STUDIES ON THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

BY

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THIS THESIS IS DEDICATED TO MUM AND DAD, JASON AND STEPH

FOR THEIR ENDLESS LOVE AND SUPPORT

AND TO BECKY FOR HER LOVING COMPANIONSHIP

DURING THE PAST THREE YEARS

ABSTRACT

The β-adrenergic receptor of sheep adipose tissue was studied in adipose tissue membranes and partially-purified preparations, to determine some of its basic structural and pharmacological properties. The work in sheep was complemented with studies on the β-adrenergic receptor of rat adipocytes, to compare the β-receptors in these two species. The key findings of the study can be summarised as follows:

1) Sheep omental adipose tissue membranes, rich in β-adrenergic receptors (about 150 fmol/mg membrane protein measured using 10 nM [^{3}H]dihydroalprenolol ([^{3}H]DHA)), were prepared from whole tissue by differential centrifugation, without any collagenase digestion of the tissue. β-Adrenergic receptors were effectively solubilised with digitonin, and partially purified by affinity chromatography on a Sepharose 4B-alprenolol column. This resulted in β-adrenergic receptor preparations with a specific activity of about 35 pmol/mg protein (> 300-fold purification), and an overall recovery of 65%.

2) β-Adrenergic receptors were photoaffinity-labelled with [^{125}I]iodocyanopindolol-diazirine ([^{125}I]ICYPD) and subjected to SDS PAGE. Photoaffinity-labelling of the β-adrenergic receptors of sheep adipose tissue membranes specifically labelled a broad protein band of approximately 58 kDa in membranes, and two minor proteins with molecular weights of 42.5 and 30 kDa. In partially-purified preparations only a 58 kDa protein was labelled. The migration of this protein on SDS PAGE was unaffected by addition of high concentrations of reducing agents capable of breaking disulphide bridge. The results were similar for both omental and subcutaneous adipose tissue. In rat adipocyte membranes, a 48 kDa protein was specifically labelled with [^{125}I]ICYPD. The

isoelectric point of photoaffinity-labelled β-adrenergic receptor was 6.15-6.25 in sheep adipose tissue membranes. Two dimensional-PAGE of photoaffinity-labelled partially-purified β-adrenergic receptors revealed nine apparently different proteins, all with a molecular weight of 58 kDa. Studies were also performed with benzyldimethyl-n-hexadecylammonium chloride PAGE (16-BAC PAGE), in an attempt to develop an alternative purification strategy. However, quantification of β-adrenergic receptors following 16-BAC PAGE was not possible, and therefore the technique was not used for receptor purification.

3) The β-adrenergic receptor of sheep omental adipose tissue possessed high affinity for the non-subtype selective radioligands [^{3}H]DHA (K_d - 3.23 ± 0.38 nM (n=4)) and [^{125}I]iodocynaopindolol ([^{125}I]ICYP) (K_d - 53.5 pM). For [^{3}H]DHA binding, B_{MAX} varied between 370 and 600 fmol/mg membrane protein. The rank order of potencies of β-adrenergic agonists for competition of [^{3}H]DHA-binding sites was (-)-isoproterenol > (-)-adrenaline > (-)-noradrenaline. For subtype selective β-adrenergic antagonists the order was ICI 118,551 (β_2)>>CGP 20712A (β_1)>atenolol (β_1). These potencies are indicative of β_2-adrenergic receptors.

4) The affinities of β-adrenergic receptor preparations from different depots from sheep and rats were compared. Displacement of [^{125}I]ICYP-binding with ICI 118,551 and CGP 20712A from sheep adipose tissue membranes prepared from three depots, revealed heterogeneity in β-adrenergic receptor affinities. Analysis of $\log_{10}$-transformed results gave an order of β_2-character of subcutaneous = popliteal > omental ($P<0.01$). Similar studies with rat adipose tissue revealed the presence of β_1- and atypical (i.e. neither β_1- nor β_2-subtypes) β-adrenergic receptors in both lumbar and parametrial depots. The parametrial depot

possessed a higher percentage (of total receptor binding) of atypical β-adrenergic receptors compared to the lumbar depot.

5) β-Adrenergic receptor affinities for ICI 118,551 and CGP 20712A were unaffected by lactation. However, lactation increased β-adrenergic receptor ligand binding in omental ($P<0.01$) and subcutaneous ($P<0.02$) depots.

6) Polyclonal antisera were raised in mice against three different immunogens containing β-adrenergic receptors from sheep omental adipose tissue. Some antisera displaced [^{125}I]ICYP binding to sheep adipose tissue membranes. However, the antisera were non-specific with regards to β-receptors, recognising many proteins on immunoblots. Production of monoclonal antibodies against the β-adrenergic receptor of sheep adipose tissue was unsuccessful. However, the polyclonal antiserum CM 13, directed against the hamster lung β_2-adrenergic receptor, recognised 61 and 59 kDa proteins on immunoblots of sheep omental adipose tissue membranes.

