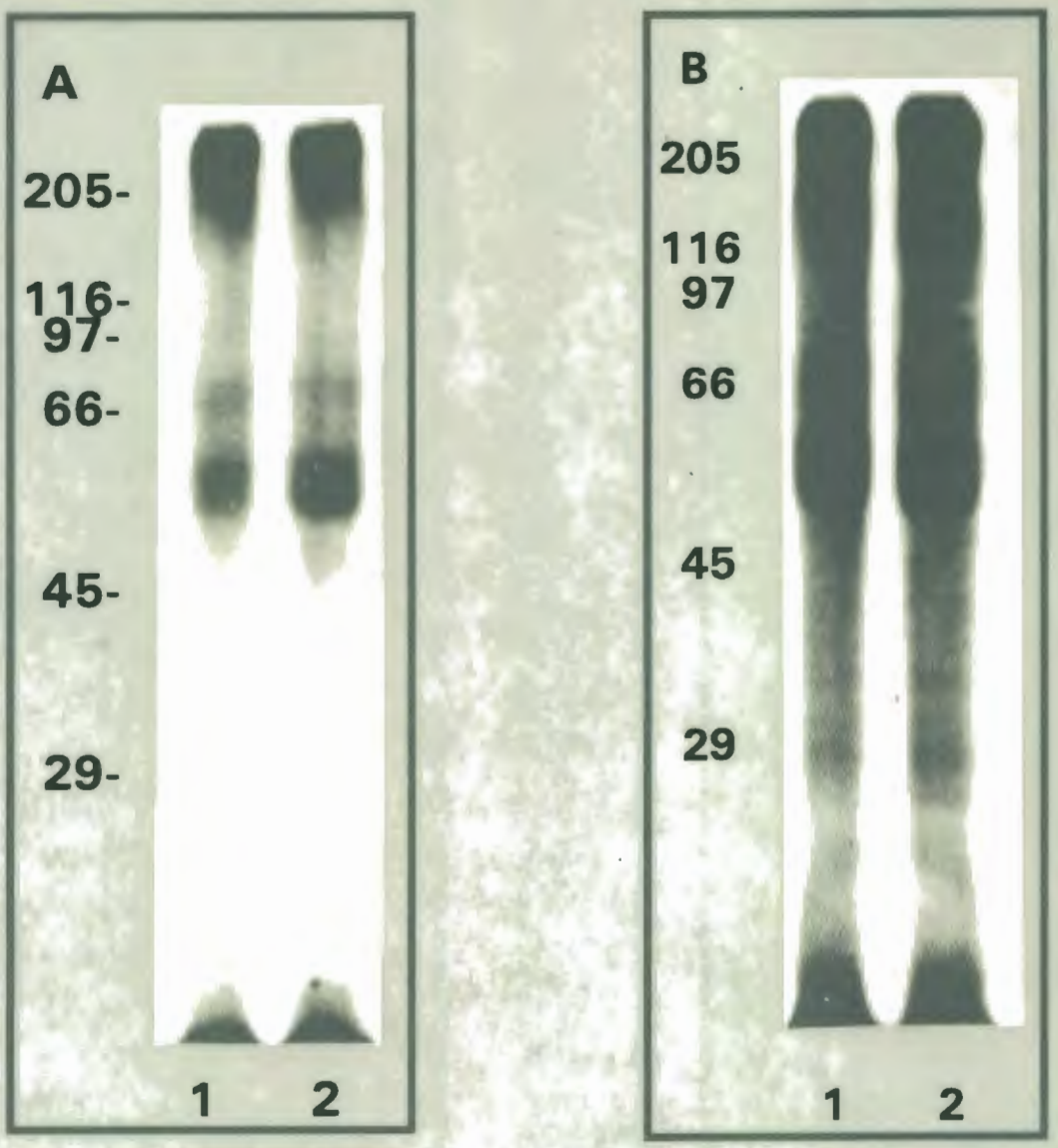


Figure 1.2.1 Probing for Insulin - Binding Proteins in Isolated Rat Liver Nuclear Envelopes.

Rat liver nuclear envelopes (200 µg) isolated by the method of Bornens and Courvalin, (1978) (A1 & B1) and Kaufmann et al., (1983) (A2 & B2) were incubated with carrier - free [^{125}I]ASD - insulin (6.3.5) and subjected to one dimensional SDS PAGE and autoradiography (6.4.7.3).

A: incubation with [^{125}I]ASD - insulin at 4°C
B: incubation with [^{125}I]ASD - insulin at 22°C.

1.2.2 Results

The biological activity of the synthesised insulin analogue was investigated using intact hepatoma cells (figure 1.1.15), since this system ensures an intact plasma membrane. However, rat liver was used to investigate the existence of intracellular insulin - docking polypeptides because the preparation of highly purified nuclei from rat liver homogenate is more convenient than it is from cultured hepatoma cells.

Attempts to probe for specific insulin binding proteins in purified rat liver nuclear envelopes were unsuccessful (a typical result is demonstrated in figure 1.2.1). There was consistently a high background, especially at temperatures above 4°C (figure 1.2.1 B). This is possibly due to increased membrane fluidity, resulting in increased insertion of the hydrophobic SASD moiety into the lipid double layer. To circumvent this problem and to avoid possible alteration of the putative insulin receptors prior to the binding studies, whole nuclei were incubated with the derivatised insulin. Subsequently the nuclear envelopes were isolated after crosslinking with u.v. radiation. A typical insulin binding study with rat liver nuclei is shown in figure 1.2.2 A. A very faint band with a molecular weight of approximately 135 kD (figure 1.2.2 A, lane 1) is absent when excess cold insulin is added to the incubation mixture (figure 1.2.2 A, lane 2). Comparative scanning also indicated that only one fraction significantly decreased in labelling (average 30%) as the result of quenching with free insulin, confirming the visual observations (figure 1.2.2 A).

In addition a further polypeptide of approximately 60 kD was specifically labelled (figure 1.2.3). This band is completely absent in control experiments, even when the

autoradiograms were overexposed (figure 1.2.3., inset). Attempts were made to emulate the results of Wong et al. (1988) by preparing wheat germ agglutinin extracts of isolated rat liver nuclei and then labelling these extracts with the derivatised insulin (figure 1.2.2 B). The resultant autoradiograms proved to be difficult to interpret because discrete bands were not obtained. Figure 1.2.2 B indicates a diffuse, labelled region in the 130 kD range. Overexposure of autoradiographs from incubates with excess cold insulin did not reveal a similar region in the gel (figure 1.2.2 B, lane 2). The result indicates that there may be an enrichment of an insulin - binding protein with a molecular weight close to that of the plasma membrane insulin subunit, however a high non - specific binding and / or proteolytic degradation obscures the result and does not allow any real conclusion to be drawn.

In Friend cell nuclei a region in the 130 kD range appears to become labelled by insulin (figure 1.2.4). Friend cells have been reported by some authors to be responsive (Ginsberg et al., 1979; Ginsberg et al., 1981; Simon et al., 1987). However, there was again heavy non - specific binding.

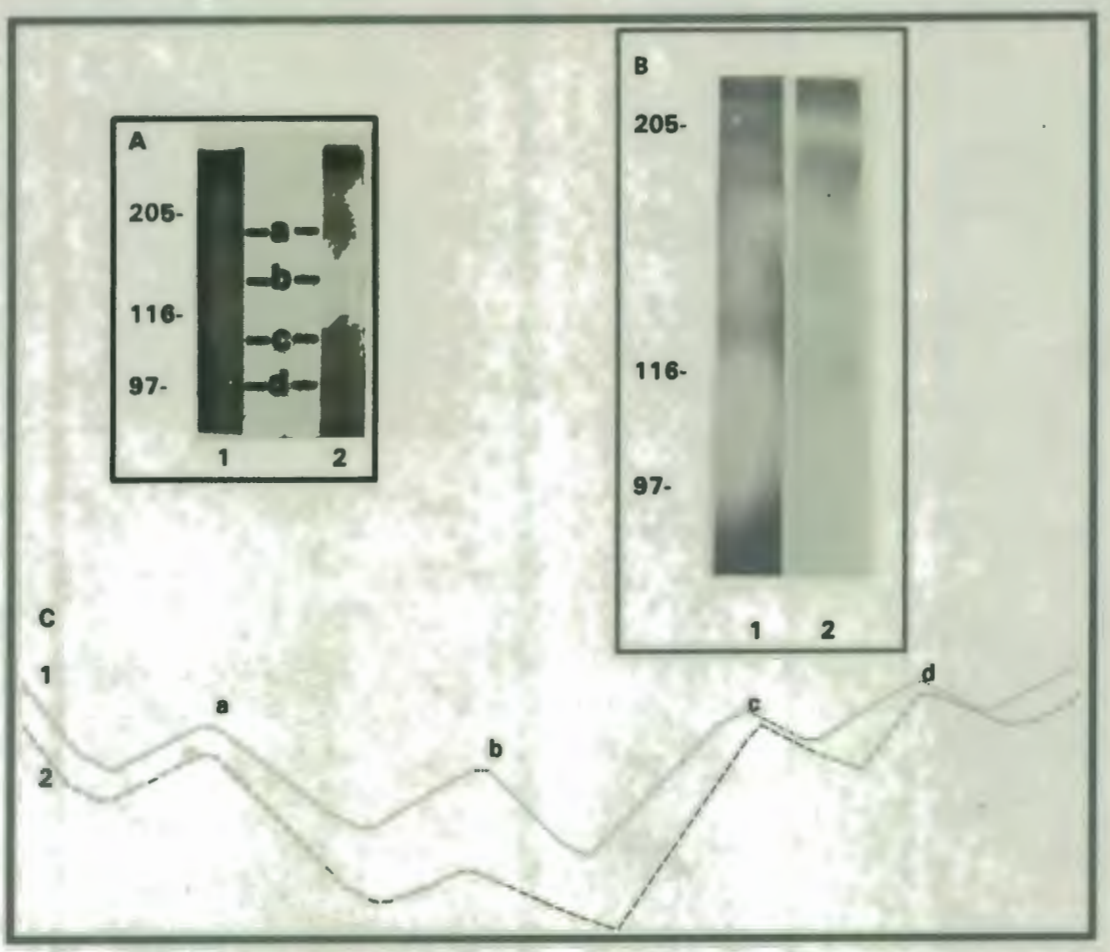


Figure 1.2.2 Probing for Insulin Binding Proteins in Isolated Rat Liver Nuclei.

Rat liver nuclei (A) isolated by the method of Blobel and Potter (1966) and wheat germ agglutinin extracts of nuclei (B) prepared as described in (6.1.7) were incubated with carrier - free [^{125}I]ASD-insulin, washed to remove unbound insulin and irradiated with u.v. radiation (6.3.5). The fractions were subjected to one dimensional SDS PAGE and autoradiography (6.4.7.3).

A: rat liver nuclei incubated with carrier - free [^{125}I]ASD -insulin in the absence (1) and presence (2) of 100 nM cold insulin.
B: 1) wheat germ agglutinin extract of rat liver nuclei incubated with carrier - free [^{125}I]ASD-insulin in the absence (1) and presence (2) of 100 nM cold insulin.
C: densitometric scan of the autoradiogram in A. The symbols 1 & 2 and a - d correspond to those in A. The scans of the two lanes were standardised by an algorithm developed by Dr. Hugh Patterton, in order to correct for any variations in the amount of material loaded onto each lane.

1.2.3 Discussion

The two major problems encountered in this study were the high background levels and the low intensity of the labelled polypeptides. This has made rigorous quantitation and interpretation very difficult.

The high background effect was more acute when isolated nuclear envelopes were incubated with [^{125}I]ASD-insulin, especially at higher temperatures (figure 1.2.2). This may reflect a higher fluidity of the nuclear membrane at these temperatures which, in turn, may result in the non - specific association of the SASD - derivatised insulin with the envelope due to insertion of the hydrophobic SASD moiety into the fluid lipid layer. Subsequent washing may not remove the unbound insulin, and if in close proximity to other membrane proteins, may crosslink non - specifically when exposed to u.v. radiation.

Furthermore, the low intensity of the labelled bands may be due to degradation or conformational changes of any putative insulin docking polypeptide as a result of the nuclear envelope isolation procedure. For example, it is recognised that nuclear envelopes isolated using heparin have a characteristic closed vesicular nature (Monneron et al., 1972). Any putative insulin binding protein of the nuclear envelope may thus be in an inverted conformation i.e. orientated toward the centre of such vesicles and thus be inaccessible to insulin.

For all the above reasons the nuclear envelope fractions were isolated after incubation of the intact nuclei with insulin. In addition all procedures to isolate nuclei and nuclear envelopes were modified to eliminate reducing agents such as ß - mercaptoethanol, thus ensuring that the insulin receptor (or receptor - like protein) was not reduced and possibly changing its solubility properties.

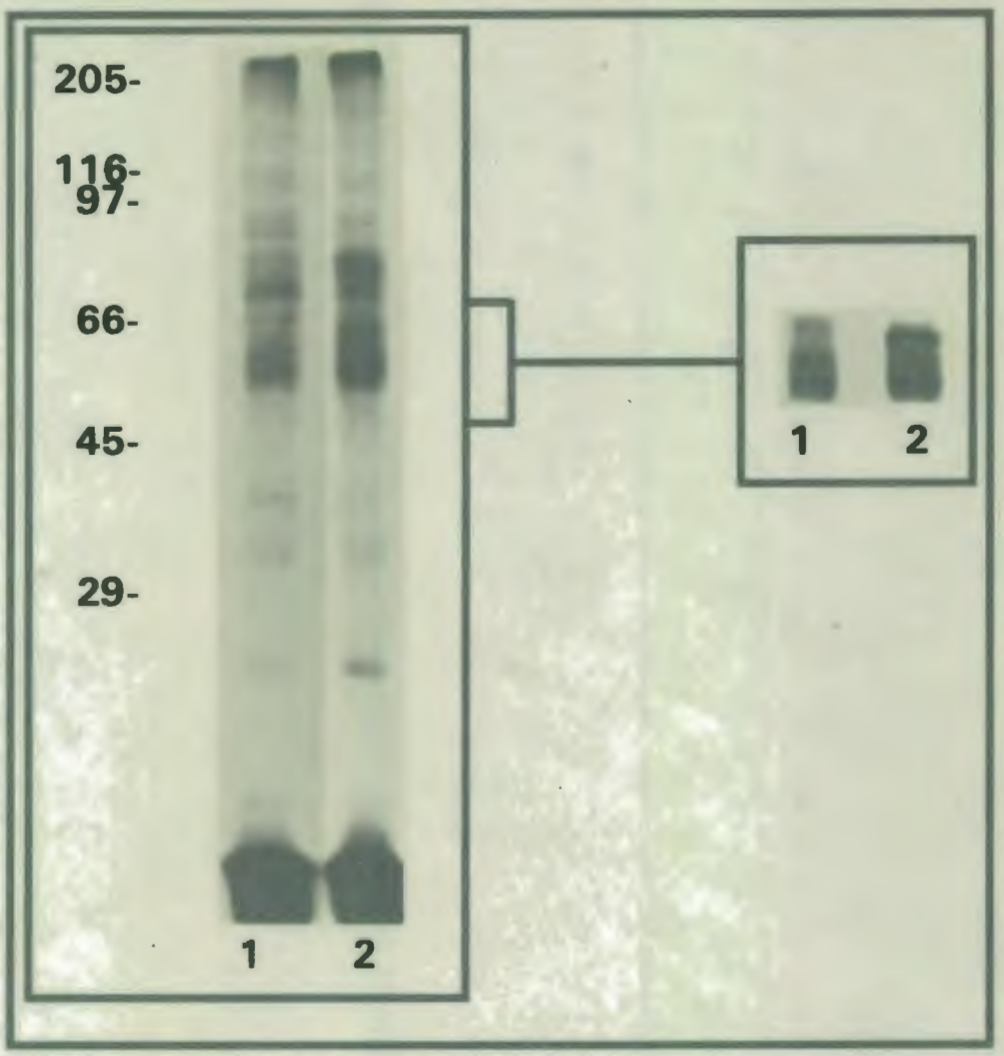


Figure 1.2.3 Demonstration of a Novel Insulin - Binding Protein in the Nuclear Envelope of Rat Liver.

Isolated nuclei (6.1.1) were incubated with carrier - free [^{125}I]ASD-insulin in the presence (1) or absence (2) of 100 nM cold insulin (6.3.5) and then subjected to one dimensional SDS PAGE and autoradiography (6.4.7.3).

The inset shows an enlarged view of the indicated area. Lane 1 has been overexposed relative to lane 2 to demonstrate the lack of the 60kD protein in the presence of excess cold insulin

I have tried to use tissue with an increased number of free hypothetical intracellular insulin - binding sites, by using streptozotocin - induced diabetic rats. However, the livers from treated rats were invariably at an advanced state of degeneration even when removed from animals with moderate increases in blood glucose (see also section 4.3).

No clear picture emerged from a wheat germ agglutinin - binding fraction of rat liver nuclear envelopes (figure 1.2.2 B), in that no discrete labelled bands were obtained. Only a diffuse labelled region in the 130 kD range is inhibited by excess cold insulin (figure 1.2.2 B, lane 2). The photoactivatable insulin analogue gave better results with Friend cell nuclei, pointing at the possible presence of a polypeptide in the 130 kD region, quenchable by excess cold insulin.
Of interest is the insulin binding by an approximately 60 kD polypeptide (figure 1.2.3). Whether this constitutes a proteolytic fragment of the insulin receptor α subunit binding to insulin, remains to be seen. Increased susceptibility of the insulin receptor to tryptic cleavage after insulin binding and even a tryptic fragment with a molecular weight of 64 kD have been reported (Pilch and Czech, 1979; Donner and Yonkers, 1983). The possibility may also exist that this 60 kD polypeptide is not a degradation product and the 135 kD and 60 kD insulin binding proteins coexist and that, indeed, both are necessary for signal induction at the level of the nucleus (see chapter 5).

Figure 1.2.4 Demonstration of a Nuclear Envelope Insulin Binding Protein in Friend Cells.

Friend cell nuclei (6.1.5) were incubated with carrier - free [^{125}I]ASD-insulin in the presence (1) and absence (2) of 100 nM cold insulin (6.3.5) and subjected to one dimensional SDS PAGE and autoradiography (6.4.7.3). The Friend nuclei were shown by electron microscopy to be pure and intact. Overexposure of lane 1 (data not shown) failed to reveal any bands in the 45 - 205 kD molecular weight range.

Chapter 2

The Enzymatic Synthesis of Carrier - Free 8-Azidoadenosine 5'-[γ^{32}P] Triphosphate

2.1. Introduction

Azido photoaffinity analogues of nucleotide mono -, di -, and triphosphates are a powerful tool in the investigation of cellular regulatory processes.
Azido-ATP (N_3ATP) has been used in investigating the structure and mechanism of action of ATPases (Haley & Hoffman, 1974; Scheiner - Bobis & Schoner, 1985), the identification of membrane - bound ATPases and protein kinases (Roth & Cassel, 1983; Clawson et al., 1984; Coughlan & Hind, 1986), and in the investigation of receptor autokinases (Hapgood et al., 1986). N_3ATP has also been employed in elucidating conformational changes in ATP - binding enzymes (Reimann et al., 1988).
Azido - derivatised guanine nucleotides, especially N_3GTP, have been particularly powerful probes in the investigation of the mechanism of action of GTP - binding proteins (G proteins) in various systems (Peter et al., 1988; Schleicher et al., 1986; Droms et al., 1987).

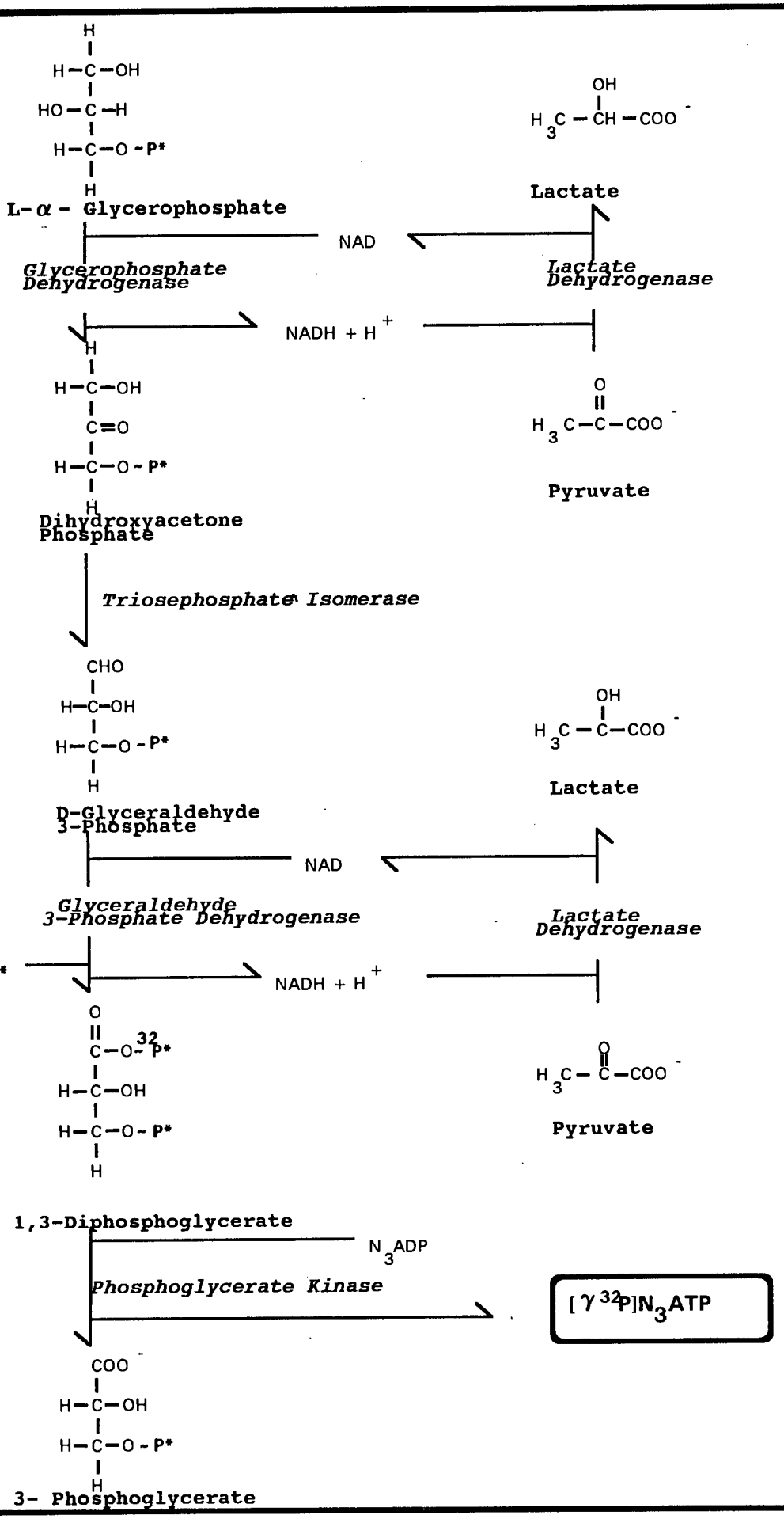

L-α - Glycerophosphate
NAD
Glycerophosphate Dehydrogenase
NADH + H+
Dihydroxyacetone Phosphate
Triosephosphate Isomerase
D-Glyceraldehyde 3-Phosphate
NAD
Glyceraldehyde 3-Phosphate Dehydrogenase
32P*
NADH + H+
1,3-Diphosphoglycerate
N3ADP
Phosphoglycerate Kinase
3- Phosphoglycerate
Lactate
Lactate Dehydrogenase
Pyruvate
Lactate
Lactate Dehydrogenase
Pyruvate
[γ 32P]N3ATP

Figure 2.1. The Enzymatic Synthesis of Carrier - Free 8-Azidoadenosine 5'-[γ^{32}P] Triphosphate.

N_3ATP: 8-azidoadenosine 5'- triphosphate
N_3ADP: 8-azidoadenosine 5'- diphosphate

P* *: phosphoryl residue*

2.1.1 The Synthesis of Azido-ATP

2.1.1.1. Chemical Synthesis

A method for the chemical synthesis of azido analogues of adenine nucleotides is well documented (Czarnecki et al., 1979) and is a modification of the method of Michelson (1964). This multi - step process entails the coupling of pyrophosphate to N_3AMP which has been previously activated by incubation with diphenyl chlorophosphate. The N_3ATP (30 - 50%) is separated from contaminating byproducts by DEAE - cellulose chromatography. To synthesise $[\gamma^{32}P]N_3ATP$, the pyrophosphate is spiked with ^{32}PPi. The disadvantages of this methodology are the low specific activity of the product, the duration of the synthesis and the potential hazard of the chemicals used.

2.1.1.2. Enzymatic Synthesis

Enzymatic methods have been reported (Glynn & Chappell, 1964; Potter & Haley, 1983), but these are based either on exchange reactions between inorganic phosphate and the gamma phosphate of ATP (Glynn & Chappell, 1964) or require purification of the end product (Potter & Haley, 1983). This purification step of the final product to a carrier - free state is particularly important when investigating low abundance proteins, such as receptors.