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ABBREVIATIONS

ATP	Adenosine triphosphate
16-BAC	Benzyldimethyl-n-hexylammonium chloride
βARK	β-Adrenergic receptor kinase
B_{MAX}	Maximum number of binding sites
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-3',5'-monophosphate
CCV	Clathrin-coated vesicles
CGP 20712A	(±)-(2-hydroxy-5-[2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1H-imidazole-2-yl-)phenoxy)propyl)amino) ethoxy]-benzamide monoethane sulfonate
CPM	Counts per minute
D.F.	Dye front
[^{3}H]DHA	(-)-[^{3}H]Dihydroalprenolol
DPM	Disintergrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
GDP	Guanosine diphosphate
G protein	Guanine nucleotide regulatory protein
GRE	Glucocorticoid response elements
GTP	Guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IC_{50}	Effective concentration of drug giving half-maximal response
ICI 118,551	Erythro-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol
[^{125}I]ICYP	(-)-[^{125}I]Iodocyanopindolol
[^{125}I]ICYPD	(±)-3-[^{125}I]Iodocyanopindolol diazirine

IEF	Isoelectric focussing
Ig	Immunoglobulin
IP_3	Inositol triphosphate
K_d	Equilibrium dissociation constant
KDa	Kilodalton
K_i	Inhibition constant
mRNA	Messenger ribonucleic acid
NMS	Normal mouse serum
NRS	Normal rabbit serum
Ori	Origin
PAGE	Polyacrylamide gel electophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pI	Isoelectric point
PKA	cAMP-dependent protein kinase (protein kinase A)
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
Rf	Relative mobility
r.p.m.	Revolutions per minute
SAPU	Scottish antibody production unit
SATM	Sheep adipose tissue membranes
SDS	Sodium dodecyl (lauryl) sulphate
SEG	Sheep erythrocyte ghosts
S.E.M.	Standard error of the mean
TBS	Tris-buffered saline
Tris	Tris(hydroxymethyl)methylamine
2D-PAGE	Two dimensional polyacrylamide gel electrophoresis

U.V.	Ultra violet
Vo	Void volume
v/v	Volume to volume
w/v	Weight to volume

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CHAPTER ONE

INTRODUCTION

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1.1. Adrenergic mechanisms

1.1.1. Catecholamines

The physiologically relevant catecholamines which exert their effects via adrenergic receptors are the hormone adrenaline, and the neurotransmitter noradrenaline. Catecholamines are synthesised from the amino acid l-tyrosine (Barrand & Callingham, 1983). Adrenaline is synthesised and released exclusively from the adrenal medulla (Barrand & Callingham, 1983), whilst noradrenaline is released from synaptic nerve endings upon sympathetic stimulation (Landsberg & Young, 1985). The half-life of catecholamines in the blood is about 20 s (Vane, 1969), primarily due to uptake by body tissues (Iversen, 1975). They are inactivated by methylation of the 3-hydroxyl group of the catechol ring by catechol-O-methyl transferase, or oxidative removal of their amino group by monoamine oxidase (Stryer, 1981; Barrand & Callingham, 1983). Generally, catecholamines are released during stress or shock in order to increase the supply of metabolic fuels (Von Euler, 1964).

1.1.2. Adrenergic receptor subtypes

Catecholamines such as adrenaline and noradrenaline regulate physiological processes via their interactions with a variety of receptors located in the plasma membrane (Lefkowitz & Caron, 1988). Ahlquist (1948) originally classified these receptors as α- and β-adrenergic receptors, on the basis of the relative potencies of various agonists in stimulating responses through these receptors. Further studies suggested that at least two major subtypes of both α- and β-adrenergic receptors existed. Using a variety of pharmacological criteria, α_1- and α_2-adrenergic receptors were distinguished (Langer,

1974; Berthelsen & Pettinger, 1977), as were β_1- and β_2-adrenergic receptors (Lands et al., 1967).

1.1.2.1. α_1-Adrenergic receptors

Binding of agonists to α_1-adrenergic receptors leads to signal transduction across the membrane, resulting in the activation of a phosphatidylinositol-4,5-bisphosphate-specific phospholipase C (reviewed in Berridge, 1984; Graham & Lanier, 1986). This transmembrane signalling process is thought to involve an as yet uncharacterised G protein (Goodhardt et al., 1982; Boyer et al., 1984; Terman et al., 1987; Im & Graham, 1989). Activation of phospholipase C leads to the generation of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol, which act as second messengers (Ambler et al., 1984). IP_3 mobilises intracellular stores of calcium (Michell, 1975; Graham & Lanier, 1986; Putney, 1987), and diacylglycerol activates protein kinase C (Nishizuka, 1984). Other studies have implicated addditional transduction mechanisms including activation of phosphatidylcholine-specific phopholipase D (Irving & Exton, 1987) and activation of phospholipase A_2 (Burch et al., 1986; Slivka & Insel, 1987).

The α_1-adrenergic class of receptors has been subdivided pharmacologically into two subtypes, namely α_{1A} and α_{1B} (Morrow et al., 1985; Han et al., 1987; Minneman et al., 1988). This heterogeneity has been corroborated by the cloning of the α_{1B}-adrenergic receptor (Cotecchia et al., 1988). Also a novel α_1-adrenergic receptor subtype has been cloned and expressed which, although it resembles the α_{1A} subtype, displays some unique pharmacological characteristics (Schwinn et al., 1990). This suggests that more α_1-adrenergic receptor subtypes may exist than those identified by pharmacological criteria.