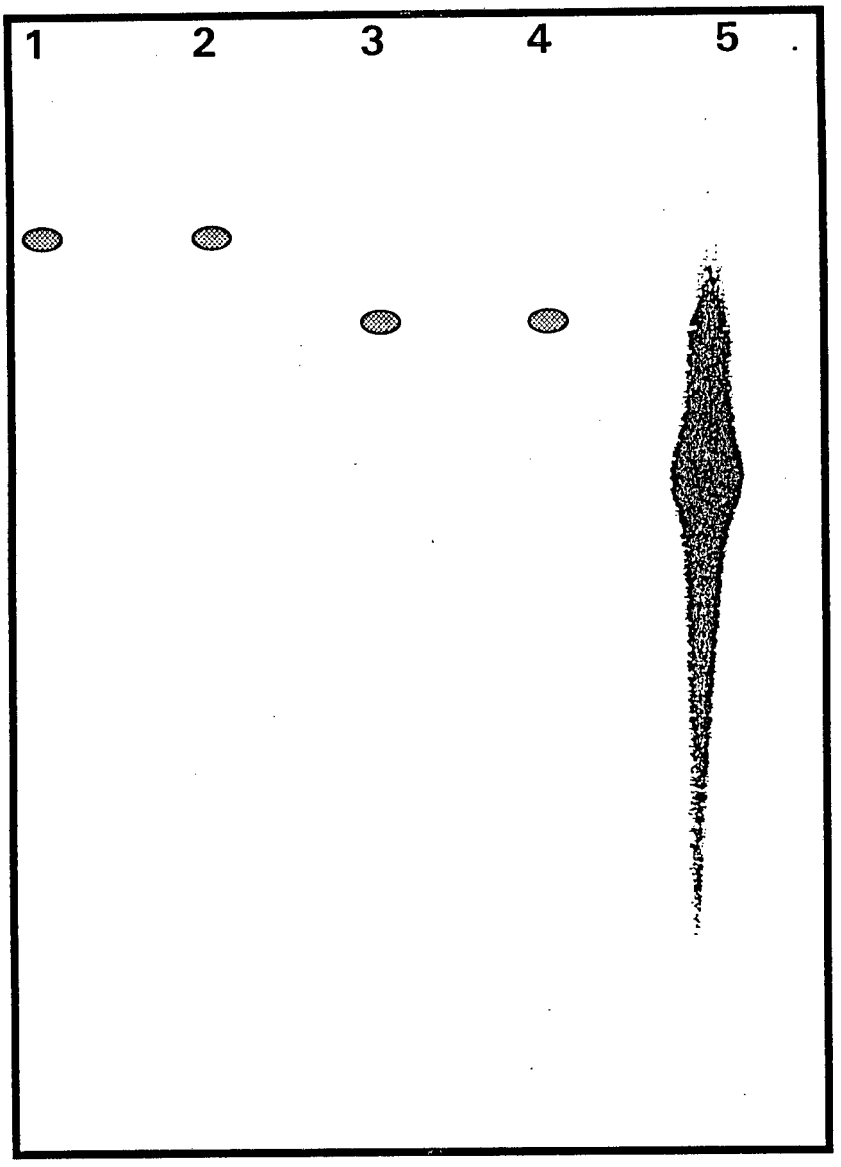


Figure 2.2. TLC Characterisation of the Azido Product Synthesised in the Presence of 6 mM Dithiothreitol.

The azido product was synthesised using the method of Johnson and Walseth (1979) for the synthesis of [γ^{32}P]ATP. This protocol is similar to that described in 6.2.1 with the major difference being the concentration of dithiothreitol (6 mM).
The reaction was monitored by a charcoal assay (6.4.11) and terminated by the addition of an equal volume of Tris - saturated phenol. After extraction with chloroform an aliquot of the product was spotted onto a PEIE - cellulose TLC plate and run as described in 6.4.13.

1) ADP; 2) N_3ADP; 3) ATP; 4) N_3ATP; 5) autoradiogram of the synthesis product. The non - radioactive standards (1 - 4) were visualised by fluorescence quenching under ultraviolet light.

This chapter describes the establishment of an enzymatic method for the synthesis of carrier - free [γ^{32}P]N_3ATP by a single step process. The method is founded on the ability of N_3ADP to act as a phosphoryl acceptor in the glycolytic pathway from 1,3,- diphosphoglyceric acid to 3 - phosphoglycerate (figure 2.1). The product has been characterised and shown to meet all the criteria for a specific, photoreactive ATP analogue.

2.2 Results

2.2.1 Synthesis

The methodology employed is as described in (6.2.1), except that the reagent mixture and the glycolytic enzyme mixture contained 15 mM and 5 mM dithiothreitol respectively. These are the amounts reported by Johnson and Walseth (1979) for the synthesis of radioactive adenine nucleotides.
Initial syntheses yielded a main radioactive product with an Rf value different from N_3ATP on TLC, (figure 2.2, lane 5).

The azido product, without further purification, was used to photoaffinity label creatine kinase, known not to be an autokinase (thus excluding the complication of autophosphorylation). Total incorporation was only 0.01%, which was far less than expected. This demonstrated that in all probability, the major product of the synthesis was not the expected azido compound and that there may have been destruction or alteration of the azido moeity during the synthesis.

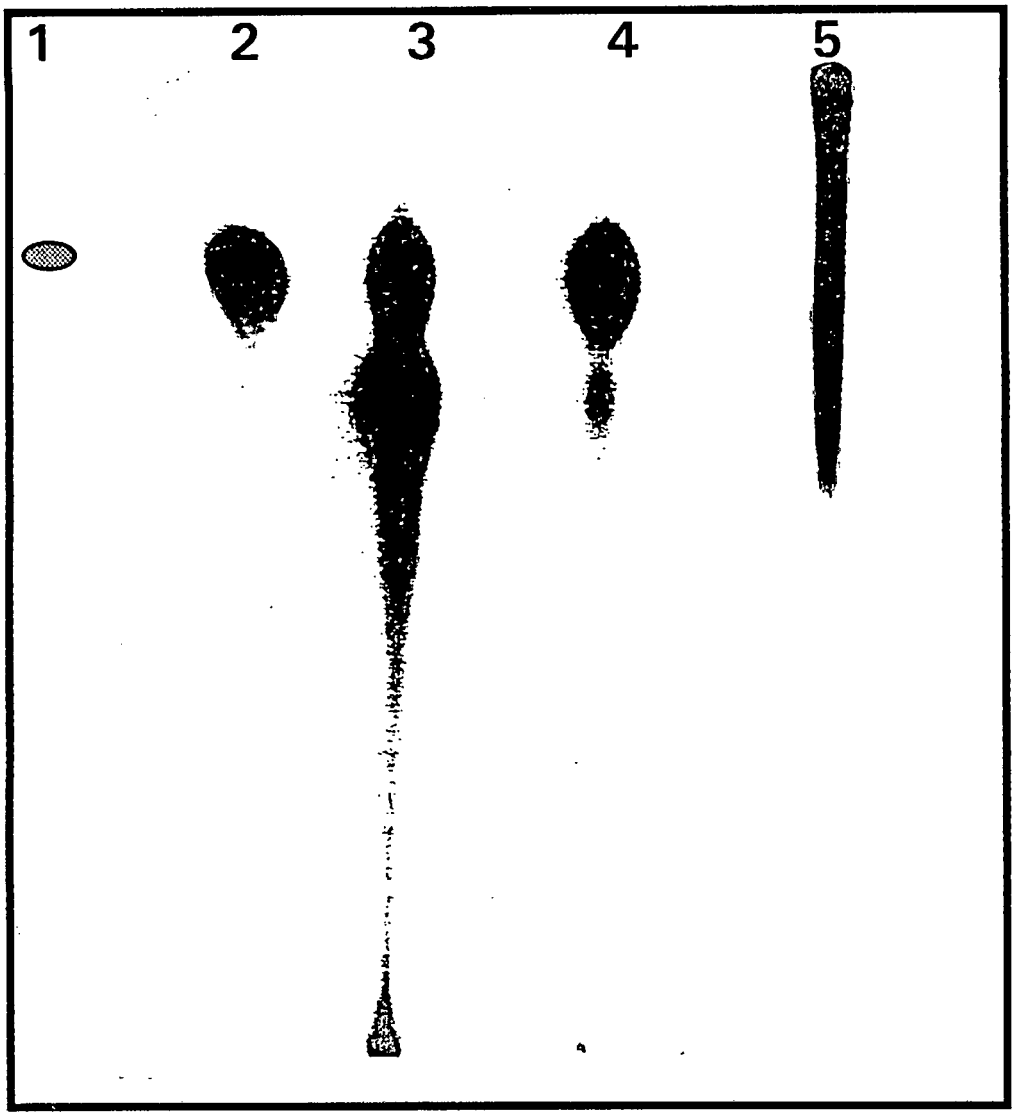


Figure 2.3. TLC Analysis of the Products Synthesised Using Different ß-Mercaptoethanol Concentrations.

The azido products were synthesised as described for figure 2.2, except that dithiothreitol was substituted by ß-mercaptoethanol.
Aliquots of the synthesised products were subjected to TLC as described in 6.4.13 and autoradiography (6.4.12). The standard non-radioactive ATP was visualised by means of fluorescence under ultraviolet light.
1) ATP standard
2) [γ^{32}P]ATP
3) Product synthesised in the presence of 6 mM ß - mercaptoethanol.
4) Product synthesised in the presence of 360 µM ß - mercaptoethanol.
5) Product synthesised in the absence of ß -mercaptoethanol.

.

The faint smudge above the main azido product (representing 3% of the total radioactivity) was potentially interesting in that its Rf value was that of non - radioactive N_3ATP. Thus, preparative TLCs were run and the corresponding area scraped (figure 2.2, lane 5). The radioactive product was eluted from the scrapings with 0.2 M ammonium bicarbonate and lyophilised. This product was found to photolabel creatine phosphokinase tenfold more effectively than the major synthesis product.
The above results demonstrate that the major radioactive spot is not the desired product and that in fact it is the minor product (representing 3% of the total radioactivty) that constitutes $[\gamma^{32}P]N_3ATP$.

The fact that the major product is not photoactivatable, focussed attention on the azido moeity which may undergo reduction to an amine derivative, rendering it inert, as a result of the presence of dithiothreitol (DTT) in the incubation mixture (used for the protection of the glycolytic enzymes). It was therefore decided to substitute the DTT with the milder reducing agent mercaptoethanol. Syntheses were performed with reagent mixtures containing no mercaptoethanol and glycolytic enzyme mixtures with varying amounts of the reducing agent. The products are dramatically different in that they consist either of 2 radioactive spots (figure 2.3, lane 3) or a single spot in the case of the product synthesised at a final mercaptoethanol concentration of 360 μM (figure 2.3, lane 4). In the total absence of mercaptoethanol (figure 2.3, lane 5) no defined product is synthesized.

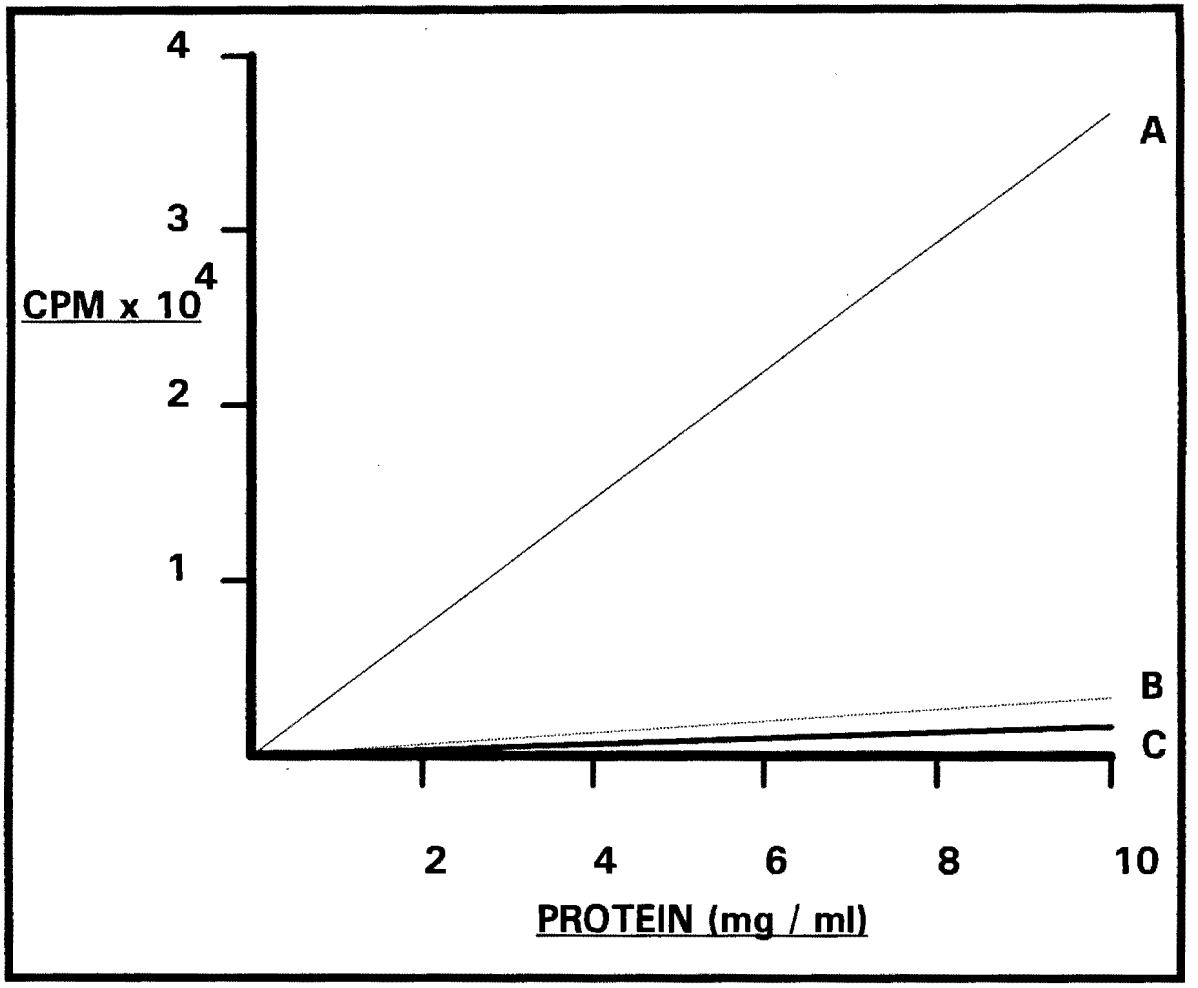


Figure 2.4. The Effect of Increasing Protein Concentration on Specific (A) and Non - Specific (B,C) Labelling by [γ^{32}P]N_3ATP

Various concentrations of the proteins were dissolved in incubation medium containing [γ^{32}P]N_3ATP and after 2 minutes of preincubation exposed to eight 10 millisecond flashes from a ultraviolet flash system as described in 6.4.15. The proteins were then precipitated with trichloroacetic acid (10% final concentration) and subjected to SDS PAGE as described in 6.4.7.3. The bands were excised, placed in 10 ml scintillation medium and counted.
A) creatine kinase; B) bovine serum albumin; C) concanavalin A.

2.3. Characterisation of the Synthesised Azido Product

2.3.1. TLC Mobility Studies

Figure 2.3 summarises the TLC characterisation of the synthesised product. The product of synthesis 2 i.e. in the presence of 360 μM mercaptoethanol (lane 4) is virtually homogeneous and has a Rf value identical to that of ATP and N_3ATP. The product of synthesis 1 i.e.in the presence of 6 mM mercaptoethanol (lane 3) is a heterogeneous mixture as in figure 2.2, lane 5, indicating that the lower mobility fraction is dependent on a critical level of mercaptoethanol. The product at 360 μM mercaptoethanol has the Rf of cold N_3ATP.

2.3.2 The Photoreactivity of the Products

The products were incubated with creatine phosphokinase as described in section 6.3.1 and the incubation media exposed to ultraviolet radiation (section 6.4.15). After TCA precipitation and subsequent SDS PAGE, the extent of labelling of the enzyme was established (see legend of table 2.1).

The data (table 2.1) reveal that both products label creatine kinase and that this labelling for the synthesis 2 product is approximately twice that for the synthesis 1 product. From this it can be concluded that the synthesis 2 product constitutes the desired photoreactive azido compound in high yield.

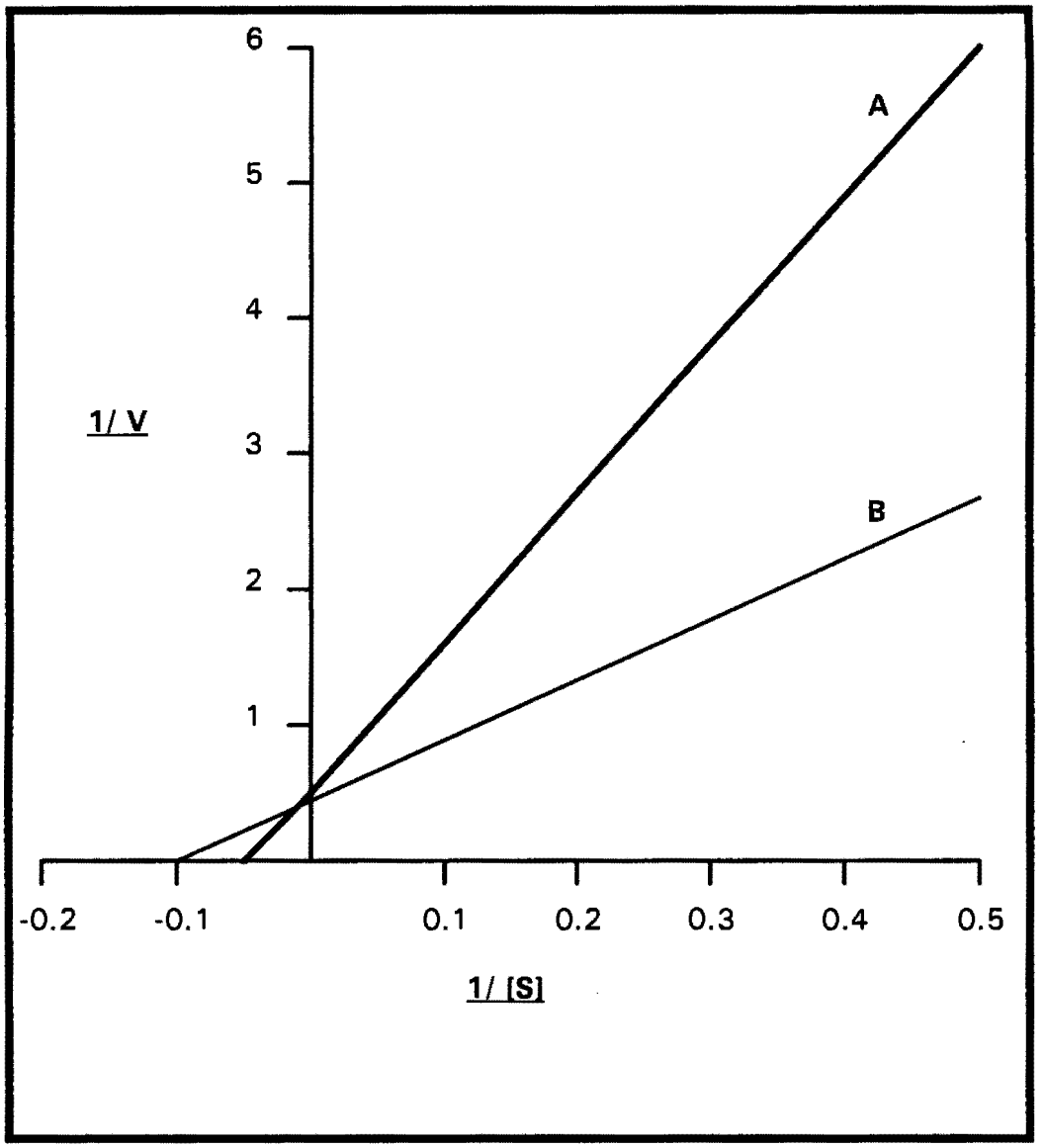


Figure 2.5. The Study of the Kinetics and Specificity of Hydrolysis of the Azido Product by Rabbit Skeletal [Na^+,K^+]ATPase.

[Na^+,K^+]ATPase (0.02 U / 250 µl) was incubated with varying amounts of the azido product and the degree of hydrolysis after 10 minutes determined by the charcoal assay (6.4.11). The rate of hydrolysis is represented as the number of moles of ATP hydrolysed / 0.02 U of enzyme / 10 minutes (6.4.4). Enzymatic reactions were executed in the absence (B) and presence (A) of cold ATP.

2.3.3 Labelling Specificity

The specificity of labelling was ascertained by incubating the synthesis 2 azido product with various non - ATP - binding proteins. The results in table 2.1 indicate that the azido product specifically labels only the kinase.
Non - specific binding as a function of protein concentration was investigated and the results in figure 2.4 clearly show that non - specific binding does not increase to any great extent with increasing concentration.

2.3.4 Kinetic And Enzymatic Studies

The kinetic studies were undertaken using a [Na^+, K^+]ATPase as described in section 6.4.4. Various concentrations of the synthesised product were incubated with the ATPase and the rate of hydrolysis was determined by measuring the amount of liberated ^{32}Pi in an aliquot after 10 minutes. Lineweaver - Burk analysis of the data (figure 2.5) reveals that the Km of hydrolysis is 10 μM and that competition with cold ATP is competitive (figure 2.5, B).

2.4 Discussion

An enzymatic protocol has been developed to synthesise carrier - free [$\gamma^{32}P$]N_3ATP in a single step, without the need for purification of the final product (Sabbatini & von Holt, 1987).
This product has been shown to meet all the criteria for a specific, photoreactive ATP analogue. It has the same TLC mobility as ATP and nonradioactive N_3ATP (figure 2.3); it is photoreactive (figures 2.4, table 2.1); it specifically

Substrate	U.V.	Incorporation (c.p.m.)
creatine kinase (1)	+	3000
	-	200
creatine kinase (2)	+	6500
	-	260
bovine serum albumin	+	600
	-	310
concanavalin A	+	600
	-	280
carbonic anhydrase	+	450
	-	290
soybean trypsin inhibitor	+	650
	-	340
alcohol dehydrogenase	+	350
	-	280

Table 2.1. Photoreactivity and Specificity of Labelling of the Synthesised Products.

Labelling studies were undertaken as described in 6.3.1.
1) synthesis 1 azido product
Except for creatine kinase (1), all incubations were with the synthesis 2 azido product.
The + and - in the u.v. column indicate the presence and absence of ultraviolet radiation.

labels a protein kinase (table 2.1); the Km of hydrolysis is very similar to that determined by Scheiner - Bobis & Schoner (1985) and the competitive inhibition by cold ATP indicates that the azido product is binding to a specific ATP - binding site.

This synthesis protocol has distinct advantages over other methods. The chemical synthesis yields (30 - 50%) N_3ATP which then has to be separated from contaminating N_3AMP and an unidentified by - product (Czarnecki et al., 1979; Michelson, 1964). This method also employs potentially harmful reagents. The method of Potter and Haley (1983) results in a contaminating by - product, which could be the contaminant we encountered when relatively high quantities of mercaptoethanol or dithiothreitol are used. This problem is circumvented in our method by substituting mercaptoethanol for dithiothreitol and by decreasing the concentration of the reducing agent from 6 mM to 360 μM (final concentration).

The carrier - free [γ^{32}P]N_3ATP has been used to probe for low quantity ATP - binding proteins in various cellular substructures (chapter 3) and to investigate direct effects of insulin on these ATP - binding proteins (chapter 4). The importance of carrier - free [γ^{32}P]N_3ATP is illustrated by its use in investigating the putative protein kinase activity of the glucocorticoid hormone receptor complex (Appendix 1). These studies revealed the presence of a very low abundance protein kinase contaminant in the receptor preparation. The use of non - carrier - free [γ^{32}P]N_3ATP may not have provided such a definitive identification.

Chapter 3

The Identification of Phosphoproteins and ATP - Binding Proteins in Intracellular Membranes

3.1. Introduction

There are indications in the literature and in the experiments presented of specific insulin binding by structures in the nucleus (see 2.2.1). Furthermore, insulin has been reported to directly modulate the phosphorylation state and activity of nuclear proteins (see 4.1). Thus, a study of the nuclear ATP - binding protein profile could make a contribution in the identification of kinases / NTPases that may be directly regulated by insulin.