1.1.2.2. α_2-Adrenergic receptors

Activation of α_2-adrenergic receptors elicits a variety of inhibitory and stimulatory responses at effector cells in the central nervous system and peripheral tissues (Graham & Lanier, 1986). In several tissues, the signal transduction mechanism utilised by this receptor is poorly defined (Isom & Limbird, 1988; Bylund, 1988). Activation of α_2-adrenergic receptors can inhibit the stimulation of adenylate cyclase and limit intracellular cAMP concentrations (Burns & Langley, 1975; Jacobs et al., 1976; Cooper et al., 1979), and hence antagonise β-adrenergic receptor action (see 1.1.2.3). This effect is mediated by the G protein, G_i (Bokoch et al., 1984; Katada et al., 1984a,b,c). However this pathway does not account for receptor-mediated inhibition of insulin release (Ullrich & Wollheim, 1984; Nilsson et al., 1988), depolarisation-induced neurotransmitter release (Hirning et al., 1988), or smooth muscle contraction (Timmermans & van Zweiten, 1982; Graham & Lanier, 1986) produced by α_2-adrenergic receptor agonists. Alternative transduction pathways have thus been proposed such as coupling to an ion channel (Hirning et al., 1988) or activation of the Na^+/H^+ exchanger (Sweatt et al., 1985; Isom et al., 1987; Cantiello & Lanier, 1989).

Like the α_1-adrenergic receptors, the α_2-adrenergic receptor class has been found to possess subtypes. At least three and possibly four α_2-adrenergic receptor subtypes are thought to exist (α_{2A}, α_{2B}, and α_{2C}) based on pharmacological data, and the genes for the α_{2A}- and α_{2B}-adrenergic receptors have been cloned (reviewed by Bylund, 1988).

1.1.2.3. β-Adrenergic receptors

β-Adrenergic receptor stimulation of the membrane-bound enzyme

adenylate cyclase is well established in many tissues including the heart (Murad et al., 1962), adipose tissue (Birnbaumer et al., 1969), liver (Marinetti et al., 1969), lung (Benovic et al., 1984), and lymphoid cells (Makman, 1971). There is also evidence for β_1-adrenergic receptor stimulation of Ca^{2+} channels in both the heart (Yatani et al., 1987) and skeletal muscle (Yatani et al., 1988). β-Adrenergic receptors have become model systems for researchers studying the nature and regulation of plasma membrane hormone and drug receptors, primarily because they are present in virtually all mammalian tissues, and their intimate coupling to adenylate cyclase. Indeed, the β-adrenergic receptor-dependent adenylate cyclase was the first transmembrane signalling system to be resolved into its components and fully reconstituted (reviewed in Levitzki, 1985, 1988). G_s is the G protein involved in this pathway (Gilman, 1984, 1987). The activation of the G_s-adenylate cyclase system by agonist-occupied β-adrenergic receptor is a catalytic event, and the receptor is not permanently associated with the G_s-adenylate cyclase complex (Levitzki, 1988). Furthermore, β-adrenergic receptor activation of G_s does not appear to require the presence of adenylate cyclase (Levitzki, 1988).

Currently, three β-adrenergic receptor subtypes have been conclusively defined. β_1- and β_2-adrenergic receptors were originally distinguished by Lands et al. (1967). However, more recent studies suggest that β-adrenergic receptors with atypical characteristics i.e. not conforming to the β_1- and β_2-receptor classification, exist in some tissues. In the first instance, white (Wilson et al., 1984; Bojanic et al., 1985; Hollenga & Zaagsma, 1990) and brown (Arch et al., 1984) adipocytes were shown to possess atypical β-adrenergic receptors.

Atypical β-adrenergic receptors have also been proposed to transduce catecholamine control of several metabolic processes in colon (McLaughlin & MacDonald, 1989) and ileum (Blue et al., 1988; Bond & Clarke, 1988) smooth muscle, skeletal muscle (Challiss et al., 1988), and the heart (Kaumann, 1989). The existence of a β_3-adrenergic receptor subtype has been confirmed with the isolation of a human gene that encodes a third β-adrenergic receptor (Emorine et al., 1989). The human β_3-adrenergic receptor shares some characteristics with the rat white adipocyte β-adrenergic receptor, but appears not to be identical (Zaagsma & Nahorski, 1990). Therefore it is very probable that more β-adrenergic receptor subtypes await discovery.

1.1.3. β-Adrenergic receptor signal transduction

Signal transduction in this system involves the sequential interactions of β-adrenergic receptor, G_s and adenylate cyclase and/or Ca^{2+} channels. Only adenylate cyclase stimulation will be discussed here due to its relevancy to adipose tissue metabolism. G_s interaction with Ca^{2+} channels is extensively reviewed in Birnbaumer et al. (1990).

1.1.3.1. G proteins

About 80% of all known hormones and neurotransmitters, as well as many neuromodulators, autocrine and paracrine factors that regulate cellular interactions, are known to elicit cellular responses by binding to specific receptors which are coupled to effector systems by G proteins (reviewed by Graziano & Gilman, 1987; Birnbaumer et al., 1990). All G proteins are heterotrimers composed of α, β and γ subunits, which dissociate upon activation into α-subunits and $\beta\gamma$ dimers (Kuhn, 1980; Northup et al., 1980; Hildebrandt et al., 1984). It is the α-subunits which bind and hydrolyse GTP, and define the receptor and effector

specificity of a G protein (Manning & Gilman, 1983). The stimulatory effect of G_s on adenylate cyclase is mediated directly, and this capacity is thought to reside in $G_{s\alpha}$ (Gilman, 1987). However, interaction of $\beta\gamma$-dimers with effector systems has not been ruled out. Indeed a direct inhibitory interaction of $\beta\gamma$-dimers with adenylate cyclase has been proposed (Katada et al., 1986); however this is controversial (Smigel, 1986; Birnbaumer et al., 1990). Therefore the active species is presumed to be $G_{s\alpha}$.GTP.adenylate cyclase. This complex is relatively long-lived (about 15 s), because of the very slow rate of GTP hydrolysis catalysed by the $G_{s\alpha}$ subunits (Gilman, 1987). Upon GTP hydrolysis to GDP, the complex dissociates and adenylate cyclase activity ceases (Gilman, 1987). $G_{s\alpha}$.GDP then reassociates with a $\beta\gamma$-dimer to form a holo-G_s protein (Hildebrandt et al., 1984; Gilman, 1984). $G_{s\alpha}$ subunits are also substrates for ADP-ribosylation by cholera toxin, which leads to their activation. ADP-ribosylation is thought to occur at an arginine residue (Van Dop et al., 1984).

1.1.3.2. Adenylate cyclase

The primary mechanism for regulation of the intracellular concentration of the second messenger adenosine 3',5'-monophosphate (cAMP) is by modulation of the activity of adenylate cyclase. This enzyme catalyses the formation of cAMP from intracellular adenosine triphosphate (Sutherland et al., 1968). Adenylate cyclase has been shown to exist in multiple forms ranging in molecular weights from 120 kDa to 150 kDa (Pfeuffer et al., 1985; Smigel, 1986). Recently, cDNAs encoding an adenylate cyclase were isolated (Krupinski et al., 1989). Analysis of the deduced amino acid sequence reveals two alternating sets of hydrophobic and hydrophilic domains. Each of the hydrophobic domains

appears to contain six transmembrane α-helices. The hydrophilic domains contain a sequence that is homologous to a single cytoplasmic domain of several guanylate cyclases, possibly a nucleotide binding site. The predicted topography resembles that of various plasma membrane ion channels and transporters. Therefore it is possible that adenylate cyclase not only synthesises cAMP, but also transports it from the cell (Davoren, 1963). This process is known to occur in the slime mold Dictyostelium discoideum, which synthesises cAMP and then exports it as an extracellular signal for chemotaxis, aggregation and differentiation (Gerisch, 1987). A role for this possible function of adenylate cyclase in higher eukaryotes is not obvious.

1.1.4. Physiological effects of β-adrenergic receptor stimulation

As stated earlier, adrenergic receptors are found on most cell types. Therefore effects of released adrenaline and noradrenaline are not restricted to a specific tissue within the body (reviewed by Lefkowitz et al., 1984; Smigel et al., 1984). Major effects mediated by β-adrenergic receptor stimulation are: i) stimulation of adipocyte lipolysis; ii) stimulation of the rate and force of cardiac contraction; iii) relaxation of smooth muscle in bronchi, blood vessels, and genito-urinary and gastrointestinal tracts; iv) increased glycogenolysis in liver and muscle. In contrast, α_2-adrenergic receptor activation antagonises many of the β-adrenergic receptor responses (Table 1.1). The number of systems regulated by β-adrenergic receptors causes many problems during the development of therapeutic drugs acting via adrenergic receptors. Major considerations in the design of such compounds are adrenergic receptor and tissue specificity, because these factors are likely to determine the number of unwanted side-effects.

1.2. Purification of β-adrenergic receptors

1.2.1. Ligand-binding studies

Study of the biochemical properties of any membrane-bound hormone or drug receptor requires the ability to measure the receptor in membranes and solubilised or purified fractions. Successful measurement of β-adrenergic receptors using direct radioligand binding methods was achieved only in 1974. The initial ligands used were (-)-[^{3}H]dihydroalprenolol (Lefkowitz et al., 1974), (±)-[^{125}I]iodohydroxybenzylpindolol (Aurbach et al., 1974), and (±)[^{3}H]propranolol (Atlas et al., 1974). Many more agonist and antagonist radioligands have subsequently been developed (Burgisser & Lefkowitz, 1982). Since 1981, the preferred ligand has been (-)-[^{125}I]iodocyanopindolol because it exhibits higher affinity and specific radioactivity than the earlier radioligands (Engel et al., 1981). These compounds are non-selective in their affinities for β_1- and β_2-adrenergic receptors (Stiles et al., 1984b; Hussain et al., 1987). However, by displacing the binding of such radioligands with β-adrenergic antagonists, whose binding is specific for either β_1- or β_2-adrenergic receptors, it is possible to quantify the number of each β-adrenergic receptor subtype present in a tissue (Minneman et al., 1981; De Lean et al., 1982). Such experiments reveal that β_1- and β_2-receptors often co-exist in the same tissue (Minneman et al., 1981; Moore & Whitsett, 1981).