Protein Kinases

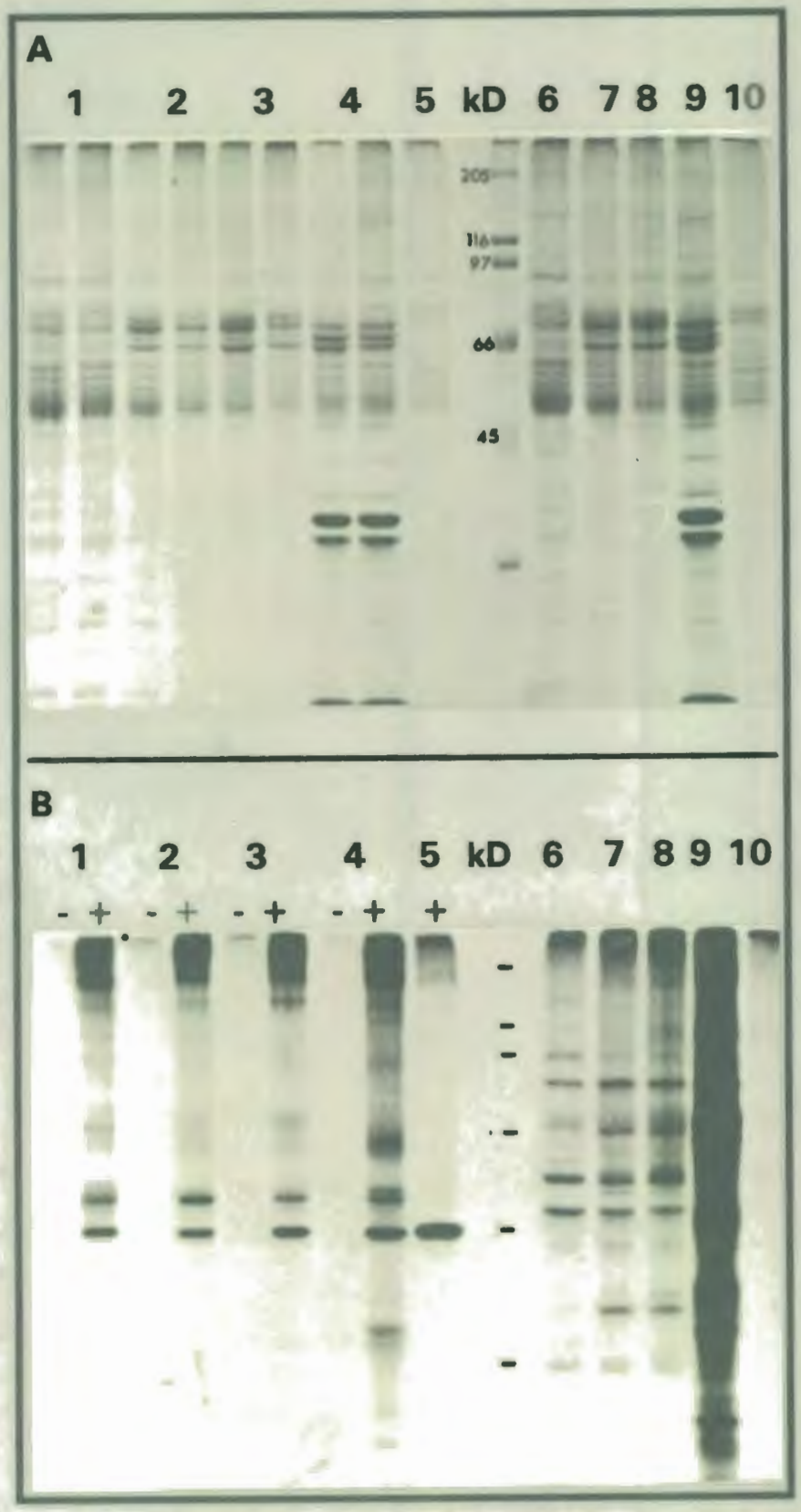
Numerous nuclear envelope substrates for endogenous protein kinases have been identified (Lam & Kasper, 1979; Kletzien et al., 1981; Smith & Wells, 1983; Dessev et al., 1988; Rottman et al., 1987; Fisher, 1987; Gerace & Blobel, 1980; Ottaviano & Gerace, 1985; Fields et al., 1988; Hornbeck et al., 1988). A number of these phosphoproteins are associated with the nuclear pore complex (Lam & Kasper, 1979; Steer et al., 1980; Smith & Wells, 1983; Ahmed & Steer, 1982; Rottman et al., 1987) or the nuclear lamina (Dessev et al., 1988; Fisher, 1987; Gerace & Blobel, 1980; Ottaviano & Gerace, 1985; Newport & Spann, 1987; Fields et al., 1988; Hornbeck et al., 1988). Smith and Wells (1983) have made a systematic

study of rat liver nuclear envelope phosphoproteins and have identified at least 12 such modified polypeptides, the most prominent (7, 20, 51, 59,and 70 kDa) being associated with the nuclear pore complex.

The role of phosphorylation / dephosphorylation of the nuclear lamins in nuclear breakdown and reassembly is extensively documented. In mitosis, hyperphosphorylation of the lamins precedes nuclear envelope breakdown (Fisher, 1987; Gerace & Blobel, 1980; Ottaviano & Gerace, 1985). This high degree of phosphorylation is thought to result in the depolymerisation of the lamina network as evidenced by the fact that lamins A and C are solubilized (Gerace & Blobel, 1980; Ottaviano & Gerace, 1985; Miake - Lye & Kirschner, 1985). Conversely, dephosphorylation of the solubilized lamins precedes polymerisation and marks the onset of nuclear reassembly (Burke & Gerace, 1986; Fisher, 1987; Peter et al, 1990).

Ottaviano and Gerace (1985) have demonstrated that phosphorylation occurs only on assembled lamins indicating that an endogenous membrane - bound kinase is probably responsible. This finding is substantiated by the fact that the nuclear lamina contains a very tightly bound protein kinase activity responsible for phosphorylating the lamins as well as several other polypeptides (Dessev et al., 1988). This kinase activity is Ca^{2+} and cAMP independent. Lamin phosphorylation has been shown to be enhanced by the maturation promoting factor (MPF) from Xenopus oocytes and the possibility exists that this factor is a protein kinase which induces mitotic nuclear breakdown by phosphorylating the lamina, resulting in its depolymerization (Fisher, 1987; Miake-Lye & Kirschner, 1985).

Recent results indicate induction of lamin phosphorylation by ligands that activate protein kinase C (Fields et al.,


A
1 2 3 4 5 kD 6 7 8 9 10
205
116
97
66
45
B
1 2 3 4 5 kD 6 7 8 9 10
- + - + - + - + +

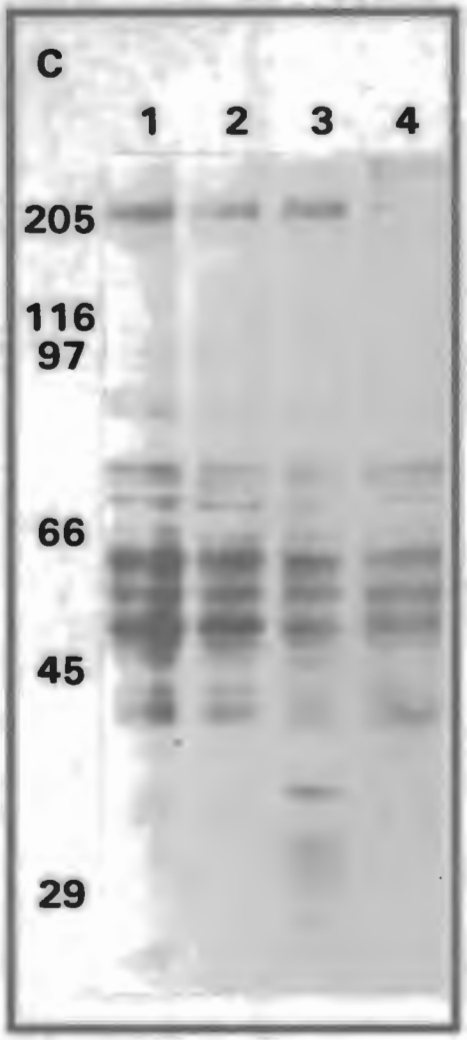


Figure 3.1. The Phosphorylation, N_3ATP - Binding and Concanavalin A - Binding Profile of Rat Liver Nuclear Envelopes Isolated by Different Methods.

Rat liver nuclear envelopes were isolated by various methods as described in 6.1.2.

A: Coomassie pattern of nuclear envelope isolated by the methods of 1) Bornens and Courvalin (1978) 2) Kaufmann et al. (1983) 3) Kay et al. (1972) 4) Kay et al. (1972) 5) Monneron et al. (1972). The nuclear envelopes in 3) are as for those in 4) except that they have undergone additional DNase 1 digestion. This not only removed additional DNA but also the histones bound to the latter i.e. the two prominent low mobility bands. The nuclear envelopes in lanes 6 - 10 correspond to those in lanes 1 - 5.

B: N_3ATP binding and phosphorylation pattern of the nuclear envelopes in A. The lane designations are as in A. Approximately 100 µg of nuclear envelope fraction was resuspended in 250 µl of incubation medium and incubated with either [γ^{32}P]N_3ATP (lanes 1 - 5) or [γ^{32}P]ATP (lanes 6 - 10) as described in 6.3.2 & 6.3.3, respectively. After incubation with [γ^{32}P]N_3ATP, the envelopes were either solubilised immediately without exposure to u.v. radiation (-) or were irradiated (+) prior to solubilisation in sample application buffer.
SDS PAGE and autoradiography were undertaken as described in 6.4.7.

C: Concanavalin A binding pattern of rat liver nuclear envelopes isolated by the methods of 1) Bornens and Courvalin (1978) 2) Kaufmann et al. (1983) 3) Kay et al. (1972) and 4) Monneron et al. (1972).
Concanavalin A studies were undertaken as described in 6.4.5.

1988; Hornbeck et al., 1988). The implication here is that the lamin kinase(s) is / are not lamina associated. There are a number of possible mechanisms of protein kinase - mediated phosphorylation of the lamins: ligands may translocate to the nucleus bound to specific complexes which then bind and activate protein kinase C (Hornbeck et al., 1988); protein kinase C is activated, resulting in a phosphorylation cascade, finally activating a membrane - bound lamin kinase with similar properties to protein kinase C; or protein kinase C may be cleaved to form a low molecular weight kinase (protein kinase M) which translocates to the nucleus. There is evidence for the latter model (Tapley & Murray, 1985). These authors have demonstrated protein kinase M - induced phosphorylation of purified lamin B.

Rottman et al. (1987) also provide evidence that the protein kinase responsible for the phosphorylation of certain nuclear pore complex lamina proteins (115, 90, 72 kDa) as well as topoisomerase 11 in the sponge *Goedia cydonium* is not a membrane - associated kinase but is instead protein kinase C which binds to the nuclear envelope upon activation by diacylglycerol.

Conversely, many nuclear envelope proteins (Lam & Kasper, 1979; Kletzien, 1981; Smith & Wells, 1983) are phosphorylated by cAMP - independent, Mg^{2+} - dependent kinases which appear to be endogenous to the nuclear envelope. Smith and Wells (1983) provide convincing evidence that this activity is confined to the nuclear pore complex. The stimulation of these kinases is independent of cyclic mono -nucleotides, calcium, and calmodulin (Lam & Kasper, 1979; Steer et al., 1980; Smith & Wells, 1983) although Steer and coworkers show a marked stimulation of kinase activity by cobalt.

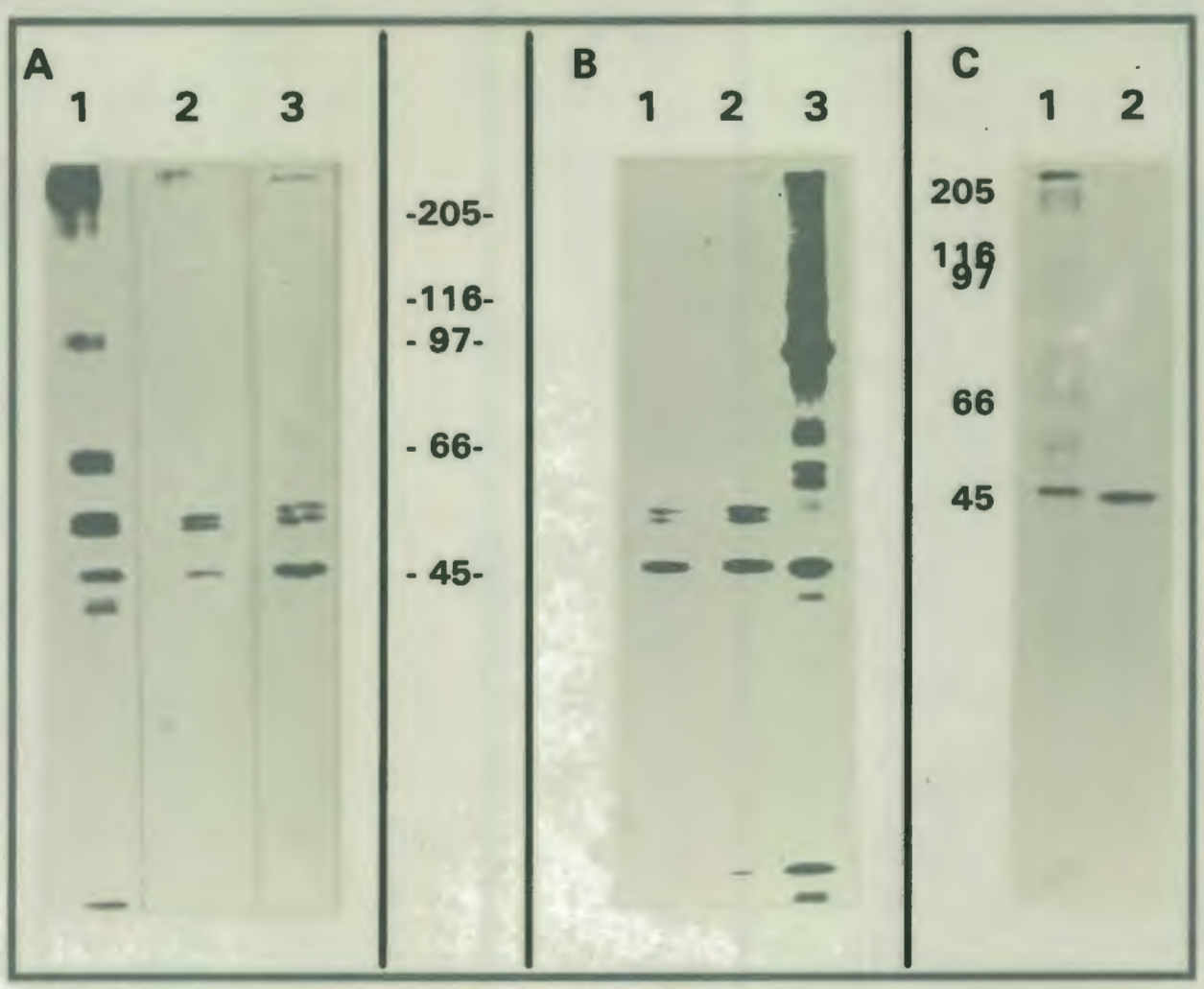


Figure 3.2 Comparison of the N_3ATP - Binding Profile of Cellular Substructures Isolated from Various Tissues.

Approximately 100 µg of each fraction was resuspended in 250 µl of incubation medium and incubated with [$\gamma^{32}P$]N_3ATP (6.3.2.) and subjected to one dimensional SDS PAGE and autoradiography (6.4.7).
The isolation procedures for plasma membrane, nuclear envelope, mitochondria, and metaphase chromosomes are detailed in sections 6.1.3., 6.1.2., 6.1.4., and 6.1.6. respectively.

A: 1) Rat liver plasma membrane 2) rat liver nuclear envelope isolated by the method of Bornens and Courvalin (1978) 3) rat liver mitochondria.

B: 1) Friend nuclear envelope isolated by the method of Bornens and Courvalin (1978) 2) Friend plasma membrane 3) Friend metaphase chromosomes.

C: 1) Hepatoma HIIE nuclear envelope isolated by the method of Bornens and Courvalin (1978) 2) hepatoma HIIE plasma membrane.

Nuclear envelope protein phosphatase activity has been reported to be Mg^{2+}-dependent and independent of cyclic mononucleotides (Steer et al., 1979 a & b). This activity is firmly associated with the nuclear envelope as witnessed by the fact that even 1.4 M sodium chloride does not reduce the envelope phosphatase activity (Steer et al., 1979 b). These authors describe the presence of multiple types of phosphatases.

Nucleoside Triphosphatases

The interpretation of experiments attempting to identify or characterize envelope proteins is difficult or often impossible because many of the published experimental details do not unequivocally exclude possible contamination with other subcellular fractions. There has recently been considerable progress toward the characterisation of the major rat liver nuclear envelope NTPase (46 kDa) which is postulated to be pivotal in insulin - stimulated mRNA efflux from the nucleus (see 4.1).
Photoaffinity probing of nuclear envelope with azido analogues of ATP has identified a 46 kDa protein thought to be a major nuclear NTPase (Clawson et al., 1984). Schröder et al. (1986) designate to the purified enzyme a molecular weight of 40 kDa and Konder - Koch et al. (1982) a molecular weight of 47 kDa.
There is conflicting evidence concerning the detergent solubility of this enzyme. Clawson et al. (1985) have shown it to be insoluble and have assigned it to the nuclear matrix. Opposing evidence does exist (Schröder et al., 1986). There is evidence however, that the enzyme is located at the nuclear pore complex (Agutter et al., 1979b), although this is not undisputed (Clawson et al., 1980a).

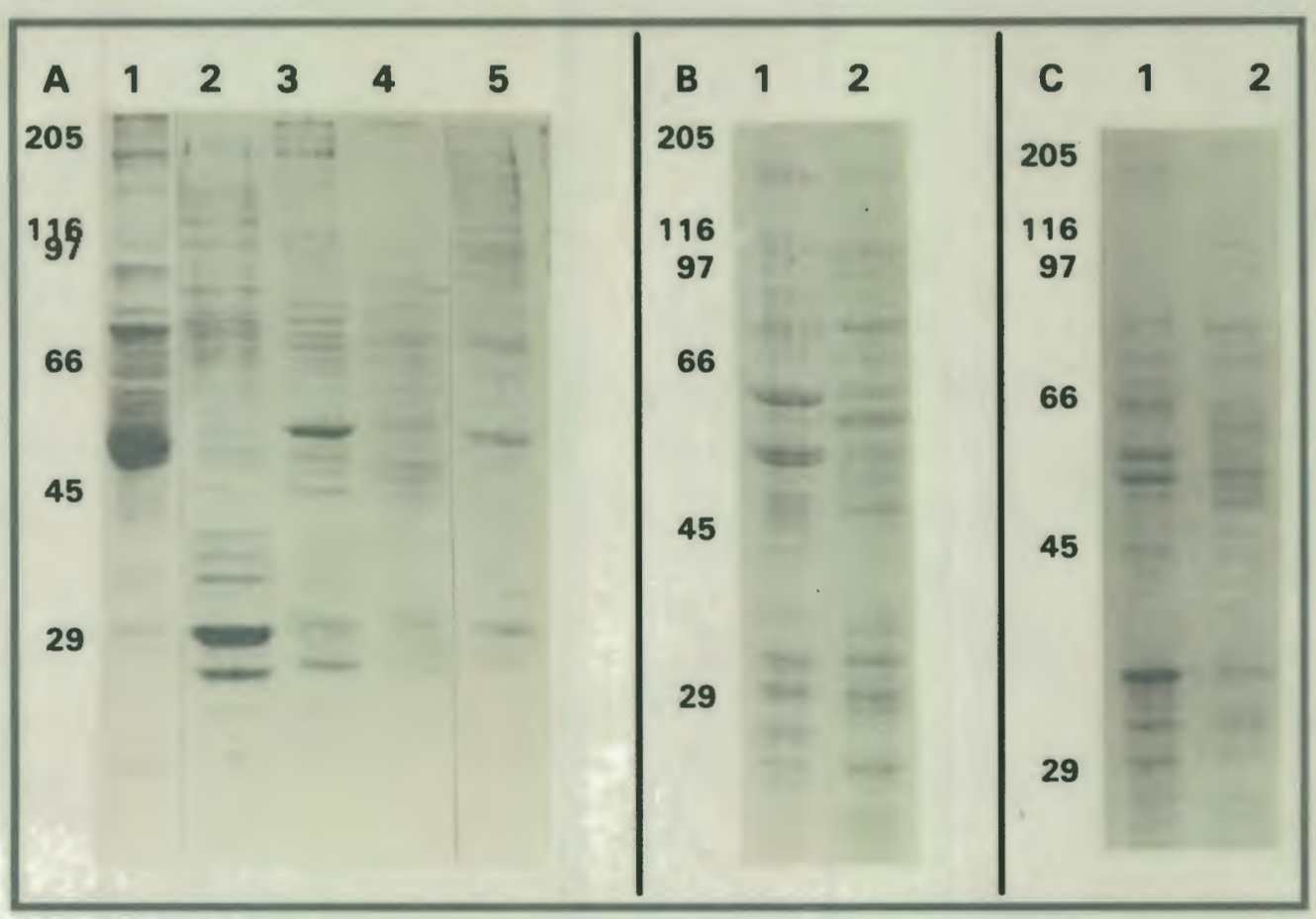


Figure 3.3 **Comparison of the Electrophoretic Pattern of Polypeptides from Cellular Substructures Isolated from Various Tissues.**

Approximately 100 µg of each fraction was solubilised in 60 µl of sample application buffer and subjected to one dimensional SDS PAGE (6.4.7).

A: Coomassie pattern of 1) rat liver nuclear envelope isolated by the method of Bornens and Courvalin (1978) 2) rat liver nuclei 3) rat liver plasma membrane 4) Friend nuclear envelope isolated by the method of Bornens and Courvalin (1978) 5) Friend metaphase chromosomes.

B: Coomassie pattern of 1) hepatoma HIIE plasma membranes and 2) hepatoma HIIE nuclear envelopes isolated by the method of Bornens and Courvalin (1978).

C: Coomassie pattern of 1) rat liver mitochondria and 2) rat liver endoplasmic reticulum.

The isolation of nuclei, nuclear envelopes, plasma membrane, mitochondria, and metaphase chromosomes is described in sections 6.1.1, 6.1.2, 6.1.3, 6.1.4, and 6.1.6 respectively.

Unlike other NTPases this enzyme has been reported to have a broad specificity, hydrolyzing ATP, GTP, UTP and even CTP (Purrello et al., 1982; Schröder et al., 1986). It has also been suggested that ADP may be utilized (Clawson et al.,1980b).

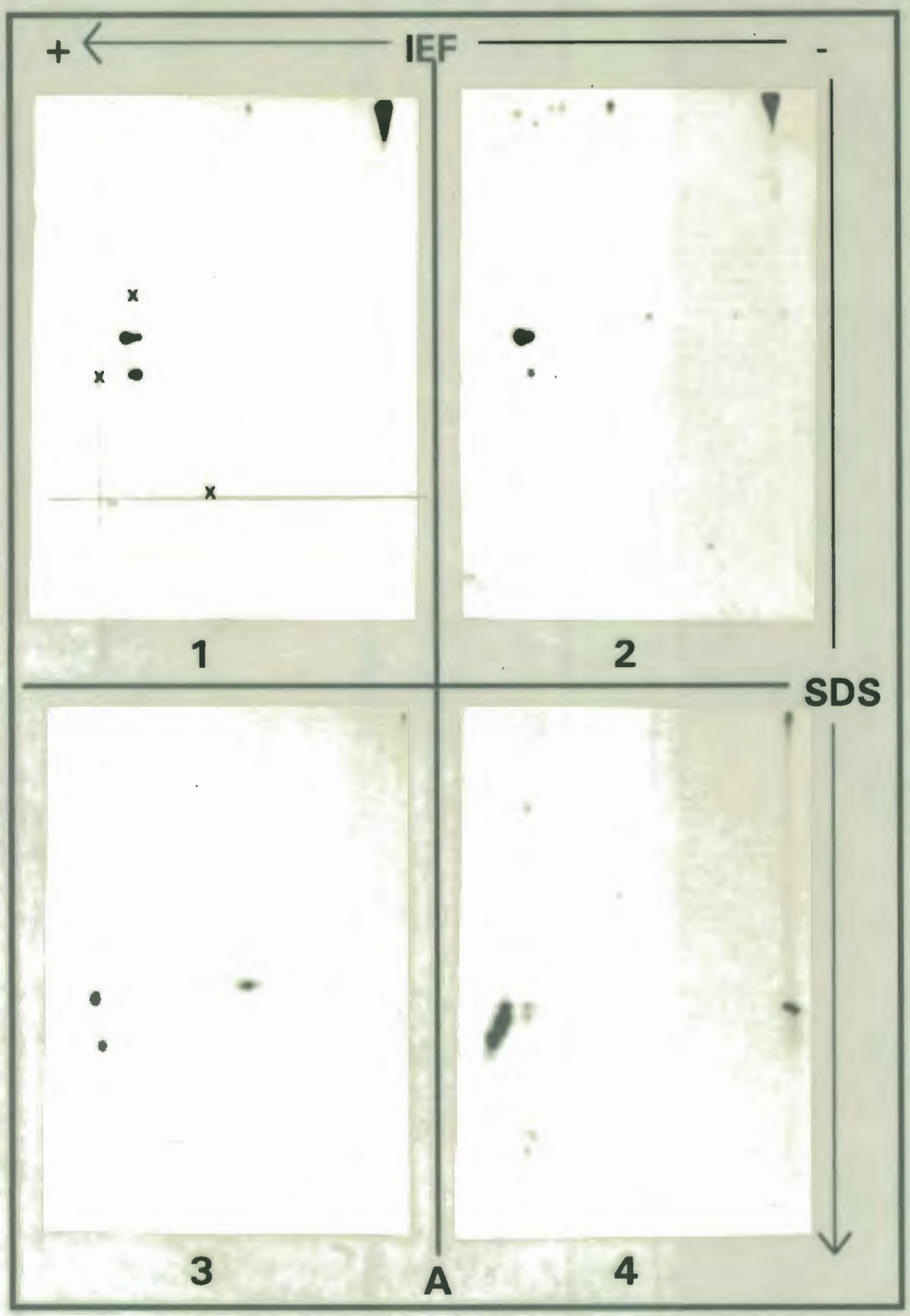
The stimulation of this ATPase by cAMP (Purrello et al., 1982) and the polyA tail of mRNA (Bernd et al., 1982; Clawson et al., 1984) has led to the conclusion that these NTPases may play a role as the energy - generator necessary for nucleo - cytoplasmic translocation of mRNA (see 4.1).

In previous investigations by other authors, the effects of insulin on the nuclear envelope protein phosphorylation state and NTPase activity has been expressed in terms of changes to the overall phosphorylation profile (Purrello et al., 1983) and to changes of the rate of ATP hydrolysis (Purrello et al., 1982), respectively. However, no individual proteins involved in the change of phosphorylation state of the nucleus has been identified. To assess the validity of the involvement of the state of phosphorylation of nuclear envelope proteins it would be important to demonstrate the effects (if any) of insulin on specific ATP - binding proteins (i.e. either protein kinases or ATPases). Any change in N_3ATP binding of these proteins in the presence of insulin would represent evidence for the direct modulation of these nuclear enzymes by insulin.

This chapter reports the results of the investigation of the N_3ATP - binding proteins of the rat liver nuclear envelope and the comparison of the identified ATPases / kinases with those of other cellular structures from rat liver and various cell lines. This is an important first step in the identification of nuclear envelope protein kinases / ATPases that may be directly modulated by insulin (see chapter 4).

3.2. Results

Prior to any studies on the effect of insulin on the rat liver nuclear envelope, it was important to determine whether the enzymatic profile of the isolated envelopes is dependent on the isolation methodology. Thus, rat liver nuclear envelopes were isolated by several different published methods and the ATP - binding and phosphorylation profiles compared (figure 3.1 A & B). Both the ATP - binding and phosphorylation pattern were found to be essentially independent of the isolation method employed. Consistently present in ATP - binding studies is a doublet at approximately 55 kD and a 46 kD species. Membranes isolated by the method of Monneron et al. (1972) (see 6.1.2.3) consistently had lower ATP - binding activity in the 55 kD range (figure 3.1 B, lane 5). The additional bands (figure 3.1 B, lane 4) present in nuclear envelopes isolated by the method of Kay et al. (1972) (6.1.2.4) represent nuclear contaminants (figure 3.1 A, lane 4) because further DNase 1 digestion eliminated these fractions (figure 3.1 A, lane 3). It is obvious from the phosphorylation pattern (figure 3.1 B, lanes 6 - 10) that the ATP - binding proteins in the 46 - 55 kD range are not autokinases. The heavy phosphorylation pattern (figure 3.1 B, lane 9) is due to nuclear contamination, as described above.
The Coomassie - staining pattern is also essentially independent of the isolation procedure (figure 3.1 A) and this is corroborated by the Concanavalin A - binding pattern (figure 3.1 C), reflecting similar qualitative glycoprotein enrichment in the nuclear envelope fractions. One significant difference is the relatively low abundance of protein with the mobility of the lamin proteins (60 - 70 kD) in envelopes isolated by the method of Bornens and Courvalin (1978) (see 6.1.2.1). This is in agreement with published observations which indicate the solubilisation of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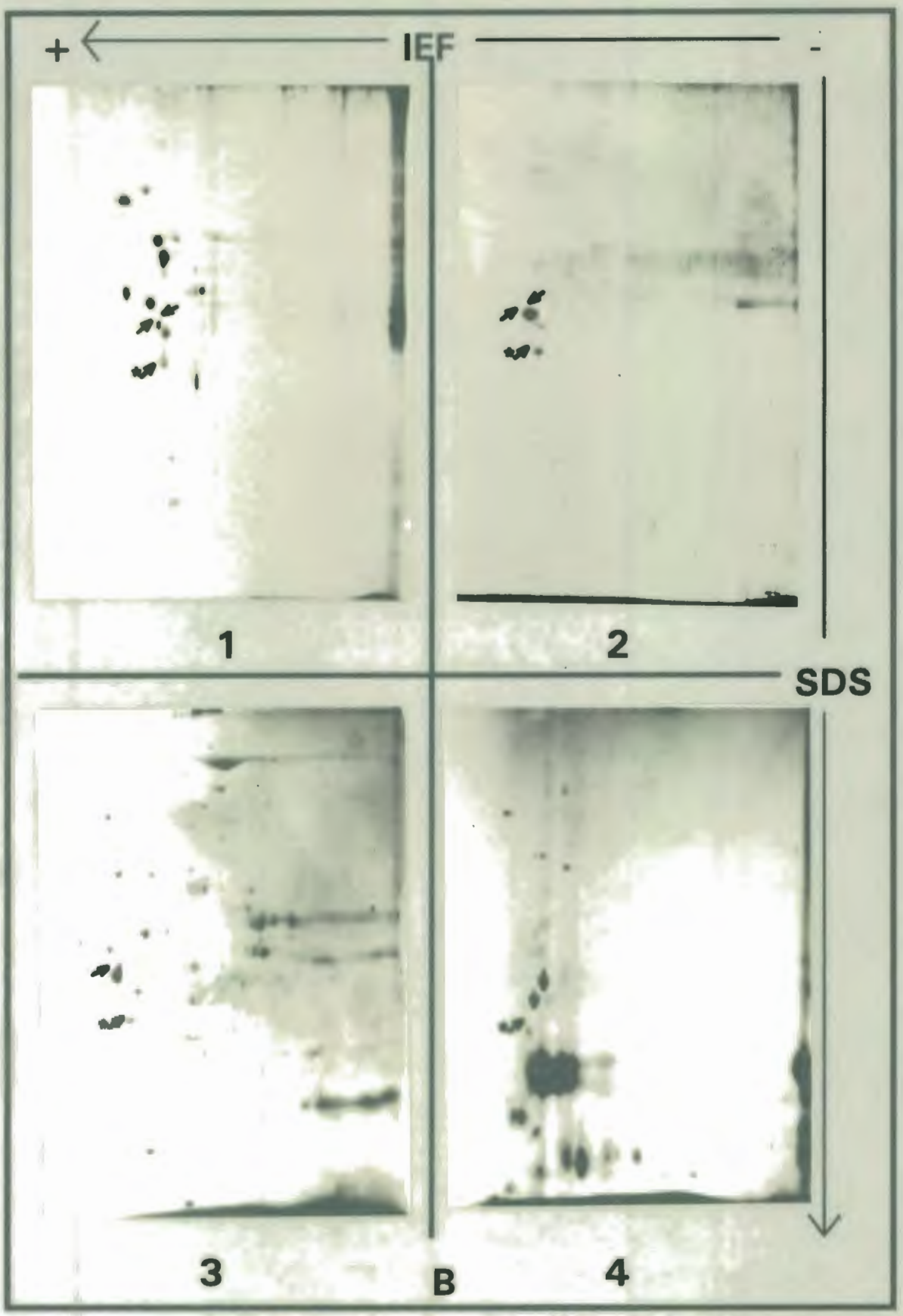
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Figure 3.4 Two Dimensional PAGE of N_3ATP - Labelled Rat Liver and Friend Fractions.

A: Autoradiographs (12 hour exposure) of [$\gamma^{32}P$]N_3ATP labelled fractions. Approximately 250 μg of each fraction was labelled (6.3.2) and subjected to two dimensional PAGE (6.4.7).
1) Rat liver nuclear envelope 2) rat liver plasma membrane 3) rat liver mitochondria 4) Friend metaphase chromosomes. The crosses (x) indicate the positions of three marker proteins - from top to bottom: bovine serum albumin (M.W. 68kD), ovalbumin (M.W. 45kD; pI 4.8), and carbonic anhydrase (M.W. 29kD; pI 6.1).

B: Coomassie pattern. 1 - 4 correspond to 1 - 4 in A. The arrows indicate the positions of the azido ATP labelled proteins in A. The arrow indicated by the asterics (*) indicates the position of the 46 kD, azido labelled protein.

For both A and B, the migration in the first dimension electrophoresis (IEF) is from right to left. Migration in the second direction electrophoresis (SDS) is from top to bottom.

the lamins by heparin.

In order to establish whether the ATP - binding pattern found in rat liver nuclear envelopes is nuclear specific, a comparative study was undertaken. The ATP - binding pattern of rat liver mitochondria and plasma membrane was found to be essentially as that for nuclear envelope (figure 3.2 A), except for the presence of additional ATP - binding proteins in plasma membranes. The similarity in ATP - binding pattern of the various membrane preparations, including the extra - nuclear ones, is all the more striking in the light of the differences in Coomassie profile of the various fractions (figure 3.3).

To ensure that these results are not due to a coincidental similarity in electrophoretic mobility (i.e. similar molecular weight), certain of the fractions were analysed by two dimensional PAGE (figure 3.4). The ATP - binding pattern for rat liver nuclear envelope, plasma membrane, and mitochondria (figure 3.4 A; 1, 2, & 3 respectively) are almost identical, indicating that these structures possess a set of ATP - binding proteins of the same molecular weight and isoelectric point. It is evident from figure 3.4 B that the N_3ATP - labelled proteins are closely related to Coomassie stained proteins. That the autoradiographic spots do not coincide precisely with a major Coomassie spot (figure 3 B) indicates that only a fraction of the protein is phosphorylated. This small phosphorylated component, being more acidic, migrates further in the first dimension isoelectric focussing gel. It was thus concluded that these subcellular fractions from various sources share a common set of ATP - binding proteins.

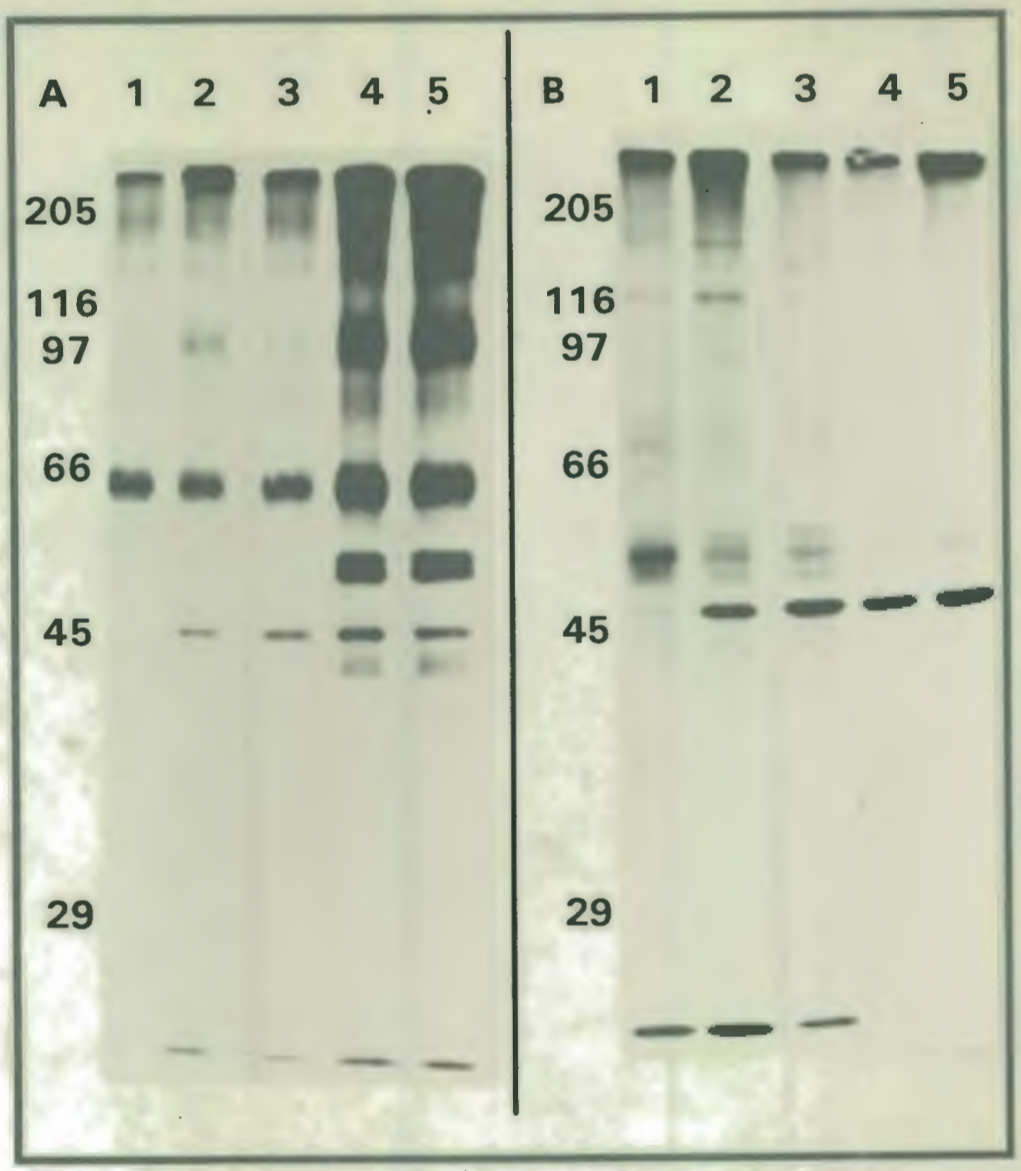


Figure 3.5 Determination of the GTP Binding Characteristics of the Rat Liver Plasma Membrane and Nuclear Envelope Azido Binding Proteins.

A: Approximately 150 µg of plasma membrane (6.1.4) was resuspended in 250 µl of incubation medium and incubated with [γ^{32}P]N_3ATP (6.3.2) in the presence of various concentrations of GTP and ATP.
1) 10 µM ATP, 2) 500 nM ATP 3) 10 µM GTP 4) 500 nM GTP 5) no GTP.
B: Approximately 150 µg of nuclear envelope isolated by the method of Kaufmann et al. (1983) was resuspended in 250 µl of incubation medium and GTP and ATP binding characteristics determined as for plasma membrane.
1) 10 µM ATP 2) 500 nM ATP 3) 10 µM GTP 4) 500 nM GTP 5) no GTP.

The GTP - binding characteristics were also investigated. This was undertaken as a first step in the possible identification of nuclear envelope G proteins. Having concluded that the 55 kD doublet and the 46 kD protein are present in all the fractions investigated, GTP - binding studies were undertaken only on rat liver nuclear envelope and plasma membranes. The results (figure 3.5 A & B) indicate that GTP is not a substrate for either the plasma membrane or nuclear envelope 46 kD protein. In contrast, at 500 nM ATP, there is a marked decrease in N_3ATP - binding (figure 3.5 A & B, lanes 2).

To further investigate the identity of the 46 kD, various membrane fractions were preincubated with quercetin, ouabain, and colchicine, and the inhibition characteristics compared. As evidenced in figure 3.6 A - C, the characteristics, namely prominent inhibition of ATP binding by quercetin, are identical, irrespective of the membrane investigated.

The possibility that the presence of this seemingly ubiquitous 46 kD protein (as well as the 55 kD doublet) in nuclear envelope and also in chromosome preparations may be a result of cross - contamination during the isolation procedure was investigated. To this end N_3ATP - labelled rat liver mitochondria, plasma membrane, and endoplasmic reticulum were added to separate non - radioactive rat liver homogenates prior to the first centrifugation and nuclei and nuclear envelopes were then isolated by established methods. The autoradiographs after comparable exposure time (3 days) of the subsequent SDS PAGE gels revealed no evidence of a radioactive 46 kD species in the nuclear envelope lanes (figure 3.7, 4 - 6). The autoradiographs were exposed for an additional 2 weeks, without any appearance of a 46 kD band. Thus cross - contamination during the isolation procedure as a cause for the ubiquitous presence of the 46 kD protein can be excluded.

Rat liver cells were the principal substrate for this investigation. However, for comparative purposes, other cells were investigated. Figure 3.2 C demonstrates the presence of the 46 kD N_3ATP - binding polypeptide in hepatoma cells. The ATP - binding profile of Friend cell nuclear envelope (figure 3.2 B) is identical to that of the rat liver. The doublet at approximately 55 kD is absent in the metaphase chromosome fraction derived from these cells (figure 3.2 B, lane 3). The presence of the 46 kD N_3ATP - binding polypeptide was confirmed by the 2D PAGE analysis (figure 3.4 A, autoradiogram 4).

3.3. Discussion

The results presented in this chapter demonstrate that there is present in all rat liver subfractions investigated, a ubiquitous, ATP - binding 46 kD polypeptide chain. All these fractions also share two polypeptides in the range 53 - 58 kD, which bind ATP.

The N_3ATP - binding 55 kD doublet present in all membrane fractions in all likelihood represents the same polypeptide because of identical molecular weight (figure 3.2 A, B, & C), isoelectric point (figure 3.4 A), and response to inhibitors (fig 3.6).

The ubiquitous 46 kD N_3ATP - binding protein exhibits identical properties in terms of molecular weight (figure 4.2), isoelectric point (figure 3.4 A) and inhibition characteristics (figure 3.6). The widespread distribution has been demonstrated not to be an artefact of the isolation procedures (figure 3.7, lanes 4 - 6), and is in accordance with the findings of Rubins et al. (1990), who have demonstrated the existence of a 47 kD polypeptide in the nuclear envelope and rough endoplasmic reticulum.

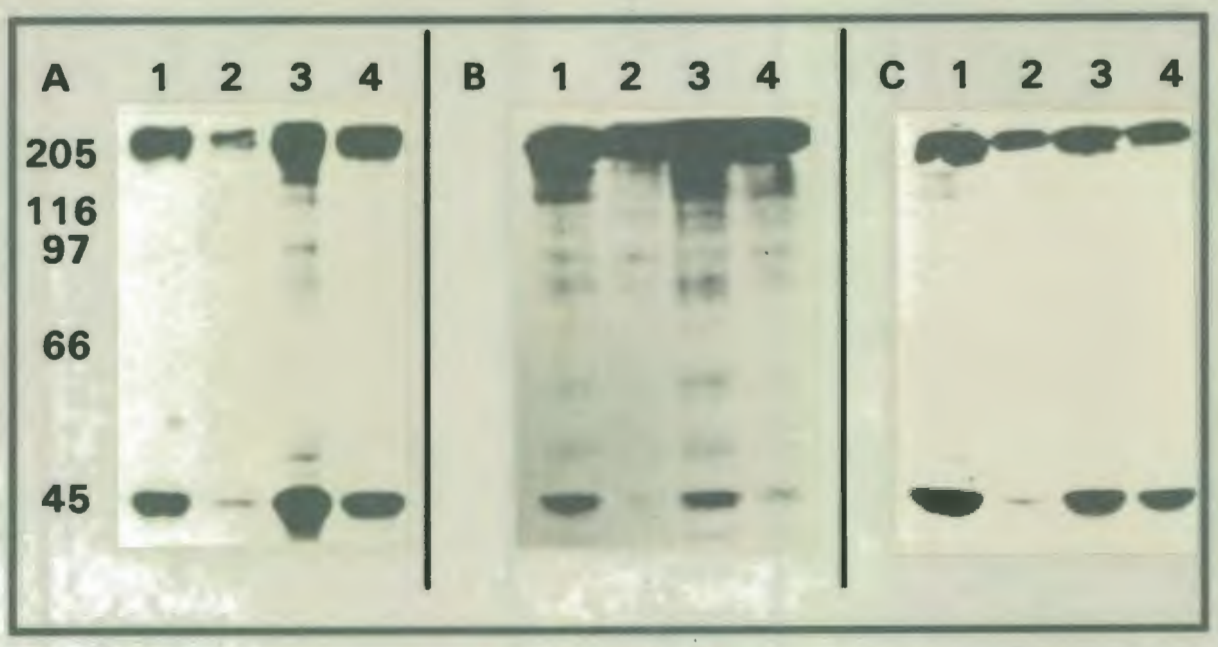


Figure 3.6 Sensitivity of the 46kD Protein from Various Subcellular Fractions to the Inhibitors Quercetin, Oubain, and Colchicine.

150 µg of each fraction was resuspended in 300 µl of incubation medium and preincubated with inhibitor for 5 minutes at 22°C. Each fraction was then labelled with [γ^{32}P] N_3ATP (6.3.2). After irradiation, the fractions were pelleted, solubilised in 70 µl of sample application buffer and subjected to one dimensional SDS PAGE and subsequent autoradiography (6.4.7).

A) Rat liver nuclear envelope B) rat liver plasma membrane C) rat liver endoplasmic reticulum
For A - C: 1) no preincubation with inhibitor 2) preincubation with quercetin (10 µg / ml) 3) preincubation with oubain (1 mM) 4) preincubation with colchicine (1 mM).

This 46 kD protein shares many of the characteristics of the NTPase reported to be involved in nucleocytoplasmic translocation of mRNA. It binds N_3ATP in a specific manner (figure 3.1 B, lanes 1 - 5); it has a molecular weight similar to that reported by Clawson et al. (1984); in agreement with the findings of the above authors, it is not phosphorylated nor solublized (Agutter et al., 1979a); and the inhibition characteristics are similar to those reported for nuclear envelope ATPases (Agutter et al., 1979b).
The 46 kD protein, despite being a minor envelope component, is highly labelled by N_3ATP (figure 3.1 A & B, lanes 1 - 5). This high affinity is in accordance with the results of Clawson et al. (1984), who reported the specific activity of this band to be up to 20 times greater than that of the more abundant 58 kD band.
The isolation procedures for nuclear envelopes, in general, do not affect the yield of ATP - binding proteins (figure 3.1 B, lanes 1 - 5). However, the phosphorylation pattern is more sensitive to the isolation parameters (figure 3.1, lanes 6 - 10) reflecting slight variations in protein kinase and / or substrate content.
The inhibition characteristics are similar to those reported for both the nuclear envelope - bound and purified NTPase (Agutter et al., 1979b; Schröder et al., 1986). As figure 3.6 illustrates, quercetin (10 μg / ml) significantly reduces labelling of the 46 kD protein. At this concentration, labelling was reduced by 80%. This inhibitory effect by quercetin is much more pronounced than that reported for the isolated NTPase (Schröder et el., 1986) and is in keeping with the observations of Agutter et al., (1979a & b) for the general nuclear envelope NTPase. The inhibition by quercetin is at odds with the report by Clawson et al. (1984), stating no inhibition of photolabelling of the 46 kD protein to occur by this inhibitor in the presence of 5 mM $MgCl_2$.

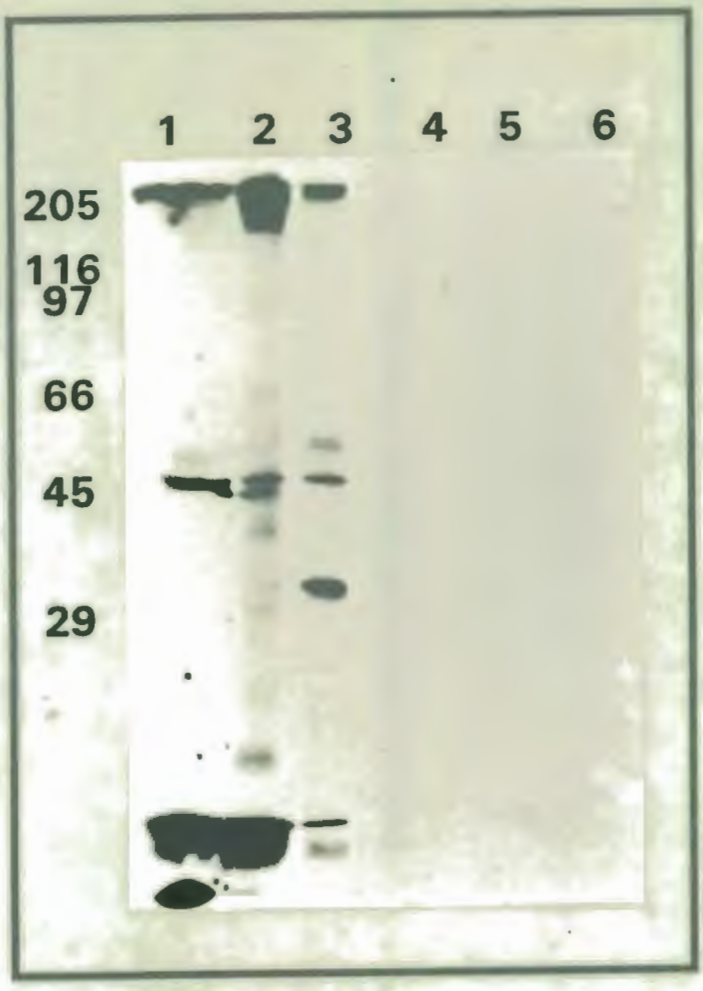


Figure 3.7 **Absence of Cross - Contamination of the Subcellular Fractions Investigated.**

[$\gamma^{32}P$]N_3ATP labelled mitochondria (approximately 2 x 10^7dpm, plasma membrane (approximately 4 x 10^6 dpm), and endoplasmic reticulum (approximately 3 x 10^6 dpm), was washed extensively to remove all excess label and pelleted. A rat liver homogenate (30 g liver) was divided in three prior to the first 1000g centrifugation (6.1.1) and to each of these fractions was added one of the N_3ATP labelled subcellular fractions. Nuclei were then isolated as described in 6.1.1 and subsequently nuclear envelopes were isolated by the method of Bornens and Courvalin (1978). These envelope fractions were then subjected to one dimensional SDS PAGE and extensive autoradiography (6.4.7).