Some of the radioligands have been modified by addition of a highly reactive azide moieties, which upon irradiation form highly reactive nitrenes that can react covalently with a variety of chemical groups (Bayley & Staros, 1984). The resulting compounds are potent photoaffinity labels for the β-adrenergic receptor, and have been used

extensively to identify receptors in many tissues (Lefkowitz et al., 1983; Burgermeister et al., 1983).

1.2.2. Purification of the β-adrenergic receptor

Successful purification of β-adrenergic receptors from several sources has been accomplished. These include receptors from avian (Shorr et al., 1981, 1982; Marbach & Levitzki, 1988), amphibian (Caron et al., 1979), and mammalian (Homcy et al., 1983; Benovic et al., 1984; Cubero & Malbon, 1984; Graziano et al., 1985; Bahouth & Malbon, 1987) species. The use of affinity chromatography appears to be critical, in particular the utilisation of Sepharose-immobilised alprenolol (Vauquelin et al., 1977; Caron et al., 1979). Alprenolol is a potent (K_d~1-10 nM) non-subtype selective β-adrenergic antagonist.

Plasma membranes have generally been solubilised with digitonin (Hubbard, 1954), a plant sterol isolated from Digitalis purpurea, since this detergent solubilises β-adrenergic receptors in a form permitting their subsequent interaction with adrenergic ligands (Caron & Lefkowitz, 1976; Caron et al., 1979). Attempts to solubilise the receptors with other detergents including Lubrol PX (Haga et al., 1977), Triton X-100 (Fraser & Venter, 1982), Tween, cholate, and deoxycholate (Shorr et al., 1985), have led to complete loss of receptor-binding activity. Recently, the detergent dodecyl-β-D-maltoside has been used to solubilise and purify the β-adrenergic receptor (Tota & Strader, 1990). This synthetic detergent was preferred because of the variable quality of digitonin preparations.

Affinity chromatography of digitonin-solubilised β-adrenergic receptors on Sepharose-alprenolol gels leads to a 100-1000 fold purification, depending on the exact method used. Elution of the

receptors with a concentration gradient of antagonist was found to be 8 times more efficient than a step elution with a single concentration (Benovic et al., 1984).

To purify the β-adrenergic receptor to homogeneity requires additional techniques. By coupling repeated affinity chromatography to ion exchange (Shorr et al., 1981) or gel filtration (Homcy et al., 1983), or affinity chromatography and several high performance liquid chromatography steps (Shorr et al., 1982; Cubero & Malbon, 1984; Benovic et al., 1984; Shorr et al., 1985; Graziano et al., 1985) the β-adrenergic receptors from several sources have been purified to homogeneity. Hydrophobic chromatography using heptylamine-Sepharose has also been used during the purification of human placenta β_1- and β_2-adrenergic receptors (Bahouth & Malbon, 1987). Importantly, the purified β-adrenergic receptors retain the affinities and specificities of 'native' receptors still embedded in plasma membranes (Shorr et al., 1981; Cubero & Malbon, 1984; Benovic et al., 1984; Graziano et al., 1985).

1.2.3. Reconstitutional studies of β-adrenergic receptors

To study the ability of purified β-adrenergic receptor to activate biological processes requires a reconstitution assay. The requirement for a lipid membrane in which the components of the β-adrenergic receptor-coupled adenylate cyclase system can interact is mandatory. In fact, restoration of the interaction between delipidated β-adrenergic receptor and G_s requires the addition of at least three specific phospholipids (Ben-Arie et al., 1988). It is likely that these added phospholipids substitute for the glycolipid tightly associated with the β-adrenergic receptor in turkey erythrocytes (Bar-Sinai et al., 1986). The coupling between the receptor and adenylate cyclase is sensitive to

detergent, and is inhibited at detergent concentrations that are one-tenth of those required to inhibit G_s and adenylate cyclase interaction (Keenan et al., 1982). It remains to be established whether glycolipids play a role in the functional coupling between β-adrenergic receptors and the components of adenylate cyclase, or have another role such as receptor stabilisation and maintainance within the lipid bilayer. Palmitoylation of cysteine^{341} residue in human β_2-adrenergic receptor has been reported (O'Dowd et al., 1989). A non-palmitoylated form of the same receptor possessed drastically reduced ability to mediate isoproterenol-stimulation of adenylate cyclase (O'Dowd et al., 1980). Insertion of the palmitic acid into a lipid bilayer may therefore be crucial for normal coupling.

Schramm et al. (1977) pioneered reconstitution studies using relatively crude preparations of β-adrenergic receptor, G_s and adenylate cyclase. Although some agonist-stimulated accumulation of cyclic AMP was achieved, it was still possible that impurites in the preparations were involved in signal transduction. Cerione et al. (1984) were also plagued by impure preparations of adenylate cyclase, since they had to use an extract of rat brain. Later, reconstitution of pure proteins into unilamellar vesicles showed that these three proteins are sufficient for the expression of hormone-stimulated adenylate cyclase (May et al., 1985). Utilisation of such systems allows direct evaluation of the stoichiometry and kinetics of the reactions leading to adenylate cyclase activation following hormone binding. Furthermore, such an approach allows definition of the role that the lipid bilayer plays in promoting and modulating these interactions.

1.3. Properties of the β-adrenergic receptor

1.3.1. Determination of β-adrenergic receptor molecular weights

The apparent molecular weight of β-adrenergic receptors has generally been measured using denaturing SDS PAGE, and appropriate protein standards. The molecular weights of purified β-receptor from many sources have been reported including those of human placenta (M_r=67 kDa)(Bahouth & Malbon, 1987); rat adipocytes (M_r=67 kDa)(Cubero & Malbon, 1984); hamster lung (M_r=64 kDa)(Benovic et al., 1984); rat liver (M_r=67 kDa)(Graziano et al., 1985); guinea-pig lung (M_r=67 kDa)(Drake & Perkins, 1984), and S49 mouse lymphoma cells (M_r=67 kDa)(George & Malbon, 1985). The canine lung β_2-adrenergic receptor has a molecular weight of 52-53 kDa (Homcy et al., 1983), and appears to be smaller than most mammalian β-adrenergic receptors, as is the frog erythrocyte β_2-adrenergic receptor (M_r=58 kDa)(Shorr et al., 1982).

The nature of β-adrenergic receptors investigated by photoaffinity-labelling in mammalian lung is even more diverse. β-Adrenergic receptors studied using this technique have suggested molecular weights of 64 kDa in hamster lung (Benovic et al., 1983); 50-55 kDa in canine lung (Homcy et al., 1983); 59 kDa in bovine lung (Fraser & Venter, 1982); 64 kDa in sheep and pig lung (J. L. Benovic, personal communication), and 64 kDa, 53 kDa and 44 kDa in rat lung (Rashibaigi et al., 1983). Other β-adrenergic receptors characterised by photoaffinity-labelling include those of human placenta (M_r=65-67 kDa)(Bahouth & Malbon, 1987; Emorine et al., 1989); human A431 cells (M_r=65 kDa)(Kaveri et al., 1987); S49 mouse lymphoma cells (M_r=65 kDa and 55 kDa)(Rashibaigi et al., 1983), (M_r=67 kDa)(Cubero et al., 1983); human, canine, porcine, rabbit, frog, and rat ventricle (M_r=62 kDa)(Stiles et al., 1983). There

appears to be little difference in the molecular weights of the three human β-adrenergic receptor subtypes (Bahouth & Malbon, 1987; Emorine et al., 1989).

Photoaffinity-labelling of avian erythrocyte β_1-adrenergic receptors identified receptors with molecular weights of 48 kDa and 45 kDa in duck (Dickinson & Nahorski, 1981; Rashibaigi & Ruoho, 1982); 52 kDa and 45 kDa in pigeon (Rashibaigi & Ruoho, 1982); 45 kDa and 40 kDa in turkey (Lavin et al., 1981; Shorr et al., 1982). The turkey erythrocyte β-adrenergic receptor consists of two distinct forms, which although differing in molecular weight, show virtually identical ligand-binding properties typical of a β_1-adrenergic receptor (Shorr et al., 1982). In contrast, molecular heterogeneity sometimes observed during photoaffinity-labelling of other β-adrenergic receptors is due predominantly to proteolysis, particularly by metal-dependent proteases (Benovic et al., 1984b).

Additional studies performed using less invasive techniques have suggested that β-adrenergic receptors may dimerise in membranes. Target-size inactivation studies of β-adrenergic receptors in mammalian lung indicates a functional molecular weight for the binding moiety in situ of about 110 kDa (Fraser & Venter, 1982). The purified β_2-adrenergic receptor from rat liver and the β-adrenergic receptor from rat adipocyte both migrate on steric exclusion HPLC in two discrete forms, with molecular weights of 140 kDa and 67 kDa (Graziano et al., 1985). Also, studies of the hydrodynamic properties of β-adrenergic receptors in extracts from digitonin-solubilised membranes suggest a native receptor molecular weight ranging from 130 kDa-160 kDa (Homcy et al., 1983; Haga et al., 1977; Caron & Lefkowitz, 1976). However, Shorr et

al. (1984) concluded that it is not the dimer but the monomeric form of the β-adrenergic receptor which demonstrates specific ligand binding.

Immunoblotting against membranes using antisera specific for β-adrenergic receptors has confirmed the molecular weights for many receptors. These include the β-adrenergic receptors from human placenta (M_r=67 kDa)(Bahouth & Malbon, 1987); human A431 cells (M_r=65 kDa)(Kaveri et al., 1987), (55 kDa)(Guillet et al., 1985); S49 mouse lymphoma cells (M_r=65 kDa)(Wang et al., 1989c); rat adipocyte (M_r=67 kDa)(Moxham & Malbon, 1985; Moxham et al., 1986); and turkey erythrocyte (M_r=42 kDa)(Chapot et al., 1989). Therefore the apparent molecular weight of β-adrenergic receptors determined by SDS PAGE appears to be unaffected by photoaffinity-labelling.