1) N_3ATP - labelled mitochondria 2) N_3ATP - labelled plasma membrane 3) N_3ATP labelled endoplasmic reticulum 4) nuclear envelope isolated in the presence of labelled mitochondria 5) nuclear envelope isolated in the presence of labelled plasma membrane 6) nuclear envelope isolated in the presence of labelled endoplasmic reticulum.

This discrepancy may reflect a difference in methodology for the inhibition studies, the details of which have not been reported by Clawson et al. (1984). The use of quercetin for the identification of protein kinases (Clawson et al., 1984) is of doubtful value in the light of reports by Cantley and Hammes (1976) and Lang and Racker (1974). The investigations by these authors suggest that quercetin binds to ATP - binding sites in general and can therefore not be used as a tool to differentiate between protein kinases or ATPases. Ouabain was found to have no inhibitory effect on the ATP - binding 46 kD polypeptide.

The effect of colchicine on N_3ATP labelling of the 46 kD protein was found to be very similar to that reported by Agutter et al. (1979a). A low level of inhibition of N_3ATP - binding by concentrations of colchicine demonstrated to inhibit nucleocytoplasmic mRNA transport (Agutter and Suckling, 1982) is in keeping with previous observations (Agutter et al., 1979a & b). Also, the isolated nuclear envelope NTPase has been shown to be unaffected by colchicine (Schröder et al., 1986), indicating that this inhibitor does not directly interact with the NTPase. A model for colchicines actions in the inhibition of nucleocytoplasmic mRNA efflux has been postulated by Agutter and Suckling (1982) in which colchicine does not interact with the NTPase, but rather with a protein in the pore lamina complex.

The GTP competition result (figure 3.5) is in keeping with the report of Rubins et al. (1990) indicating that the ATP - binding of the 46 kD polypeptide is inhibited only by ATP and not GTP. Parenthetically, the 55 kD doublet of the nuclear envelope is not ATP specific. This experimental agreement reinforces the conclusion that the 46 kD polypeptide investigated in this thesis is indeed the NTPase putatively involved in nucleocytoplasmic translocation of mRNA.

The postulated role of the 46 kD polypeptide as the specific energy generator for mRNA nucleocytoplasmic translocation in the rat liver (see 4.1) is difficult to reconcile with its widespread occurrence. These findings call into question the assumption that this protein is a specific nuclear envelope / nuclear matrix component (Clawson et al., 1984; Clawson et al., 1986; Clawson et al., 1985), facilitating nucleocytoplasmic translocation of mRNA. I have demonstrated that it is found in diverse cellular membranes. Thus, this polypeptide may rather be a general ATPase, and as such, a component in an energy generating system possibly resulting, when it is located in the nuclear envelope, in the facilitation of mRNA efflux.

The other alternative, that the 46 kD protein is not an ATPase but rather an ATP binding protein of a different nature, is open to investigation. A possible candidate may be the ubiquitous G actin. However, it has a molecular weight of only 42 kD and an isoelectric point of 5.8 versus 5.2 of the protein under investigation here (Elzinga et al, 1973). Furthermore, the monomeric G actin exchanges ATP only once its metal binding site is free - if occupied by Ca^{2+} or Mg^{2+} the ATP is fixed (Strezelecka-Golaszewska, 1973). Under the conditions chosen in my experiments i.e. 5 Or 10 mM Mg^{2+} all binding sites in the putatively present G actin should be fully occupied by metal, making the observed exchange against azido ATP unlikely. In addition, G actin is only partially saturated at 200 μM ATP, whereas the protein described here has its ATP binding site occupied at 10 μM.

Chapter 4

The *In Vitro* Effects of Insulin on the ATP - Binding and Phosphorylation of Rat Liver Nuclear Envelope Proteins

4.1. Introduction

There is evidence from *in vitro* studies that intracellular insulin binding leads to a decrease in the phosphorylation of nuclear envelope protein (Purrello et al., 1983); the stimulation of nuclear envelope NTPase activity (Purrello et al., 1982); and the stimulation of mRNA efflux (Bolander et al., 1981; Purrello et al., 1983). The concomitant increase in dephosphorylation has been interpreted to be the result of a stimulation of endogenous nuclear envelope phosphatases and not an inhibition of protein kinases (Purrello et al., 1983).

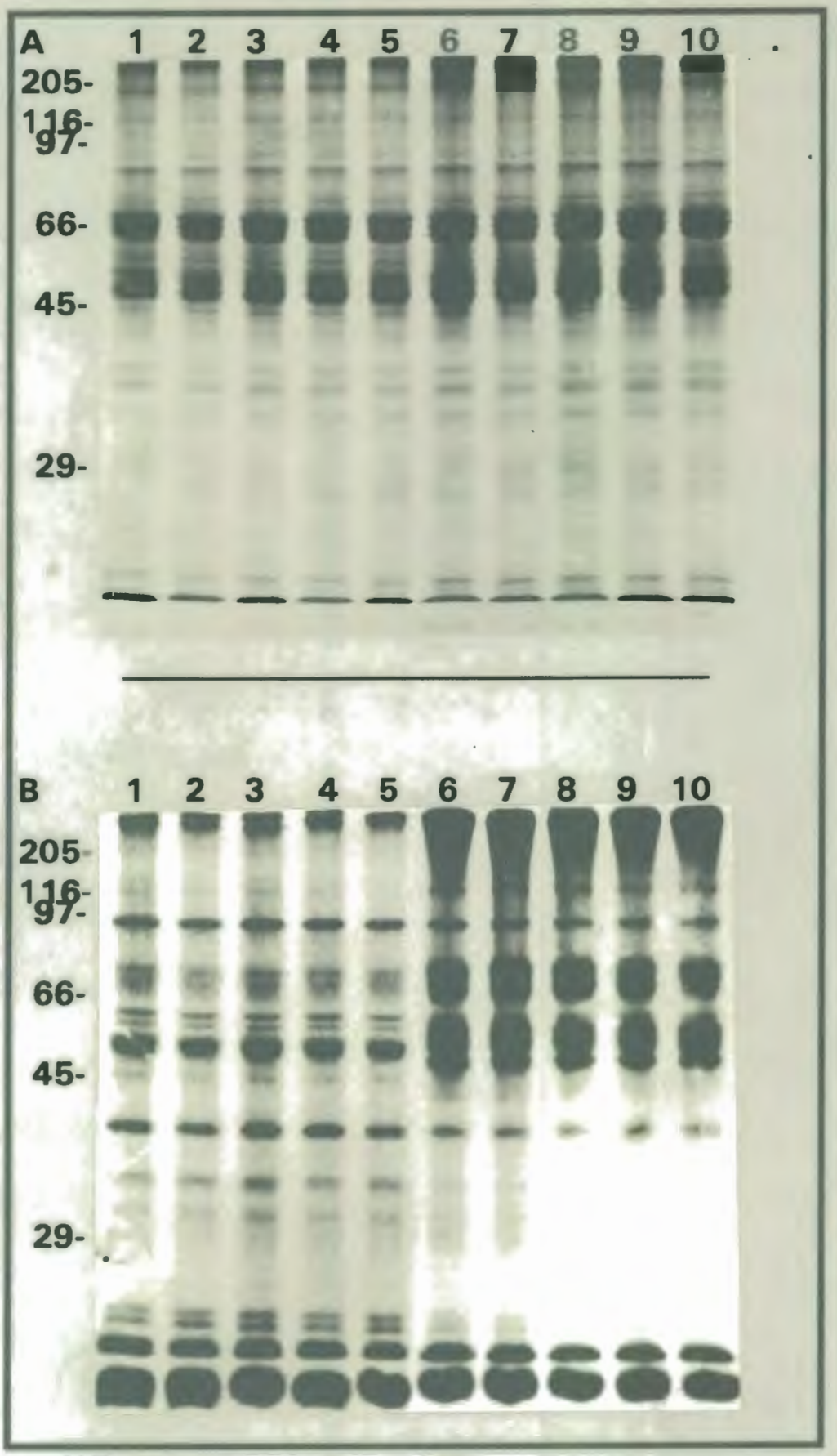
mRNA efflux is an ATP - dependent process (Ishikawa et al., 1978), the ATP being hydrolysed by the nuclear envelope NTPase (Clawson et al., 1980a & b; Agutter, 1979a & b; Agutter et al., 1976). The NTPase putatively responsible for mRNA efflux is thought to be a nuclear pore complex component, an almost inevitable location for an enzyme involved in nucleocytoplasmic translocation.

That the stimulation of nuclear envelope dephosphorylation, NTPase activity, and mRNA efflux, are all maximal at an insulin concentration of 10 pM, has suggested a link between all these phenomena and has led to the development of a model for the direct regulation of the functional state of nuclear envelope proteins and mRNA efflux by insulin (Purrello et al., 1983). Briefly, this model proposes that

nuclear - bound insulin stimulates nuclear envelope phosphatases, which in turn dephosphorylate the NTPase. The activated NTPase is then capable of increased ATP hydrolysis, thus providing the required energy for mRNA efflux. This regulation of mRNA efflux by insulin has been extensively reviewed (Goldfine et al., 1982a & b; Goldfine et al., 1985).
This empirical model is based on 3 separate observations i.e. the stimulation of dephosphorylation, NTPase activity, and mRNA efflux. To obtain direct evidence of the modulation of the NTPase activity by insulin, purified rat liver nuclear envelopes were labelled with [γ^{32}P]N_3ATP, after preincubation with varying concentrations of insulin. Any increase in ATP - binding, especially in an insulin concentration dependent manner, would represent, to my knowledge, the first unequivocal evidence of a direct effect of insulin on this NTPase activity. This chapter summarises the results of such an investigation.

4.2. Results

Rat liver nuclear envelopes isolated by the method of Kaufman et al. (1983) were preincubated with varying concentrations of insulin, prior to incubation with [γ^{32}P]-N_3ATP. The labelled membrane fractions were then subjected to SDS PAGE and autoradiography. A typical result is presented in figure 4.1 (A - D).
Other conditions of insulin preincubation time, insulin concentration, and [γ^{32}P]N_3ATP incubations times were used, with no effect on the labelling pattern, either quantitatively or qualitatively (results not shown). The insulin preincubation times in figure 4.1 i.e. 5 minutes and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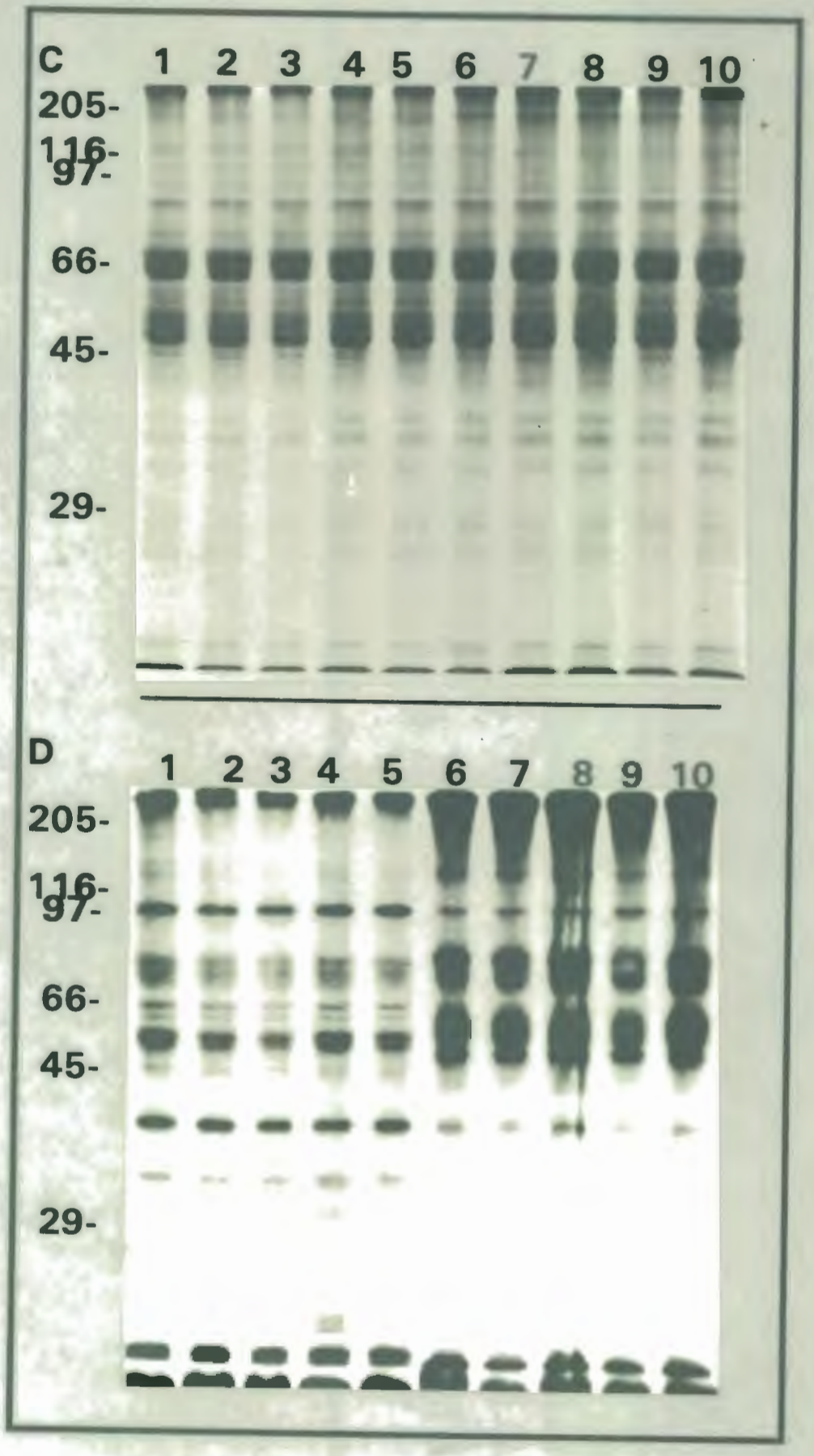
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D
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Figure 4.1. The Effect of Insulin on the Phosphorylation And [γ^{32}P]N_3ATP - Binding Pattern in Isolated Rat Liver Nuclear Envelopes.

Rat liver nuclear envelope (150 µg in 250 µl of incubation medium), isolated by the method of Kaufman et al.(1983) were incubated for 2 minutes with [γ^{32}P]ATP or [γ^{32}P]N_3ATP (made to 5 µM with cold ATP) after preincubation with varying concentrations of cold insulin for 30 minutes or 5 minutes. After incubation, the phosphorylation reactions were terminated by pelleting the membranes and immediately solubilising in 70 µl of sample application buffer. The N_3ATP incubations were terminated by pelleting and washing the envelopes with cold incubation medium (3x). These envelopes were then resuspended in incubation medium, irradiated (see 6.3.2.) and solubilised in 70 µl sample application buffer.

A: Coomassie pattern of envelopes preincubated with insulin for 30 minutes.
B: Autoradiogram of gel A.
C: Coomassie pattern of envelopes preincubated with insulin for 5 minutes.
D: Autoradiogram of gel C.

Lanes 1 - 5: nuclear envelopes incubated with [γ^{32}P]ATP
Lanes 6 - 10: nuclear envelopes incubated with [γ^{32}P]N_3ATP.

Lanes: 1 & 6) no insulin; 2 & 7) 10 pM insulin; 3 & 8) 100 pM insulin 4 & 9) 10 nM insulin; 5 & 10) 100 nM insulin.

30 minutes were chosen to represent short and medium term effects of insulin, respectively.
It can be concluded from the results, that under these experimental conditions, insulin has no direct *in vitro* effect on either the N_3ATP labelling or phosphorylation pattern in rat liver nuclear envelopes. The slight variations in labelling intensity are due to small differences in the quantity of material loaded onto the gels, and does not represent an insulin concentration - dependent change.

4.3. Discussion

The results shown in figure 4.1. provide no evidence that insulin directly modulates nuclear envelope phosphorylation activity nor increases ATP - binding by the NTPase. This does not, however, preclude insulin stimulation of NTPase activity (section 4.1). The (putative) model of insulin action on the nuclear envelope is based on 3 *independent* observations of insulin effect i.e. a general decrease in phosphorylated proteins due to a stimulation of phosphatase activity (Purrello et al., 1983); an increase in ATP hydrolysis due to an increase in Vmax (Purrello et al., 1982); and an increase in mRNA efflux (Purrello et al., 1983; Bolander et al., 1981).It is generally accepted that nuclear envelope NTPase activity is due to a single class of protein and this is borne out by the labelling patterns obtained in this investigation (figure 3.2.). Histochemical studies (Clawson et al., 1980a) suggest that the NTPase may be evenly distributed over the entire nuclear envelope. The NTPase involved in the mRNA efflux mechanism, situated at the

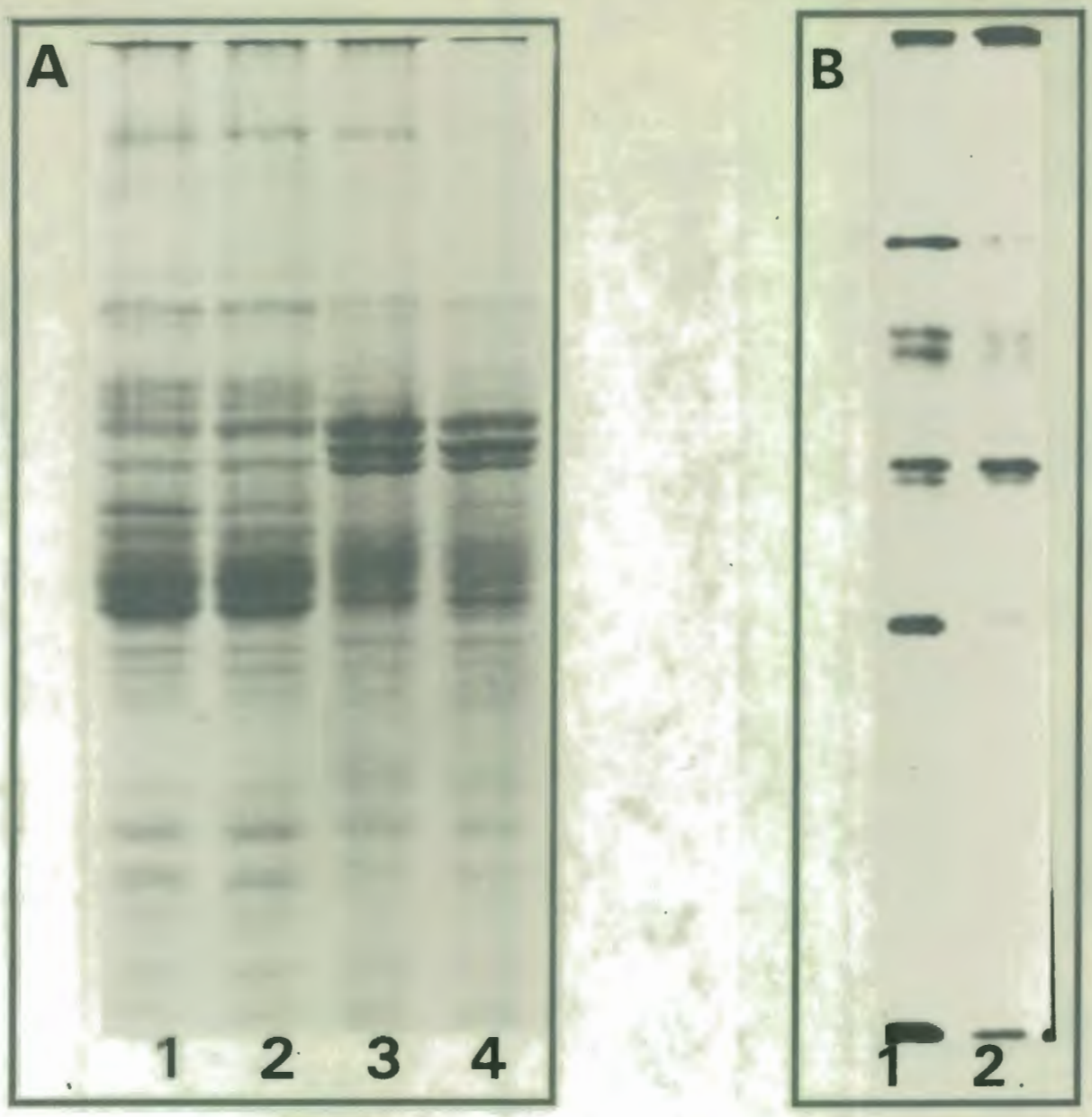

Figure 4.2 **The Effect of Streptozotocin on the Protein Profile and Enzymatic Activity of Rat Liver Nuclear Envelope.**

A: Nuclear envelopes from normal (1 and 3) and streptozotocin - treated rats (2 and 4) were isolated by the methods of Bornens and Courvalin (1978) (1 and 2) and Kaufmann et al. (1983) (3 and 4). Approximately 150 µg of each fraction was subjected to one dimensional SDS PAGE as described in (6.4.7.3).
B: Nuclear envelopes isolated by the method of Kaufmann et al. (1983) from normal (1) and streptozotocin treated rats (2) were resuspended in 250 µl incubation medium and incubated with 10 µM [γ^{32}P]ATP as described in 6.3.2. The labelled membranes were subjected to one dimensional SDS PAGE and autoradiography (6.4.7.3).

nuclear pore complex, may thus represent only a fraction of the total NTPase content. Thus any insulin - induced change in ATP - binding in the pore complex - located fraction of the NTPase may be masked by the overwhelming presence of the non - pore complex associated NTPase. This could explain the observation that insulin stimulates NTPase activity, as assayed by hydrolysis of ATP (Purrello et al., 1983), but not ATP - binding (figure 4.1.). The primary target of insulin would, however, have to be sought in a nuclear pore - specific mediator which activates only the pore located NTPase. Accepting such an explanation would make the findings in figure 4.1 reconcilable with the model proposed by Purrello et al. (1983). However, there is no evidence at this stage that insulin interacts with any of the core complex components.

The degree of stimulation may also be insufficient to be detected by the direct labelling method. A concentration of 10 pM insulin has been demonstrated to stimulate the NTPase activity by approximately only 30% (Purrello et al., 1982). If this level of stimulation is accepted as reality, then the lack of an insulin effect may indicate that insulin affects the NTPase at the post ATP - binding stage i.e. Vmax is increased due to an increase in the rate of hydrolysis or an increase in ADP - dissociation. These could arise from subtle conformational changes due to insulin induced phosphorylation / dephosphorylation of specific residues. In either case, there is a strong possibility that this would not result in a detectable change in ATP - binding.

The autoradiograph of the N_3ATP - binding profile (figure 4.1) has been overexposed and printed on low contrast photographic paper in order to accentuate any differences in the 46 kD band. A consequence of this is the pronounced background, indicting that the $[\gamma^{32}P]N_3ATP$ is being utilised as a phosphate donor by endogenous protein kinases. This is in accordance with previous findings (Sabbatini & von Holt, 1987).

The lack of discernable stimulation of dephosphorylation by insulin (figure 4.1. B & D, lanes 1 - 5) is not in accordance with the findings of Purrello et al. (1983) who reported a 40% decrease in phosphorylated proteins with 10 pM insulin, and may represent differences in methodology. These authors used nuclear envelopes isolated by the method of Monneron et al. (1972) from streptozotocin treated rats. This isolation method produces nuclear envelopes high in the 46 kD NTPase activity but relatively low in phosphorylation activity (figure 3.1.). The experiments reported here utilised nuclear envelopes isolated by the method of Kaufmann et al. (1983) from untreated, healthy rats. These envelopes display a slightly different phosphorylation pattern (figure 3.1.). More importantly, these envelopes were isolated from non - streptozotocin treated rats. I have executed intensive trials involving this treatment in order to obtain envelopes with reproducible enzymatic pattern. In the majority of animals the livers were very enlarged with mottled lipid deposits all over the tissue, giving the morphological impression of a generally toxic liver damage. Rather than use such damaged tissue, it was deemed preferable to investigate "non-diabetic" rats at higher insulin concentrations (figure 4.1.). The result represented in figure 4.2 is of interest since it clearly illustrates that despite very similar Coomassie patterns, the N_3ATP - binding profile is quantitatively very different, possibly indicative of the diseased state of the tissue from streptozotocin - treated rats. To accept these differences as a specific result of insulin deficiency would have required investigations beyond the scope of my project. In conclusion, the results reported here show no effect of insulin on ATP - binding in liver cell nuclei from rats not poisoned with streptozotocin.

Chapter 5

Discussion And Summary

There are a number of experimental results which have been interpreted to demonstrate direct control of nuclear envelope biological activity by insulin, for example, the stimulation of phosphatase (Purrello et al., 1983) and NTPase activity (Purrello et al., 1982). At the insulin concentration at which this stimulation is maximal (10 pM), mRNA efflux from isolated nuclei is also maximally stimulated (Purrello et al., 1983). Thus a model for the direct modulation of mRNA efflux by insulin has been formulated, with the nuclear envelope NTPase the energiser of this process (Purrello et al., 1983, Goldfine et al., 1982a & b).

Presented in this thesis are the results of my investigation into the putative direct modulation by insulin of mRNA efflux from the nucleus, thought to be facilitated through an effect on nuclear phosphatases. The principal focus of this investigation has been the determination of the direct biological effects of insulin on nuclear envelope NTPases *in vitro*. This target has been chosen since it is the NTPase which is thought to provide the motive force for this mRNA efflux. Much of the present evidence in the literature is based on the measurement of the overall rate of *in vitro* hydrolysis of [γ^{32}P]ATP by NTPases of purified nuclear envelopes incubated with insulin (Purrello et al., 1982). My objective has been to demonstrate the presence of ATP - binding membrane - associated polypeptides, as the mediators of such an ATP hydrolysis. Furthermore, the investigations were aimed at ascertaining whether insulin directly affects the ATP - binding by certain of these putative NTPases.

In addition, I have attempted to identify changes in the phosphorylation pattern of nuclear envelope polypeptides as a result of insulin. The second focus of this investigation has been the probing for a specific insulin docking polypeptide in nuclear envelopes. This is important since one of the first stages of a direct insulin effect on the nucleus must be binding to a nuclear envelope receptor. Demonstration of the internalisation of the insulin - receptor complex and the specificty of *in vitro* binding to the nuclear envelope has been extensively reported in the literature (see 1.2.1). The kinetics of this binding has been demonstrated to differ from that of plasma membrane binding, thus implying that the specific nuclear envelope receptor differs from that of the the plasma membrane. To date, many of the investigations have focussed on kinetic studies of the association of ^{125}I - labelled insulin with isolated nuclei (Goldfine et al., 1982a & b; Goldfine 1987) or morphological studies employing insulin conjugated to electron - dense or fluorescent moieties (Carpentier et al., 1979, Schlesinger et al., 1978). These experiments have yielded valuable information but have not identified specific nuclear insulin - docking polypeptides *in situ*. The direct visualisation and identification of a nuclear envelope - associated receptor would corroborate the findings of Wong et al. (1988) who isolated a protein very similar to the plasma membrane receptor, from nuclei. The demonstration of a docking protein in intact nuclei i.e. still associated with a membrane structure, would constitute strong support for the view that some of the biological effects of insulin are the result of a direct interaction with the nucleus.

To accomplish the objective of probing for insulin and ATP - binding polypeptides, use has been made of insulin and ATP analogues which can be covalently attached to those polypeptides with which they specifically interact, thereby

specifically labelling them.
To facilitate this it has been necessary to develop protocols for the synthesis of carrier - free, radioactive and photoactivatable analogues of both insulin and ATP. These have been fully characterised and used to probe for their respective binding polypeptides and ultimately to determine whether the putative ATP - binding proteins are directly modulated by insulin *in vitro*.
An in depth discussion of the synthesis and use of these analogues has been made (see sections 1.1.3, 1.2.3, 2.3, 3.3, 4.3), but various aspects require further mention.

The enzymatic protocol for the synthesis of $[\gamma^{32}P]N_3ATP$ represents an innovative approach to the synthesis of photoactivatable, radioactive ATP analogues (Sabbatini & von Holt., 1987). This protocol allows for the synthesis, in less than 60 minutes, of carrier - free $[\gamma^{32}P]N_3ATP$ in a single step without the need for extensive purification. This is a significant advantage over the exchange reaction of Glynn and Chappel (1964) or the method developed by Potter and Haley (1983) which requires subsequent more complex purification of the product. The advantage of this enzymatic method over the chemical protocol (Czarnecki et al., 1979), with its low yield, long reaction time, and potentially harmful starting reagents, is even more marked. The resultant product has been characterised and shown to be fully biologically active (see 2.3). This has enabled its use to probe for ATP binding proteins in intracellular membrane structures (see 3.2) and glucocorticoid receptor extracts (see appendix 1).

The mapping studies of the intracellular ATP - binding polypeptides proved both fruitful and interesting. The key finding is the widespread occurrence of a 46 kD ATP - binding polypeptide, having very similar properties to those attributed to the NTPase involved in mRNA efflux from the

nucleus. It is generally accepted that the NTPase activity of the nuclear envelope is due to a single class of NTPase and that this activity resides in the nuclear pore complex (Purrello et al., 1983). The fact that this polypeptide is present in such diverse membrane structures as mitochondria, nuclear envelopes, plasma membrane and even chromosomes (see 3.2) is interesting and negates the *assumption* that it is a specific nuclear envelope component. Its widespread occurrence indicates that it may be a general membrane - associated NTPase, and that its putative involvement in the nucleocytoplasmic translocation of mRNA may be due to its association, in the nuclear pore complex, with other possible insulin modulated components. One such component may be the phosphatase which is postulated to dephosphorylate the NTPase, thus activating it. This phosphatase may be specific to the nuclear envelope, since it has been demonstrated that there is no insulin stimulated dephosphorylation in plasma membranes (Purrello et al., 1983). Such a nuclear envelope specificity of the phosphatase would permit a general cellular NTPase, such as the 46 kD polypeptide, to become indirectly insulin - modulated when associated with the nuclear envelope. This widespread occurrence of the NTPase may also indicate that it does not possess the mRNA - binding site, since this would be evolutionarily "wasteful" from a complexity point of view. Rather, it may be that the NTPase is closely associated with the phosphatase and a mRNA - binding protein in the nuclear pore complex. The model could be modified in that the activation of the phosphatase by insulin leads to the dephosphorylation, and thus, activation of both the NTPase and the mRNA binding polypeptide. Alternatively, activation of the NTPase may result in conformational changes in its structure, with a secondary effect on mRNA - binding binding site of the closely associated polypeptide. This would not conflict with the proposal that mRNA activates the NTPase activity (Purrello et al, 1982).

The investigation of the direct effects of insulin on the ATP - binding by the NTPase did not reveal any increase in ATP - binding as a result of preincubation with insulin (figure 4.1). Thus, at face value it appears that insulin has no effects on the NTPase. However, if specific nuclear pore complex association of a NTPase - modulating phosphatase, which in turn is the insulin - sensitive target, is postulated it is feasable to argue that my findings do not contradict the model of insulin action on the nucleus. However, it cannot be postulated that the nuclear pore complex - associated NTPase differs from that dispersed throughout the nuclear envelope, since there is evidence that the NTPase activity is due to a single class of NTPase. Though the experiments presented do not disprove the validity of the model proposed to explain effects of insulin on the nucleus, they have not yielded any supporting evidence. Equally, the general phosphorylation pattern from normal rats is not influenced by exposure of the nuclei to insulin *in vitro*.

The protocol for the synthesis of the insulin analogue represents an improvement on existing protocols, since only the SASD moiety is iodinated and the coupling of this carrier - free moiety is only to the N - terminal of the insulin molecule only. The avoidance of the iodination of the insulin molecule and the minimisation of steric hindrance by the SASD moeity, by coupling to only one amino group, ensure that adverse effects on the biological activity are minimised. That this is so, is clearly demonstrated by the characterisation studies, both *in situ* and *in vivo*, which clearly demonstrate that the analogue possesses a biological activity very similar to that of native insulin (see 1.1.2). The analogue binds specifically to the plasma membrane receptor (figure 1.1.15); induces hypoglycaemia in mice to a level comparable with that of native insulin (figure 1.1.16); and induces specific genes

in a hepatoma cell line, also at levels similar to those for native insulin (figure 1.1.17). It can therefore be concluded that this protocol yields a defined and well characterised product. Considering the bulkiness of the SASD moeity and the number of manipulations involved in the synthesis, the resultant high biological activity of the final product is surprising.

Of interest are the results of the probing for intracellular insulin - docking polypeptide(s). The positive identification of the insulin receptor in the plasma membrane of intact hepatoma cells is in keeping with the gene induction studies (figure 1.1.15) and the literature.

The presence of a 60 kD insulin - binding polypeptide in rat liver nuclear envelope is of special interest and could, assuming it not to be a degradation product of a larger molecule, represent an important interface for the direct modulation of nuclear activity by insulin. As discussed in 1.2.3, this finding need not represent a contradiction of the findings of Wong et al. (1988) on the presence of a receptor virtually identical to that in the plasma membrane. Rather, such a polypeptide may play a mediating role in that it limits the initially receptor - bound insulin to a not yet identified enzyme, thereby initiating a series of specific events. Alternatively, certain effects of insulin may be dependent on binding to both the 60 kD and 130 kD nuclear envelope receptors, either sequentially or concomitantly. The alternative may also apply, that is, the plasma membrane insulin receptor constitutes a shuttling protein between the cell periphery and the nucleus, in much the same way as the cytoplasmic glucocoticoid receptor and the observed 60 kD binding polypetide may represent a proteolytic breakdown product of the shuttling protein.

In summary, novel protocols have been developed for the synthesis of carrier - free, photoactivatable ATP and insulin analogues. These have been successfully employed to identify ATP as well as insulin - binding polypeptides in various intracellular membrane structures. In applying these tools to test the model of insulin action promoted in the literature by a number of authors (see Fig 1.1), I could not find in my experiments evidence in direct support of this hypothesis. The major findings of this investigation has been the ubiquitous occurrence of the 46 kD ATP - binding protein and the presence, in the nuclear envelope, of an approximately 60 kD insulin - binding polypeptide.

Chapter 6

Methods and Materials

6.1 Isolation Procedures

6.1.1 Rat Liver Nuclei

Rat liver nuclei were isolated by the method of Blobel and Potter (1966). Rats were starved overnight and sacrificed by cervical dislocation and immediate exsanguination. The excised livers were trimmed of connective tissue and weighed. After finely chopping, the livers were homogenised in two volumes of 0,25 M sucrose / TKM (0,05 M Tris-HCl, pH7,5; 0,025 M KCl; 0,0015 M $MgCl_2$) using a glass / teflon rotary homogeniser. The homogenate was filtered through one layer and then four layers of cheesecloth and the resulting filtrate spun at x750g for 10 minutes. The supernatant was then decanted and the loose pellet resuspended in 9 - 10 volumes 2,3 M sucrose / TKM. This suspension was centrifuged at x39000g for 45 minutes. The resultant pellet of purified nuclei were resuspended in 2,3 M sucrose / TKM and stored under liquid nitrogen.

6.1.2 Rat Liver Nuclear Envelope

6.1.2.1 Method of Bornens and Courvalin (1978)

Nuclei were suspended in 0,25 M sucrose / 2 mM sodium phosphate, pH 7,85 to a protein concentration of 1,5 mg / ml. While gently stirring, heparin was slowly added to a final heparin : DNA ratio of 1 (w / w). After stirring for 60 minutes the solution was centrifuged at x48000g for 45 minutes. The crude nuclear envelope pellet was resuspended in a minimum volume of 0,25 M sucrose / 2 mM sodium phosphate, pH 7.85 by gently homogenising in a 2 ml glass homogeniser. This suspension was then layered over a 25 - 50% sucrose / 2 mM sodium phosphate, pH 7.85 continious gradient and centrifuged for 180 minutes at x230000g. The purified nuclear envelope banding at approximately 37% was fractionated and stored under liquid nitrogen.

6.1.2.2 Method of Kaufmann et al. (1983)

Nuclei were suspended in 0,25 M sucrose / TMP (50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 1 mM PMSF) to a concentration of $5x10^8$ nuclei / ml. The nuclei were incubated with DNase 1 and RNase A (1 mg / 4 ml) for 60 minutes and then centrifuged at x800g for 10 minutes.
The resultant pellet was resuspended in TMP to a concentration as above and 4 volumes of 2 M NaCl, TMP was added dropwise with stirring. Mercaptoethanol was added to a final concentration of 1% and the solution was allowed to incubate for 15 minutes. The solution was centrifuged at x1600g for 30 minutes and the resultant pellet further purified by reextracting with 2 M NaCl / TMP (minus mercaptoethanol).
The isolated material was stored under liquid nitrogen.

6.1.2.3 Method of Monneron et al. (1972)

The nuclear pellet (60 mg DNA) was resuspended in 25 drops of glycerol followed by 24 ml of 1,8 M sucrose / TM (50 mM Tris-HCl, pH 7.5; 500 mM $MgCl_2$) and mixed vigorously for 60 seconds using a vortex mixer. The viscous solution was then centrifuged at x1000g for 5 minutes and 3 ml aliquots of the resultant supernatants were placed in rotor tubes. Over these aliquots were poured 20 - 50% sucrose / TM gradients and the samples were then centrifuged at x230000g for 90 minutes in a SW 40 rotor.
The resultant discrete bands were pooled and stored under liquid nitrogen.

6.1.2.4 Method of Kay et al. (1972)

Purified nuclei were suspended in 0,1 mM $MgCl_2$ at a concentration of 7 mg DNA / ml. To this was added DNase 1 (10 μg / ml) and 4 volumes of 0,3 M sucrose / TMM (10 mM Tris-HCl, pH 8.5; 0,1 mM $MgCl_2$; 5 mM mercaptoethanol) and the solution was then incubated for 15 minutes at 22°C. The incubation was terminated by the addition of an equal volume of ice cold water followed by centrifugation at x38000g for 15 minutes.
The resultant pellet was further purified by repeating the DNase 1 digestion but this time in 0,3 M sucrose / TMM, pH 7.4. The nuclear envelope was stored under liquid nitrogen.

6.1.3 Rat Liver Plasma Membrane

Plasma membranes were isolated essentially as described by Aronson and Touster (1974). Rats were starved overnight and sacrificed by cervical dislocation and exsanguination. The livers were immediately perfused with 50 ml of 0,25 M sucrose / 5 mM Tris-HCl, pH 8.0 and trimmed of connective tissue prior to being finely chopped up. The liver was homogenised in 3 volumes of perfusion buffer per gram of liver in a teflon / glass Potter homogeniser. The homogenate was filtered through four layers of cheesecloth and then centrifuged at x1000g for 10 minutes.
The resultant supernatant was spun at x33000g for 8 minutes and the pellet retained. The supernatant was then centrifuged at x78000g for 100 minutes and the resultant pellet homogenised with 3 volumes of 57% sucrose / 5 mM Tris-HCl, pH 8.0 (w / w) per gram of liver using a Dounce homogeniser. Twelve ml of this homogenate was placed in a rotor tube and overlayered with 15 ml of 34% sucrose / 5 mM Tris-HCl, pH 8.0 (w / w). The tube was then topped with 0,25 M sucrose / 5 mM Tris-HCl, pH 8.0. The samples were centrifuged at x100000g for 16 hours in a SW 28 rotor.
The plasma membrane fraction was obtained at the 34% / 0,25 M sucrose interface.
Plasma membranes were also obtained from the nuclear pellet by resuspending the pellet in 3 volumes of 0.25 M sucrose and centrifuging at x33000g for 10 minutes. The packed nuclei were then resuspended in 1.5 volumes 57% sucrose per gram of liver and approximately 15 ml placed in the bottom of a SW 28 tube. This was overlayered with 15 ml of 37.2% sucrose and finally 0.25 M sucrose. The samples were then subjected to centrifugation at x100000g for 16 hours. The plasma membrane fraction at the 37.2 / 0.25 M sucrose interface was removed.
All plasma membrane fractions were pelleted, resuspended in 57% sucrose, and stored under liquid nitrogen.

6.1.4 Rat Liver Mitochondria

Mitochondrial fractions were isolated essentially as described by Aronson and Touster (1974) for the isolation of plasma membrane (7.1.4). The mitochondrial pellet obtained after the x33000g spin was resuspended in 0.25 M sucrose and recentrifuged. The resultant pellet was stored in 57% sucrose under liquid nitrogen.

6.1.5 Friend Cell Nuclei / Hepatoma Nuclei

Nuclei were isolated essentially either as described by Smith et al. (1988) or Gasser and Laemmli (1987). Using the former method, cells were pelleted and rinsed twice in Dulbecco's phosphate buffered saline (DPBSA) before resuspending in DPBSA at a concentration of $1x10^8$ cells / 20 ml. The cell solution was vortexed with melittin (1 μg / $1x10^6$ cells), and then centrifuged on a bench top centrifuge for 5 minutes.
The pellet was resuspended in 10 ml of 0,34 M sucrose / TKM and homogenised with 5 strokes of a tight - fitting Dounce. To the homogenised solution was added 9 volumes of 2,3 M sucrose / TKM and the solution centrifuged at x39000g for 45 minutes.
The alternative method used was that based on the report of Gasser and Laemmli (1987) for the isolation of metaphase chromosomes, with the obvious exception that the first Ficoll paque gradient to separate out metaphase cells is omitted. Briefly, cells were pelleted and resuspended in 20 ml solution 1* and pelleted at x800g for 5 minutes. This hypotonic procedure was repeated twice at 22°C and the pellet then resuspended in 20 ml of ice-cold solution 2**. The cells were then homogenised until microscopic investigation revealed that the vast majority of the cells

were lysed. The homogenate was then centrifuged at x1000g for 10 minutes and the resultant pellet resuspended in 2 M sucrose in solution 2. This suspension was then centrifuged x33000g for 45 minutes and the resultant pellet gently rehomogenised in 6 ml of solution 2. Two ml of this was applied to a percoll gradient (4.5 ml percoll; 2 ml 70% glycerol; 0.5 ml buffer B stock (x20); and 1 ml solution 3***) and centrifuged at 25000 r.p.m. for 30 minutes in a 75 Ti rotor.
Nuclei were stored under liquid nitrogen.
_ 100 mM Tris-HCl, pH 7.4; 400 mM KCl; 400 mM EDTA; 5 mM spermidine.
* 7.5 mM Tris-HCl, pH 7.4; 40 mM KCl; 1 mM EDTA, 0.1 mM spermine; 0.25 mM spermidine; 1% TDG; 0.1 mM PMSF.
** 15 mM Tris-HCl, pH 7.4; 80 mM KCl; 2 mM EDTA; 0.2 mM spermine; 0.5 mM spermidine; 1% TDG; 0.1 mM PMSF.
*** 20 mM spermine; 8 mM spermidine

6.1.6. The Isolation Of Metaphase Chromosomes.

All solutions for the isolation of metaphase chromosomes are as described in 6.1.7.
Metaphase arrested Friend cells were separated from non-metaphase cells on a Ficoll Paque gradient, the metaphase cells banding at 75%.
The metaphase cells were resuspended in 20 ml of solution 1 and gently pelleted for 5 minutes. This hypotonic washing step was repeated twice at room temperature. The washed cells were then resuspended in ice cold solution 2 and homogenised until the bulk of the cells were lysed (checked microscopically). The homogenate was then centrifuged at x1200g for 5 minutes and the pellet resuspended in 2 ml of solution 2 . The resuspended pellet was then added to 8 ml of a Percoll solution (4.5 ml Percoll; 2 ml of 70% glycerol;

0.5 ml buffer B; and 1 ml solution 3) and centrifuged at 25000 r.p.m. for 30 minutes in a 75 Ti rotor. The chromosomes banded in the second layer.

6.1.7 The Isolation of Crude Rat Liver Nuclear Envelope Insulin Receptors by Affinity Chromatography.

Crude rat liver nuclear envelope insulin receptor extracts were isolated as described by Wong et al. (1988). Essentially, nuclei (3 mg / ml) isolated by the method of Blobel and Potter (1966), were incubated at 22°C in 0.25 M sucrose, 2% Triton LE, 0.2 mM PMSF, and 50 mM Tris, pH 7.4. Triton LE was used due to its low uv absorbance - important when monitoring the effectiveness of washing away non - bound proteins from the affinity column. After 45 minutes, 20 ml of the solubilised receptor mixture was added to a 40 ml centrifuge tube containing 10 ml of wheat germ agglutinin - derivatised agarose (binding capacity 5 mg RNase / 3 ml matrix). The tube was sealed and incubation allowed to proceed for 2 hours at 22°C with constant agitation. The mixture was then poured as a column and extensively washed with the solubilisation buffer. The washings were continuously monitored spectrophotometrically and when a stable baseline was achieved, the column was washed with the receptor elution buffer (0.3 M N - acetylglucosamine, 150 mM NaCl, 0.1% Nonidet LE, 0.1 mM PMSF, and 50 mM Tris - HCl, pH 7.6. Fractions of 200 µl were collected and the relevant fractions pooled.
The wheat germ agglutinin - derivatised agarose was kindly prepared by Dr. Robert Warren.

6.2 Preparative Techniques

6.2.1 The Enzymatic Synthesis of 8-Azidoadenosine 5-[$\gamma^{32}P$] Triphosphate

The [$\gamma^{32}P$]N_3ATP was synthesised according to the method of Sabbatini and von Holt (1987).
All procedures were performed in subdued light.
To 40 μl of reagent mixture (125 mM Tris-HCl, pH9.0; 30 mM $MgCl_2$; 4,5 mM spermine; 0,3 mM L-α-glycerolphosphate; 0,5 mM NAD; 0.125 mM N_3ADP; and 2,5 mM pyruvate) was added 20 μl of the glycolytic enzyme mixture (0,34 U glycerolphosphate dehydrogenase; 0,1 U triosephosphate isomerase; 0,16 U glyceraldehyde-3-phosphate dehydrogenase; 0,09 U 3-phosphoglycerate kinase, and 1,9 U lactate dehydrogenase in 50 mM Tris-HCl,pH 9.0 and 2 mM ß-mercaptoethanol). To this mixture was added 50 μl ^{32}P orthophosphate and the mixture was incubated for 30 minutes. The degree of incorporation of orthophosphate into N_3ADP was determined by an activated charcoal assay (see 6.4.11.). The reaction was terminated by the addition of an equal volume of Tris - saturated phenol, pH 8.0. The aqueous phase was then extracted with chloroform and the resultant product stored at -20°C after analysis by TLC (see 6.4.13.).

6.2.2 The Preparation of the Diboc - Insulin Derivative, N-(t-Boc-Gly^{A1}, N-t-Boc-Lys^{B29}) Insulin

The method employed was essentially as that described by Grant and von Holt (1987).
Batches of 228 mg (40 μmoles) of insulin were dissolved in 2,4 ml of 75% pyridine in water and to this was added 19,6

mg (80 μmoles) of BOPA in 0,6 ml of DMF containing 200 μl of 1 N sodium bicarbonate. The reaction was allowed to proceed for 16 hours at 22°C and terminated by the addition of 16 ml of 10% acetic acid. The precipitate was pelleted by centrifugation on a bench top centrifuge and the supernatant discarded. The pellet containing mono -, di -, and tri - boc derivatives was solubilised in acetonitrile and subjected to reverse phase HPLC on a μ - Bondapak C18 Radial Pak column using the following gradient solutions and profile:

solution A:	0,05% TFA
solution B:	70% acetonitrile in 0,05% TFA
Gradient profile:	0% B for 10minutes 0 - 35% B over 5 minutes 35 - 45% B over 30 minutes 45 - 100% B over 5 minutes 100 - 0% B over 10 minutes

The flow rate was 1 ml / minute and samples were collected as 1 ml aliquots.

The fractions were lyophilised and their identity as di - boc insulin confirmed by acid - urea PAGE.
The sample's identity as N-t-boc gly A1, N-t-boc lys B29 insulin was confirmed by amino acid sequencing, the sole product of the first cleavage step being phe.

6.2.3 The Synthesis of SASD - Derivatised Insulin

Diboc insulin refers to N-t-boc gly A1, N-t-boc lys B29 insulin.
The reaction was performed in in a 2 ml stoppered, silanised glass vessel.
All procedures involving SASD were performed under stringent red light conditions to avoid photoactivation of the azido moiety.

Diboc insulin (200 μg) was dissolved in 120 μl of reaction buffer (300 parts DMSO : 20 parts triethylamine) and to this was added 50 μg of SASD dissolved in 5 μl DMSO. The reaction vessel was flushed with nitrogen and firmly stoppered. The reaction was allowed to proceed for 16 hours at 37°C, after which the buffer was evaporated under vacuum.
To the residue was added 100 μl of redistilled TFA and debocing allowed to proceed for 5 minutes. The TFA was then removed under vacuum and the dry mixture of deboced derivatised insulin redissolved in 50 μl DMSO. The mixture was sujected to reverse phase HPLC on a μ - Bondapak C18 Radial Pak column to separate out the derivatised insulin from uncoupled insulin and SASD.

The gradient solutions were as in 6.2.2 and the gradient profile as follows:
0% B for 10 minutes
0 - 35% B over 5 minutes
35 - 55% B over 40 minutes
55 - 100% B over 5 minutes

The flow rate was 1 ml / minute and the relevant peaks were collected as 1 ml fractions. The fractions were lyophilised redissolved in a minimum of DMSO and pooled according to content. After removal of an aliquot for analysis by acid - urea PAGE, the remainder was dried under vacuum and stored at minus 20°C.

6.2.4 The Synthesis of [^{125}I]SASD Derivatised Insulin

The protocol was identical to that employed in (6.2.3), except that carrier - free iodinated SASD was coupled to the diboc insulin.