1.3.2. Structure of the β-adrenergic receptor

1.3.2.1. Cloning of β-adrenergic receptors

The β_2-adrenergic receptor cDNA and/or gene from hamster (Dixon et al., 1986), human (Kobilka et al., 1987a; Emorine et al., 1987), and mouse (Nakada et al., 1989), the human β_1-adrenergic receptor cDNA (Frielle et al., 1987), the rat β_1-adrenergic receptor gene (Machida et al., 1990), and the human β_3-adrenergic receptor (Emorine et al., 1989) have been cloned and sequenced. In addition, β-adrenergic receptor cDNAs which have not been expressed and characterised, have been isolated from turkey erythrocyte (Yarden et al., 1986), human brain (Chung et al., 1987), and rat heart (Gocayne et al., 1987). Data from genomic Southern blot and gene dosage analyses indicates that β-adrenergic receptor genes are single copy genes within the genome. The chromosomal organisation of the genes encoding adrenergic receptors has also been determined. Human α_2- and β_1-adrenergic receptor genes are located on chromosome

10, while α_1- and β_2-adrenergic receptor genes are on chromosome 5 (Yang-Feng et al., 1990).

While the genes encoding these β-adrenergic receptors are distinct and intronless, they are quite homologous and are members of the growing family of cloned G protein-coupled receptors, which share many structural features (Dohlman et al., 1987b). This family also contains the α_1-adrenergic receptor (Cotecchia et al., 1988), α_2-adrenergic receptor (Kobilka et al., 1987b; Regan et al., 1988; Lomasney et al., 1990), muscarinic cholinergic (Kubo et al., 1986a,b; Peralta et al., 1987; Bonner et al., 1987), serotonin (Julius et al., 1988; Fargin et al., 1988), angiotensin II (Jackson et al., 1988), substance K (Masu et al., 1987), lutropin-choriogonadotropin and thyrotropin (Lomasney et al., 1990), and dopaminergic (Giros et al., 1989; Monsma, Jr et al., 1989) receptors, as well as the opsins (Nathans et al., 1986).

1.3.2.2. Topography of the β-adrenergic receptor

The recent cloning of the genes or cDNAs encoding the three β-adrenergic receptor subtypes has revealed the primary structure of these G-protein-linked receptors. Perhaps the most striking similarity in the topography of all these receptor proteins is that each contains seven regions of 20-28 hydrophobic amino acids, postulated to be membrane-spanning α-helices. Hydropathicity analysis of the primary amino acid sequence using the method of Kyte & Doolittle (1982) yields remarkably similar profiles (Kubo et al., 1986a,b; Dohlman et al., 1987b). Each of the transmembrane α-helices is proposed to be linked with hydrophilic loops exposed (alternately) intracellularly and extracellularly (Dohlman et al., 1987b). This arrangement bears great structural homology to the model originally proposed for bacteriorhodopsin, the purple membrane

protein of Halobacterium halobium. The presence of a bundle of transmembrane helices in this protein has been established by high resolution electron diffraction (Henderson & Unwin, 1975). An analogous arrangement in the photoreceptor pigment rhodopsin is supported by a variety of spectroscopic techniques (Applebury & Hargrave, 1987).

Receptor subtypes exhibit the greatest degree of homology within the proposed transmembrane regions, with the hydrophilic loop regions being more divergent (Dixon et al., 1986; Dohlman et al., 1987b). The amino-terminal region is exposed on the extracellular face of the membrane, whilst the carboxyl terminal region is cytoplasmic. Studies using site-directed anti-peptide antibodies support this hypothetical topography (Wang et al., 1989c). Average molecular weights for polypeptides encoded by the cloned genes of β-adrenergic receptor subtypes are 51 kDa (466-483 amino acids), 46 kDa (413-421 amino acids) and 43 kDa (402 amino acids) for β_1, β_2, and β_3 adrenergic receptors, respectively. β_1-adrenergic receptors are therefore composed of larger polypeptide chains compared to β_2- and β_3-adrenergic receptors, primarily due to the presence of a larger third intracellular loop and longer cytoplasmic tail (cf. Kobilka et al., 1987b; Frielle et al., 1988).

Note: In the following sections, reference to results obtained using the technique of site-directed mutagenesis will be made. It should be made clear that although the specific changes made to the primary amino acid sequence of β-adrenergic receptors using this technique are often small, they can affect protein trafficking and folding. Therefore the presence of artifacts in the results obtained cannot be ruled out due to non-specific effects of the mutations.

1.3.2.3. Glycosylation

G-protein-linked receptors are glycoproteins, with the β-adrenergic receptor in particular containing both complex and high mannose type oligosaccharides (Boege et al., 1988), accounting for about quarter of its native molecular weight. All of these receptors whose primary amino acid sequence is known, possess 1-3 concensus sites for N-linked glycosylation (via asparagine residues) near the extracellular amino-terminal (Dohlman et al., 1987b). Site-directed mutagenesis of the β-adrenergic receptor has demonstrated that these amino-terminal sites are glycosylated in the wild-type receptor (Rands et al., 1990).