6.2.5 The Preparation of Carrier - Free Iodinated SASD

The iodination of SASD was performed in iodogen tubes prepared by aliquoting 1 ml of an iodogen solution (1 mg / 50 ml chloroform) into Eppendorf vials and allowing the chloroform to evaporate.
To an iodogen tube was added 100 μg SASD in 50 μl DMSO and 10 μl ^{125}I. Iodination was allowed to proceed for 1 minute, after which the sample was subjected to reverse phase HPLC (C18 column) to separate [^{125}I]SASD from non - iodinated SASD and free ^{125}I.
The gradient solutions were as in 6.2.2 and the gradient profile as follows:

0% B for 10 minutes
0 - 40% B over 5 minutes
40 - 60% B over 45 minutes
60 - 100% B over 5 minutes

The flow rate was 1 ml / minute and the relevant peaks collected as 1 ml fractions. The fractions were lyophilised, redissolved in a minimum of DMSO and pooled . After evaporation under vacuum, the samples were stored at -20°C.

6.3 Binding, Labelling and Biological Induction Studies

6.3.1 Photoaffinity Labeling of Proteins with [γ^{32}P]N_3ATP

The proteins to be labeled were dissolved in 250 μl of incubation medium (50 mM Tris-HCl, pH 9.0; 25 mM KCl, and 5 mM $MgCl_2$) and transferred to acid - cleaned quartz silica cuvettes. To these were added 1 μM [γ^{32}P]N_3ATP (10 Ci / mmol) and the samples were then irradiated by means of eight 10 millisecond flashes of a quartz xenon lamp connected to a 500 V capacitor discharge system (section 6.4.15.). The solutions were removed from the cuvettes and the protein precipitated with trichloroacetic acid at a final concentration of 10%. The resulting precipitates were neutralized and solubilized in sample application buffer. The samples were subjected to SDS-PAGE (6.4.7.3.) and the resultant band excised and placed in 10 ml of liquid scintillation fluid.
When labelling creatine kinase, 5 mM creatine was included in the incubation medium.

6.3.2 Photoaffinity Labelling of Membranes with [γ^{32}P]N_3ATP

All manipulations prior to photoflashing were performed in subdued light.
Membranes (100 - 250 μg) to be labelled were suspended in 250 μl of 0,25 M sucrose / TMEN (40 mM Tris-HCl, pH7.8; 10 mM $MgCl_2$; 2 mM EDTA, and 100 mM NaCl). These were incubated with 1 μM [γ^{32}P]N_3ATP (50 Ci / mmol) and after an initial

incubation period of 5 minutes were pelleted and the pellets resuspended in fresh incubation medium. The suspensions were transferred to acid-cleaned quartz cuvettes and irradiated as described in 6.4.15. The suspensions were then transferred to 1,5 ml Eppendorf vials and the membranes pelleted by centrifugation in a microfuge. The labelled membrane pellets were prepared for PAGE and subsequent autoradiography as described in section 6.4.7.4.
Control samples were incubated exactly as described except that the samples were not irradiated (- u.v.).

6.3.3 Phosphorylation by Endogenous Protein Kinases

Incubations were performed at 22°C in 1,5 ml Eppendorf vials.
Nuclei or membrane fractions (100 - 250 μg) were suspended in 250 μl of 0,25 M sucrose / TMEN (see 6.3.2). The fractions were then incubated with 5 μM [γ^{32}P]ATP (10 Ci / mmol) for 5 minutes (unless otherwise stated) and the labelling was rapidly terminated by the addition of sodium metabisulphite to a final concentration of 50 mM. The samples were pelleted by centrifugation in a bench - top microfuge and the supernatants removed.
The phosphorylated nuclei / membranes were then solubilized for PAGE and autoradiography.

6.3.4 In Situ Binding of [^{125}I]ASD-Insulin to Hepatoma Cells

Hepatoma cells (H411E) were grown to confluency (1 x 10^7 cells / flask) and starved of insulin the day prior to binding studies. The medium was removed from the flasks and replaced with 4 ml of binding medium (MEMS, 0.1% BSA, 20 mM

Hepes, pH 7.4). To each flask was added the synthesised [^{125}I]ASD-insulin and 100 μM cold insulin in the case of displacement studies and the flasks were incubated at 4°C for 2 hours in the dark. The incubation medium was then removed and the cells were thoroughly washed with cold PBS. To the washed cells was added 4 ml of PBS and the flasks were irradiated on a transilluminator for 3 minutes. The cells were then detached and pelleted. The pellet was resuspended in 1 ml of 0.32 M sucrose / TKM and homogenised with a hand held homogeniser. The plasma membranes were isolated as in 6.1.4, solubilised in SDS sample buffer and run on a 10% polyacrylamide gel. Labelled bands were visualised by autoradiography (6.4.7.3).
Labelling studies with Friend cells were performed essentially as for the hepatoma cells except that, being free - floating, the cells did not require detachment.

6.3.5 [^{125}I]ASD-Insulin Binding to the Cellular Subfractions

This protocol was employed for *in vitro* labelling studies using [^{125}I]ASD-insulin and is applicable for plasma membrane, nuclei, nuclear envelope, and chromosomes.
The fractions (aproximately 200 μg) were suspended in 400 μl incubation medium (0.25 M sucrose / TMEN) to which was added [^{125}I]ASD-insulin and 100 μM cold insulin in the case of displacement studies. The fractions were thus incubated for 2 hours at 22°C with constant rotation in the dark and then pelleted. The pellets were washed three times by resuspension and centrifugation and finally resuspended in 250 μl of incubation mixture. The samples were then subjected to ultra violet radiation for 3 minutes and the resultant labelled fractions pelleted. The pellets were solubilised in SDS sample application buffer and subjected to polyacrylamide gel electrophoresis (6.4.7.3.). Labelled bands were visualised by autoradiography.

6.3.6 In Vivo Transcription

In vivo gene induction studies were undertaken on H4IIE hepatoma cells which had been starved overnight in serum - free medium. The induction studies were undertaken by Dr. Brenda Stickells.
Prior to induction studies the medium was decanted and the cells refed with serum - free medium containing various concentrations of insulin or ASD-insulin. The cells were incubated for 180 minutes, after which the medium was decanted and the cells detatched with DPBSA. The cells were harvested and RNA isolated by the method of Chirgwin et al.(1979) using CsCl gradient fractionation.
The isolated RNA was quantitated by measuring OD_{260nm} , denatured using formamide and finally cross - linked to nylon membrane (Hybond N) by means of u.v. radiation. Specific mRNA probes (pyruvate kinase and tyrosine amino transferase) were labelled by nick translation and hybridised to the bound RNA by the method of Wahl et al. (1981). The level of hybridisation was quantitated by means of autoradiography and densitometric scans of the resultant autoradiograms.

6.3.7 Test for Biological Activity of Insulin (Mouse Drop Test)

This test was employed to compare the in vivo effect of the synthesised insulin analogue with that of native insulin and was performed essentially as described by Thompson (1946). The mice were divided into four groups of 20 each and maintained in these groupings for the duration of the experiment. The mice were starved for 6 hours and maintained at 37°C for 1 hour prior to being injected intraperitoneally and placed on wire mesh racks inclined at 60° (one rack per

group). This acted as an early warning system since hypoglycemic mice would slide off and convulse and could all be resuscitated by intraperitoneal administration of 0.5 ml of 15% glucose.
The injection protocol was as follows:
Group 1: native insulin, low dosage (18 mU / 30 g body weight).
Group 2: native insulin, high dosage (36 mU / 30 g body weight).
Group 3: SASD insulin, low dosage (0.8 µg / 30 g body weight).
Group 4: SASD insulin, high dosage (1.6 µg / 30 g body weight).
Two hours after insulin administration, the mice still clinging to the rack were counted to determine the efficacy of the insulin.
To improve the statistical significance of the assay, further experiments were undertaken using intermediate dosages (29 mU native insulin, 1.2 µg ASD-insulin) and by rotating the insulin formats such that all groups were exposed to low and high dosages of native insulin and SASD-insulin.

6.4 Analytical Procedures

6.4.1 DNA Determination

The method was that of Schneider (1957). Aliquots of the samples to be determined were pipetted into acid - cleaned bench top centrifuge tubes and made to 1 ml with distilled water. To these were added 1 ml of 20% (v / v) perchloric acid, 2 ml of 4% diphenylamine in glacial acetic acid, and 0,1 ml of aqueous acetaldehyde (0,2 ml / 100 ml). The tubes

were vigorously vortexed and incubated at 56°C for 60 minutes. After this incubation time 1 ml of amyl acetate was added and the contents vortexed. The two phases were separated by centrifugation and the amyl acetate phase removed to determine the absorbance at 578 nm. The absorbance of samples and DNA standards were determined in triplicate.

6.4.2 Protein Determination

The method employed was essentially as described by Lowry et al. (1951).

Reagent A: 2% sodium carbonate in 0,1 N sodium hydroxide.
Reagent B: 1% copper sulfate
Reagent C: 2% sodium, potassium tartrate
Reagent D: reagent A : reagent B : reagent C (100 : 1 : 1)

Aliquots of samples to be determined were pipetted into acid cleaned tubes and made to 1 ml with distilled water. To each tube was added 5 ml of reagent D followed by mixing. The samples were incubated at 22°C for 10 minutes, after which was added 0,5 ml of Folin reagent (diluted 1 : 2 with distilled water). The tubes were shaken and incubated at 22°C for 30 minutes and the absorbance then measured at 492 nm. The quantitation of samples and standards was determined in triplicate.

6.4.3 Protein Determination (Micro - Determination)

This assay was employed to accurately determine protein concentrations in the 1 - 10 μg range and is based on the method of Bensadoun and Weinstein (1976). Samples to be assayed were placed in 1,5 ml Eppendorf vials and made to 1 ml with distilled water. To this was added 10 μl of 1,66% sodium deoxycholate and the mixture allowed to stand for 15 minutes, after which was added 333 μl of 24% trichloroacetic acid. The vials were thoroughly vortexed and centrifuged at x39000g for 30 minutes at 4°C. To the resultant pellets was added 1 ml of reagent D (as in 6.4.2) and 0,1 ml of Folin reagent (diluted 1 : 1 with distilled water). After 75 minutes the absorbances were measured at 660 nm. Samples and standards were assayed in triplicate.

6.4.4 Km of Hydrolysis of [γ^{32}P]N_3ATP by [Na^+,K^+]ATPase

The experiments were undertaken in subdued light so as to avoid photoactivating the N_3ATP which could then cross - link the ATP - binding site of the ATPase.
0,02 U of [Na^+,K^+]ATPase (Sigma) in 250 μl incubation medium (50 mM Tris-HCl, pH7.4; 25 mM KCl; 5 mM $MgCl_2$;, and 30 mM NaCl) was incubated with varying amounts of either [γ^{32}P]N_3ATP or [γ^{32}P]ATP for 10 minutes at 22°C. The rates of hydrolysis of ATP or the analogue were determined by the activated charcoal method (6.4.11.). The data was then subjected to Lineweaver - Burk analysis to determine the Km value.
V was defined as:
nmol of ^{32}Pi released / 0,02 U [Na^+,K^+]ATPase / 10 minutes.
Competition studies were performed by the addition of 20 mM cold ATP to the incubation medium. The mode of inhibition was determined from Lineweaver - Burk plots.

6.4.5 Identification of Glycosylated Proteins

Nuclei and the various membrane fractions were subjected to SDS - PAGE and the resultant gels blotted onto nitrocellulose. These blots were then incubated with 3% BSA / PBS (phosphate - buffered saline) for 16 hours in order to avoid non - specific binding by ^{125}I. This was followed by 2 washes each in PBS, PBS plus 0,1% Triton-X100, and finally PBS. Each wash was 30 minutes in duration and was performed with constant agitation.
The treated blots were then incubated with ^{125}I - labelled lectins in a minimum of PBS for 120 minutes and then subjected to the same wash cycle as described above, followed by autoradiography.

6.4.6 Iodination of Proteins and Lectins

The samples were dissolved in 500 μl of PBS in a 1,5 ml Eppendorf vial and to this was added 2 μl of ^{125}I (specific activity 3000 Ci / mM. Two Iodo -Beads (Pierce) were then added and the solution incubated for 15 minutes. The iodinated samples were separated from unbound ^{125}I by dialysis on a 0.025 μm, type VS membrane (Millipore) floating on 200 ml of PBS. The dialysis was repeated twice by which stage >99% of unbound ^{125}I was removed. The iodinated samples were then stored at -20°C.

6.4.7 SDS - Polyacrylamide Gel Electrophoresis

6.4.7.1 Two Dimensional Polyacrylamide Gel Electrophoresis

Two dimensional PAGE was performed essentially as described by O'Farrell (1975).

Solutions

First dimension gel

Solution A	28,38% acrylamide 1,62% NN - methylenebisacrylamide
Solution B	10% (v / v) Nonidet P-40

First dimension tray buffers

IEF top tray	0,02 M sodium hydroxide
IEF bottom tray	0,01 M phosphoric acid

First dimension gel overlay solution

8 M urea

First dimension sample overlay solution

9 M urea
1% ampholytes (3 / 10 range)

First dimension application buffer

9,5 M urea
2% (v / v) Nonidet-P40
5% (v / v) ß - mercaptoethanol
2% ampholytes (3 / 10 range)

Second dimension gel

solution A	29,2% acrylamide 0,8% NN-methylenebisacrylamide

Solution B
1,5 M Tris-HCl, pH 8,8
0,4% SDS

Solution C
0,5 M Tris-HCl, pH 6,8
0,4% SDS

Second dimension tray buffers

0,025 M Tris
0,192 M glycine
0,1% SDS

Second dimension application buffer

10% (w / v)glycerol
0,0625 M Tris-HCl, pH 6,8
2,3% SDS
5% (v / v) ß - mercaptoethanol

Gel Preparation

First dimension gels

The gel solution was prepared as follows:

2,75 g urea
665 µl solution A
1 ml solution B
250 µl ampholytes (3 / 10 range)
990 µl distilled water

To this solution was added 10 µl of 10% ammonium persulphate and 7 µl of TEMED. After thorough mixing the gel solution was drawn up into a 5 ml syringe to which was attached a 12 cm piece of flexible tubing. This was used to fill four 10 cm long - glass tubes (internal diameter 2 mm) which were stoppered at one end with parafilm. After ensuring that no air bubbles were introduced, the tube gels were overlayered with the gel overlay solution and allowed to set. The overlay solution was replaced 120 minutes later with first dimension application buffer.

Second dimension gels

To make 140 ml of a 10% running gel:

47 ml	solution A
35 ml	solution B
58 ml	distilled water
1 ml	10% (w / v) ammonium persulphate
150 µl	TEMED

To make 40 ml of stacking gel:

6 ml	solution A
10 ml	solution C
24 ml	distilled water

Agarose - sealed plates with 1,5 mm spacers were filled to within 2 cm of the top with the running gel solution and overlayered with 1 ml of isopropanol.
When the gel had polymerised the isopropanol was poured off and the gel topped with the stacking gel.

Running The Gels

First dimension

Isoelectric Focussing (IEF)

The parafilm was removed and the tubes mounted through the top chamber grommets such that the bottom of each tube was immersed in the bottom chamber. To the bottom chamber (positive polarity) was added 0,01 M phosphoric acid and to the top chamber (negative polarity) 0,02 N sodium hydroxide. The first dimension application buffer was removed from the top of the tube gels and replaced with 50 µl fresh buffer. The tubes were then topped up with sample overlay buffer.

The gels were then subjected to a pre - electrophoretic cycle as follows:

200 V for 15 minutes
300 V for 30 minutes
400 V for 30 minutes

The top tray buffer was decanted and all buffers removed from the top of the tube gels. The sample solubilised in first dimension application buffer was then applied to the gels and overlayed with 30 µl sample overlay solution. The top tray was then refilled with tray buffer and the gel run at 400 V for 16 hours.

Non - Equilibrium pH Gradient Electrophoresis (NEPHGE)

The tubes were mounted in the electrophoresis apparatus as described for IEF gels except that the tray polarities were reversed i.e.the top tray was positive and contained 0,01 M phosphoric acid. The gels were not pre-electrophoresed and the gels were run at 400 V for 480 minutes.

Second dimension

The first dimension gels were extruded on top of the second dimension gels and immediately bonded with a hot solution of 1% agarose dissolved in second dimension application buffer. To the bottom chamber was added tray buffer and to the top tray was added tray buffer containing 2% SDS (cf. 0,1% for normal tray buffer). After electrophoresis at 150 V for 30 minutes the top tray was emptied and refilled with normal buffer and the gels subjected to electrophoresis at 150 V for 12 hours.
The resultant gels were stained, photographed and subjected to autoradiography as required.

6.4.7.4 Sample Preparation for One Dimensional SDS PAGE

Pelleted samples were dissolved in 70 µl of second dimension application buffer containing dextran blue as tracker dye, and boiled for 10 minutes. The samples were then centrifuged in a bench top microfuge to pellet insoluble material and the supernatants applied to the wells of the gels.

6.4.8 Acid Urea Polyacrylamide Gel Electrophoresis

Acid urea polyacrylamide gel electrophoresis was performed essentially as described by Panyim and Chalkley (1969).

Solutions

Solution A :	60% acrylamide 0,4% NN-methylenebisacrylamide
Solution B :	43,2% acetic acid 4% TEMED 0,8 mM Tris
Solution C:	0,1% riboflavin
Tray buffer;	0,9 M acetic acid
Sample application buffer;	50% (v / v) acetic acid 1 µg / ml pyronine Y

Gel preparation

The gel solution was prepared by dissolving 14,4 g of urea (5 M) in 12 ml of solution A, 6 ml of solution B, and sufficient distilled water to make up the final volume to 48 ml. On addition of 100 µl of solution C , the solution was cast as 1,5 mm slabs in agarose - sealed gel plates

Running the gels

Samples were dissolved in 70 µl of sample application buffer and applied to the gels.
The samples were electrophoresed at a constant current of 20 mA for 240 minutes. The migration was from positive to negative.
The resolved gel was then stained, photographed, and autoradiographed as required.

6.4.9 Staining of Gels

Coomassie stain

The gels were stained for 180 minutes in staining solution (0,1% Coomassie brilliant blue; 50% methanol; and 10% acetic acid) with constant agitation. The stained protein bands were then visualised by destaining in destaining solution (25% ethanol, 7% acetic acid).

Silver staining

Silver staining was performed as described by Ansorg (1982) All steps were performed at 22°C with agitation.

A:	50% methanol, 12% TCA, 2% copper chloride
B:	10% ethanol, 5% acetic acid
Developer:	0,01% formaldehyde, 2% potassium carbonate

The gel was immersed sequentially into the following solutions: A for 30 minutes, B for 10 minutes, 0,01% potassium permanganate for 10 minutes, A for 10 minutes, 10%

ethanol for 10 minutes, water for 10 minutes, 0,1% silver nitrate for 10 minutes, water for 30 seconds, 10% potassium carbonate for 1 minute, and finally developer until the bands were visualised.

6.4.10 Streptozotocin - Induced Hyperglycemia in Rats

Rats were injected intrapertoneally with streptozotocin at a dosage of 10 mg / 100 g body weight.
Blood glucose levels were monitored daily with the aid of the Haemo - Glukotest strips (Boehringer) and the rats were deemed to be hyperglycemic when the glucose level rose above 300 mg / 100 ml. At this stage the rats displayed agitated behaviour and urinated excessively. The rats were sacrificed by cervical dislocation and exsanguination.

6.4.11 Charcoal Assay

To 250 µl of 50 mM KH_2PO_4 was added 1 µl of the sample and the mixture thoroughly mixed. A 25 µl aliquot was removed and added to 10 ml of liquid scintillation fluid for counting (A). To the remainder of the solution was added 50 mg of finely ground activated charcoal and the mixture was thoroughly vortexed. After pelleting the charcoal by centrifugation for 1 minute, 25 µl of the supernatant was removed and added to 10 ml of liquid scintillation fluid (B).
% incorporation = B / A x 100

6.4.12 Photography and Autoradiography

Destained gels were placed on an illuminated box and photographed using Kodak Technical Pan and a red filter. When photographing silver stained gels no filter was used. The films were developed according to the manufacturer's specifications.
Gels were flat - dried by heating on an evacuated heating tray and placed in X - ray cassettes to expose Cronex 4 X-ray film.

6.4.13 Thin Layer Chromatography

For monitoring N_3ATP reactions, samples were applied to PEIE - cellulose plates and run in 1,2 M lithium chloride.
For monitoring SASD reactions, samples were applied to silica plates and run in a benzene : amyl alcohol : chloroform : acetic acid solvent system (1 : 1 : 1 : 0,1). Plates were dried, covered in plastic film and autoradiographed.

6.4.14 Tissue Culture

Friend cells were grown in suspension in RPMI 1640 with 10% fetal calf serum at 37°C (humidity 95%; CO_2 5%).
Hepatoma cells (H4IIE) were cultured in EMEM containing 10% fetal calf serum at 37°C (humidity 95%, CO_2 5%). At 80% confluency, the cells were detached by washing for 3 minutes with DPBSA and replated.
HTC hepatoma cells were cultured in Spinner culture with Jokliks EMEM (Earles Modification of Eagles Medium) for

suspension culture, containing 0,005 M tricine. The flasks were constantly rotated on a Coulter mixer.
Prior to use in hormonal studies or subcellular fractionation, the cells were starved by refeeding with fetal calf serum - free medium for 24 hours.

6.4.15 Construction of the Ultra Violet Photoflash Unit

The photoflash unit is a capacitor discharge system, consisting of 4 100 V capacitors in series which were connected to a quartz - silica xenon lamp (Heimann GmbH, Wiesbaden - Dotzheim. Type EG 9902, 500V). The design is based on that described by Frimmer and Ziegler (1986). This system was capable of discharging a broad spectrum, high intensity flash (500 V) in 10 milliseconds.
The xenon tube is attached to the under - side of the sliding lid of the light - proof housing. Samples (either culture dishes or quartz silica cuvettes) are placed on specially constructed adaptors at the base of the box and the lid then slid into position. This configuration ensures that all samples are equidistant from the overhead lamp.

Chapter 7

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Appendix

The Use of Carrier - Free 8-Azidoadenosine 5'[γ^{32}P] Triphosphate to Investigate the Putative Intrinsic Protein Kinase Activity of the Purified Glucocorticoid Hormone Receptor Complex.

A.1 Introduction

Work in this laboratory has also focussed on the isolation and characterisation of the rat liver glucocorticoid hormone receptor complex. Dr. Hapgood has succeeded in purifying the receptor complex 9000 fold (Hapgood & von Holt, 1987) and an investigation of the protein kinase activity of the preparation using the synthesised azido-ATP (N_3ATP) was undertaken (Hapgood et al., 1986). This investigation was of interest owing to the contradictory evidence on the putative protein kinase activity of the glucocorticoid steroid receptor.

The progesterone receptor has been demonstrated to possess intrinsic kinase activity (Garcia et al., 1983) and to be phosphorylated by cAMP - dependent protein kinases (Weigel et al., 1981). The estrogen receptor is tyrosine phosphorylated (Migliaccio et al., 1984) but no evidence for intrinsic kinase activity was available.

Protein kinase activity in glucocorticoid receptor preparations had been reported (Kurl & Jacob, 1984; Singh & Moudgil, 1984; Miller-Diener et al., 1985) but the authors provided no direct evidence of receptor autokinase activity.

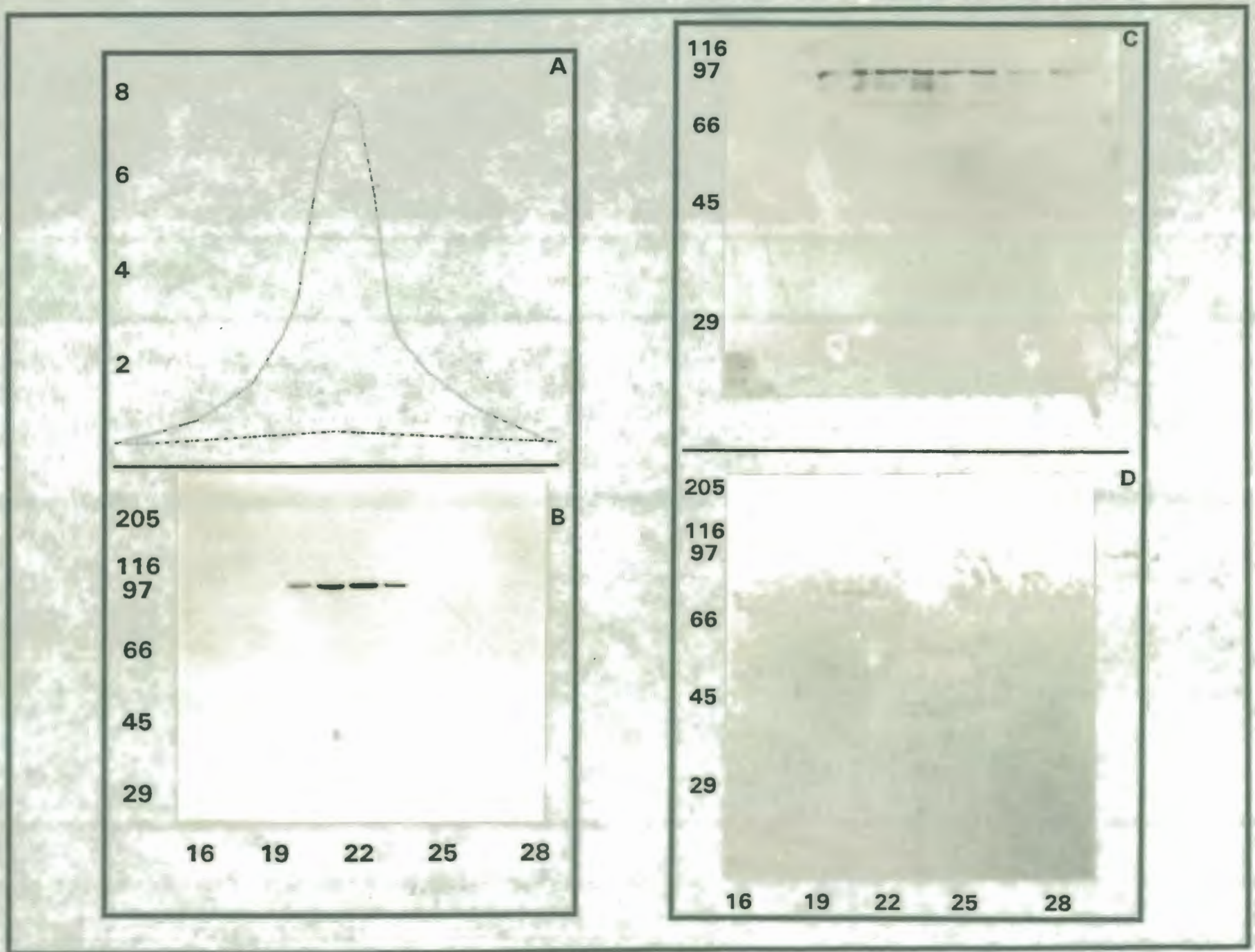
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Figure 1 Characterisation of DEAE - Sephacel Purified GHRC. Comparison with Control Mock Isolations.

The untransformed molybdate - stabilised GHRC was purified as described by Hapgood and von Holt (1987). Briefly, rat liver cytosol was subjected to phosphocellulose chromatography as described by Govindan and Gronemeyer (1984). The phosphocellulose flow - through was then incubated with the affinity matrix (synthesised by coupling the the 17B-carboxylic acid of dexamethasone to Affi-Gel 102 (Bio-Rad) via the N-hydroxybenzotriazole ester of dexamethasone). After thorough washing of the matrix, the receptor was eluted by incubation with 2 µM of tritiated triamcinolone acetamide ([^{3}H]TA) (5 Ci / mmol) for 16 hours. The resultant eluate was then subjected to DEAE-Sephacel chromatography and the untransformed GHRC eluted in the presence or absence of 10 mM molybdate with either a 0 - 500 mM sodium chloride gradient in buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM ß-mercaptoethanol, and 10% glycerol), or a 50 - 500 mM potassium phosphate gradient in buffer B (50 mM potassium phosphate, 1 mM EDTA, 5 mM ß-mercaptoethanol, and 10% glycerol, pH 7.0).
Control mock isolations were performed by incubation of cytosol with the affinity matrix in the presence of 4 µM unlabelled dexamethasone.
A: [^{3}H]TA elution profile of experiment (dotted line) and mock (dashed line) isolations. The preparations were eluted with a 50 - 500 mM potassium phosphate gradient in buffer B.
B: SDS - PAGE of individual fractions of the eluate of the experimental isolation.
C and D: anti - glucocorticoid receptor polyclonal antibody binding pattern of individual fractions for experimental (C) and mock control (D) isolations.

All that could be concluded was that the glucocorticoid receptor was a substrate for protein kinases.
This chapter summarises the published results (Hapgood et al., 1986) into the investigation of the kinase activity associated with the glucocorticoid receptor preparations under varying conditions of receptor steroid - binding activity and transformation state.

A.2 Results

The glucocorticoid receptor complex (GHRC) was isolated as described by Hapgood and von Holt (1987), and a typical final elution profile from DEAE - Sephacel is demonstrated in figure 1. The receptor elutes as a 92 kDa steroid - binding protein at 0.25 M potassium phosphate (figure 1A), and its identity confirmed by anti - receptor antibodies (figure 1C).
Incubation of GHRC preparations with [γ^{32}P]ATP after elution from DEAE - Sephacel in the absence (figure 2A) or presence (figure 2B) of molybdate, resulted in the phosphorylation of a number of proteins. The kinase activity is Mg^{2+} - dependent (figure 2B, lane 6).
Of particular interest is the phosphorylation of proteins with molecular weight of approximately 90 kDa (figures 2A & B), although these proteins are phosphorylated to a higher degree in the GHRC preparation eluted in the presence of molybdate (figure 2B). This difference in labelling was shown not to be due to the presence or absence of molybdate, but rather to the different gradients used to elute the two GHRC preparations, since the phosphorylation profiles of receptor preparations eluted from the DEAE - Sephacel in buffer 2 (minus molybdate) were identical to those in figure 2B. The phosphorylation profile of the receptor preparations after ammonium sulfate precipitation was also investigated

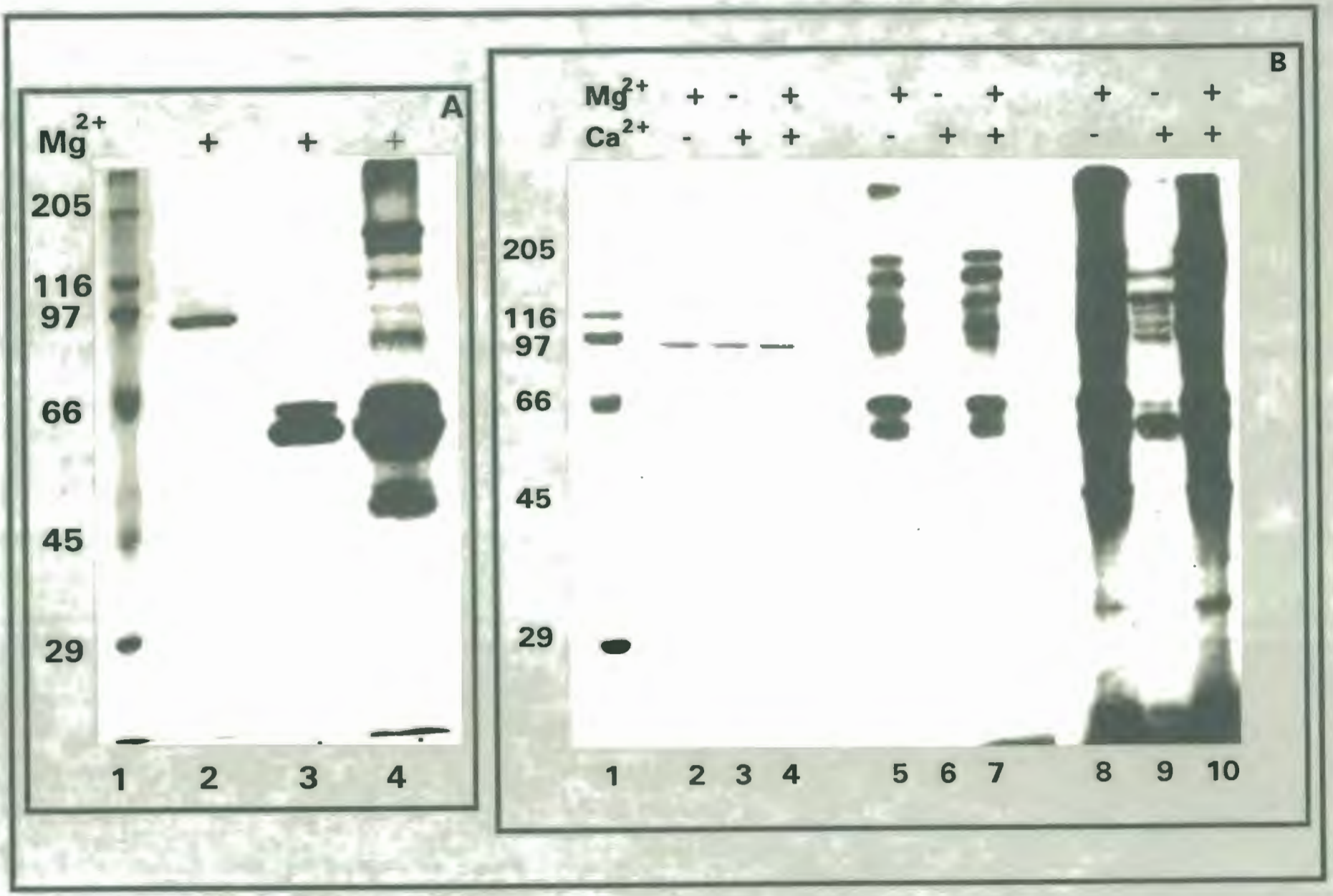
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Figure 2 Protein Kinase Activity of DEAE - Sephacel Purified GHRC.

GHRC eluted from DEAE Sephacel in buffer B minus molybdate (figure 2A) or buffer A plus molybdate (figure 2B) was diluted to a final concentration of 1 μg / ml and incubated with [γ^{32}P]ATP in the presence of 5 mM magnesium or calcium.
Coomassie stain: A, lane 2 and B, lanes 2 - 4.
Autoradiograms, 2 days exposure: A, lane 3 and B, lanes 5 - 7.
Autoradiograms, 10 days exposure: A, lane 4 and B, lanes 8 - 10.
Standards lane: A, lane 1 and B, lane 1.

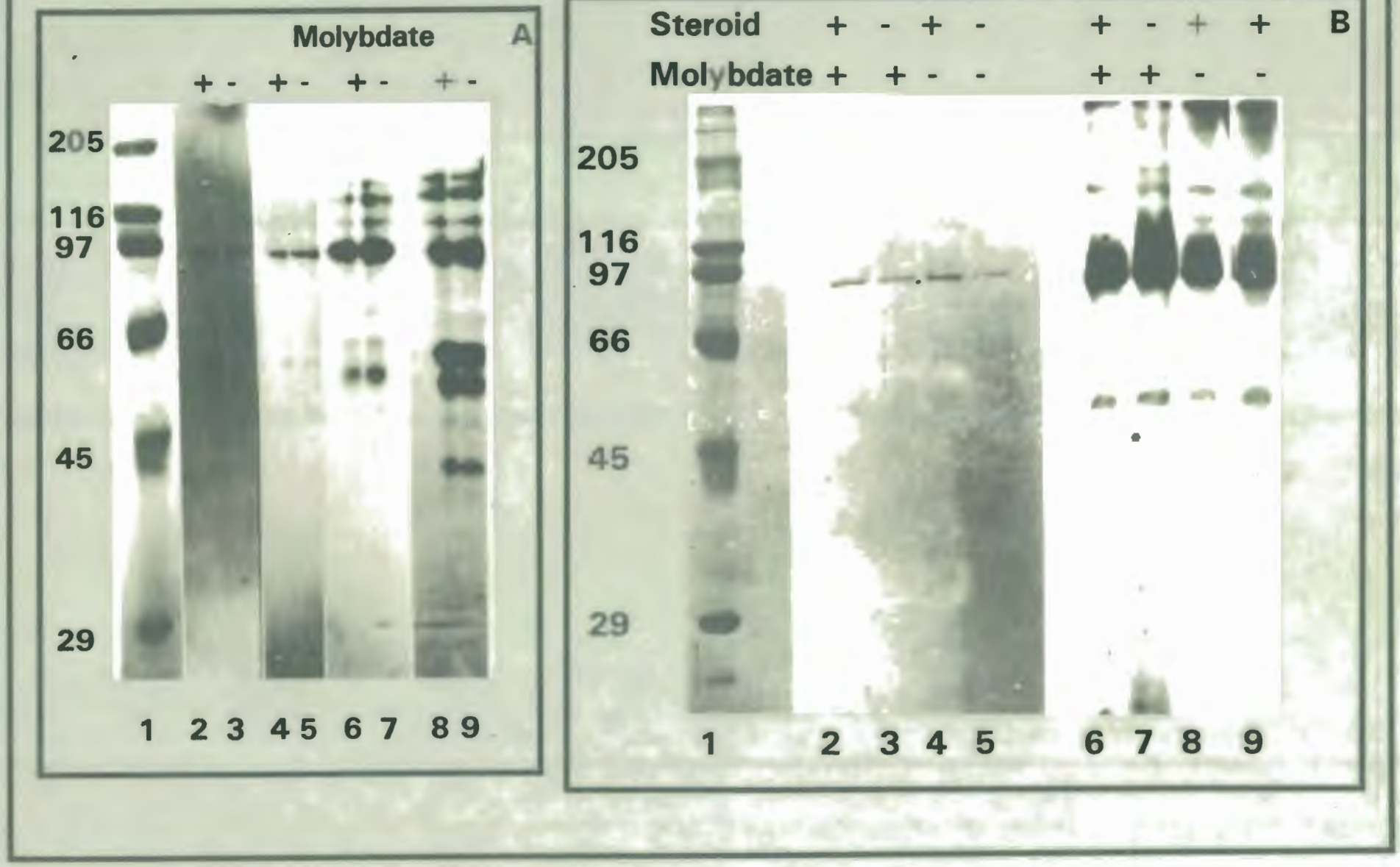
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Figure 3 Protein Kinase Activity of DEAE - Sephacel Purified GHRC After $(NH_4)_2SO_4$ Precipitation.

GHRC eluted from DEAE - Sephacel in buffer A minus molybdate (A, lanes 2 and 3), buffer B minus molybdate (A, lanes 4 and 5), or buffer A plus molybdate (B, lanes 2 - 5) was precipitated with ammonium sulfate. The precipitate was resuspended in buffer A containing 50 mM sodium chloride and 5 mM magnesium chloride and of this, 0.5 µg of GHRC incubated with [γ^{32}P]ATP under varying conditions of molybdate and steroid.
Coomassie: A, lanes 2 - 5 and B, lanes 2 - 5.
Autoradiograms: A, lanes 6 - 9 and B, lanes 6 - 9.
Standards lane: A, lane 1 and B, lane 1.

(figure 3). The 90 - 92 kDa fraction is again phosphorylated, but to a higher degree than is the case for non - precipitated receptor preparation (figure 2). The phosphorylation pattern is unaffected by molybdate or excess steroid (figure 3), although the overall phosphorylation profile is different to that of non - precipitated preparations. This probably reflects a selective precipitation of protein kinases.

These results demonstrate that the GHRC preparations have protein kinase activity and the fact that this activity is independent of molybdate, steroid - binding activity or transformation state, indicates that the GHRC receptor may not be responsible for the phosphorylation. However, the possibility that the receptor is an autokinase, could not be ruled out.

In order to positively identify any ATP - binding proteins (and thus potential protein kinases), the receptor preparations were incubated with [γ^{32}P]N_3ATP which was synthesised according to the method of Sabbatini and von Holt (1987). Incubation of $(NH_4)_2SO_4$ - precipitated receptor preparations with [γ^{32}P]N_3ATP and subsequent photoactivation identified a single N_3ATP - binding protein (48 kDa) (figure 4). The possibility that the receptor may be an autokinase but is somehow inhibited by the photoactivating conditions was discounted by the fact that the phosphorylation pattern and intensity during photoactivation was identical to that in figure 3 (Hapgood et al., 1986). These results show that the receptor does not bind ATP, and thus cannot possess kinase activity.

For further characterisation, fractions for both experiment and mock isolations, eluting close to the receptor in DEAE - Sephacel, were investigated for protein kinase activity (figure 5). The phosphorylation pattern in figure 5B demonstrates that it is independent of receptor presence since the pattern is identical for both experiment and mock isolations, as is the N_3ATP labelling pattern (figure 5C).

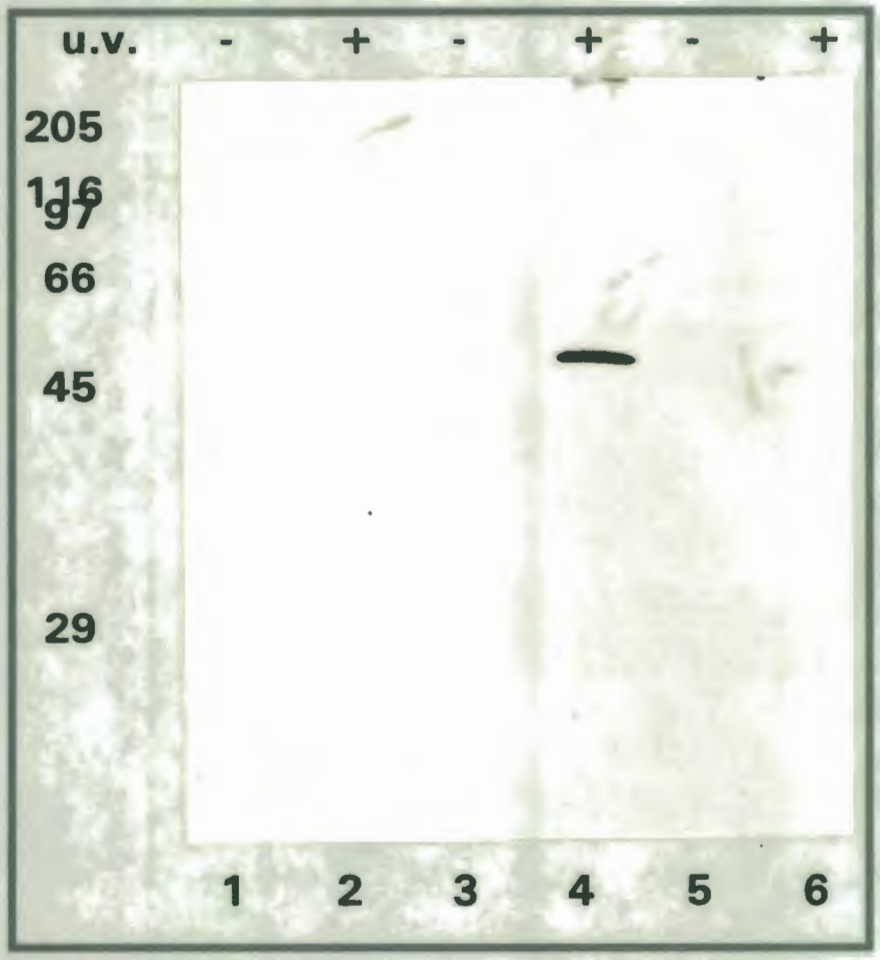

Figure 4 **[γ^{32}P]N_3ATP Photoaffinity Labelling of a Contaminant Protein Kinase in Purified $(NH_4)_2SO_4$ Precipitated GHRC Preparations.**

Precipitated GHRC was resuspended (0.5 µg / 200 µl) in buffer A containing 50 mM sodium chloride, 5 mM magnesium chloride, and 1 µM unlabelled triamcinolone acetonide and incubated with carrier - free [γ^{32}P]N_3ATP. Incubations in the presence of 1 mM cold ATP (lanes 1 and 2) or 1 µM unlabelled N_3ATP (lanes 5 and 6) were also performed. Lanes 1,3,and 5: incubations in the absence of ultraviolet light.
The autoradiogram was exposed for 2 days.

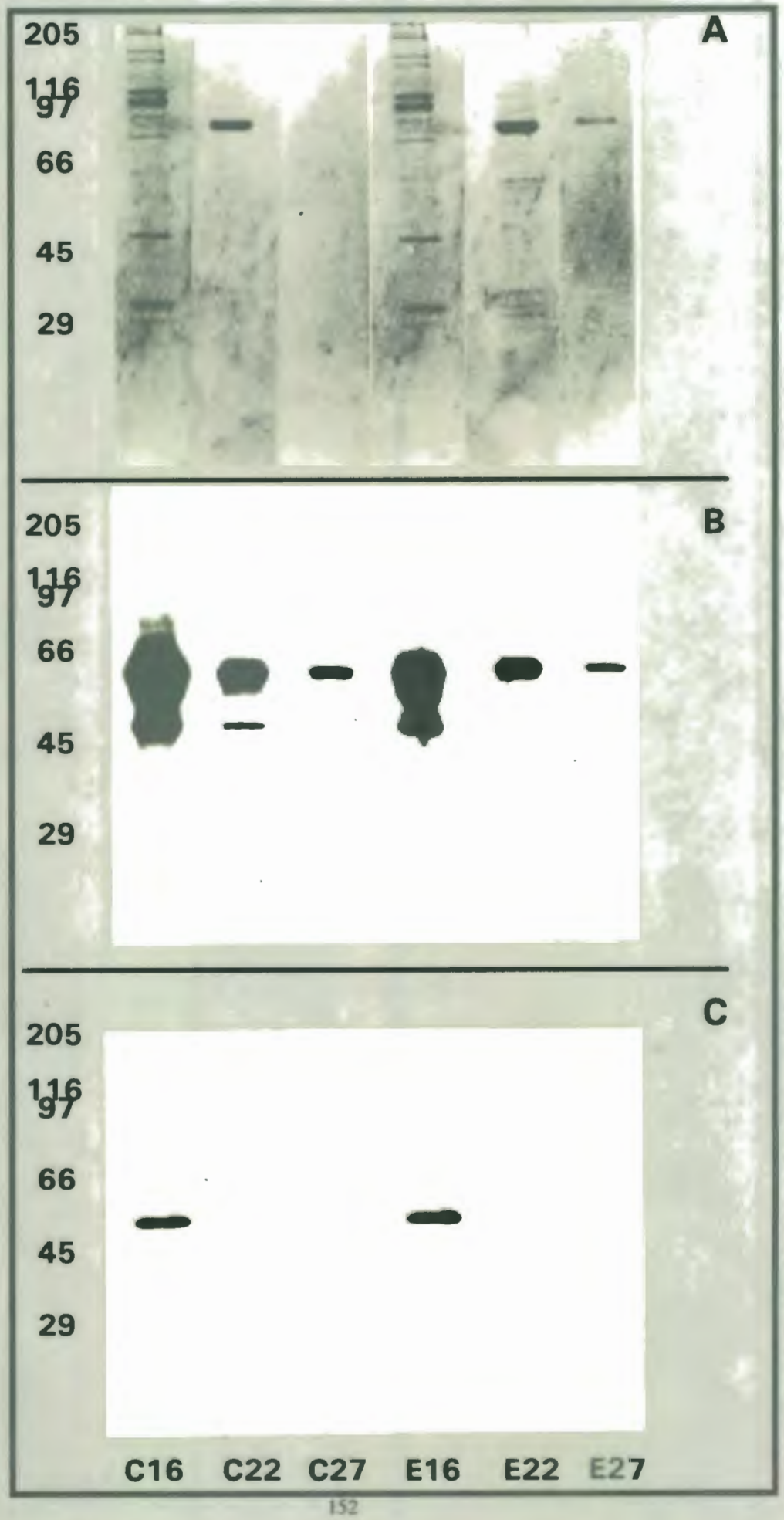
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Figure 5 Protein Kinase Contaminant in DEAE - Sephacel Purified GHRC.

Protein kinase and [γ^{32}P]N_3ATP binding activity in fractions 16, 22, and 27 of the DEAE - Sephacel eluate (buffer B minus molybdate) as defined in figure 1. Three equal volumes of each of fractions 16, 22, and 27 for experimental (E) and control (C) isolations were diluted to 600 µl to give a final GHRC concentration of 1 µg / 600 µl of GHRC for fraction 22 of the experimental isolation. Unlabelled triamcinolone acetonide (1 µM) and $MgCl_2$ (5 mM) were added to each.
A: Coomassie pattern.
B: autoradiogram after incubation with [γ^{32}P]ATP for 30 minutes at 20 ℃.
C: aliquots (2 x 600 µl)of each fraction were heat transformed by incubation for 30 minutes at 20 ℃. Six hundred microliters of each was then incubated for 15 minutes at 20 ℃ in the presence of [γ^{32}P]N_3ATP either in the presence or absence of ultraviolet light. In the absence of uv, samples analysed as for panel C showed no detectable labelled bands (data not shown).
Exposure of the gel for 5 days (as opposed to the 24 hours for panel C) did not reveal labelling of proteins in the 90-92 kDa region. However, a 55 kDa band became visible, coinciding with the major phosphorylated band in panel B. This probably indicates that the N_3ATP is being utilised by a protein kinase to phosphorylate the 55 kDa protein.

It is the fractions containing the 48 kDa ATP - binding protein (C16 and E16) that are most actively phosphorylated. In the presence of Ca^{2+}, the 48 kDa protein binds ATP with a much lower affinity (data not shown).

A.3 Discussion

These results demonstrate that neither the GHRC nor the 90 - 92 kDa non - steroid binding protein associated with the molybdate - stabilised GHRC is a Mg^{2+} - dependent protein kinase. Rather, a 48 kDa protein kinase co-isolates with the receptor. This kinase phosphorylates a number of contaminating proteins and probably the receptor itself. The phosphorylation of the 90 - 92 kDa protein appears to be unrelated to the presence of molybdate or the steroid - binding properties of the receptor.
The presence of a contaminating protein kinase could account for the conflicting reports on the putative kinase activity of the GHRC, especially if the recptor preparations of the various authors contained differing amounts of the contaminant. Our findings that the GHRC is not an autokinase is in agreement with previous findings (Sanchez & Pratt, 1986).