This glycosylation causes anomalous migration of β-adrenergic receptors on polyacrylamide gels (Benovic et al., 1984), whilst aglycoprotein receptors resolve well with reduced molecular weights (Boege et al., 1988). Glycosylation of the β-adrenergic receptor does not appear to be essential for ligand binding. When purified β-adrenergic receptor was treated with endoglycosidases to remove the sugar residues, no changes in the ligand binding properties of the receptor were noted (Stiles et al., 1984b; Dohlman et al., 1987a; Benovic et al., 1987d). In cells treated with tunicamycin, a glycosylation inhibitor, a lowering of coupling efficiency of the β-adrenergic receptor to G_s was seen, but no effect on β-adrenergic receptor expression at the cell surface was observed (George et al., 1986; Boege et al., 1988). In contast, Rands et al. (1990) found that in β-adrenergic receptor mutants (created by site-directed mutagenesis) lacking concensus sites for glycosylation, and hence lacking oligosaccharides, were fully capable of coupling to G_s and stimulating adenylate cyclase. In addition, they found that glycosylation was important for correct trafficking of the β-

adrenergic receptor through the cell to the plasma membrane. It is therefore possible that the effects of tunicamycin were not mediated by inhibition of glycosylation of the receptor.

1.3.2.4. Ligand binding domain

Many studies have suggested that the ligand binding domain of the β-adrenergic receptor is located within a protein domain embedded in the phospholipid bilayer, and involves parts of the seven putative transmembrane α-helices. Site-directed mutagenesis of cloned β-adrenergic receptor genes has been used extensively to identify sequences and amino acid residues required for ligand binding. Deletion of any of the transmembrane helices or of regions predicted to form junctions between transmembrane helices and extracellular loops results in altered protein folding (Dixon et al., 1987a). However, most of the hydrophilic loops of the β-adrenergic receptor can be deleted without affecting the structure or the ligand binding characteristics of the receptor (Dixon et al., 1987a). Therefore regions in the hydrophobic core of the β-adrenergic receptor are implicated in forming the ligand binding domain.

Analysis of chimeric α_2/β_2-adrenergic receptors indicates that helices 1-5 influence agonist specificity between α_2 and β_2 receptors, but not antagonist specificity to any great degree (Kobilka et al., 1988). Also helix 7 is the major determinant of α_2 and β_2 agonist and antagonist binding character (Kobilka et al., 1988). Only helices 6 and 7 of the β_2-adrenergic receptor are required to confer β_2-ligand binding characteristics on the chimeric α_2/β_2-adrenergic receptor, however alone they were not capable of binding ligands or activating adenylate cyclase (Kobilka et al., 1988).

Chimeric β_1/β_2-adrenergic receptors were used to define structures which confer β-subtype-selective ligand binding properties. Helix 4 was found to determine β_1 versus β_2 agonist binding characteristics i.e. a chimeric receptor containing helix 4 from the β_1-adrenergic receptor possesses β_1 ligand binding properties, and vice versa (Frielle et al., 1988). In contrast, helices 6 and 7 were found to be important for β_1 versus β_2 antagonist binding (Frielle et al., 1988).

The amino acid residue aspartate^{113} (Asp^{113}) within helix 3 is essential for the binding of both agonists and antagonists to the β-adrenergic receptor (Strader et al., 1987c, 1988). This negatively charged residue is presumed to bind the protonated amino group of the catecholamine, as a counter-ion. Substitution of Asp^{113} with a glutamate residue results in a mutant β-adrenergic receptor which recognises several well characterised β-adrenergic antagonists as partial agonists (Strader et al., 1989a). This phenomenon requires the presence of a carboxylate side-chain on the amino-acid at position 113, and is not observed when asparagine is situated at this location (Strader et al., 1989a). These observations suggest the overlapping of binding sites for agonists and antagonists on the β-adrenergic receptor.

Substitution of Asp^{79} in helix 2 results in a 10-fold decrease in the affinity of the receptor for agonists, but produced no change in the antagonist binding properties of the receptor, and may therefore be a specific counterion for agonists (Strader et al., 1988). Substitution of the highly conserved Asp^{130} residue with asparagine in helix 3 results in a mutant β-adrenergic receptor with normal antagonist binding, but unusually high-affinity agonist binding (Fraser et al., 1988). In addition, the mutant receptor: i) couples to G_s; ii) possesses an affinity

for agonist which is less sensitive to guanine nucleotides relative to wild-type β-adrenergic receptor; iii) is unable to mediate isoproterenol-stimulation of adenylate cyclase. This data coupled to previous findings (Chung et al., 1988) suggests the existence of two distinct counterions for the amine portion of catecholamines (i.e. aspartate 79 and 130), that are associated with high and low affinity agonist binding states of β-adrenergic receptors.

Structure-activity mapping of adrenergic ligands indicates the necessity of structural components, other than the amino group, for optimal receptor binding. Most importantly, β-adrenergic agonist activity depends on the presence of a catechol ring, which might interact with amino acid side-chains within the ligand binding domain via hydrogen bonding and hydrophobic interactions (Bilezikian et al., 1978a,b; Main & Tucker, 1985). Site-directed mutagenesis was again used to investigate the molecular basis for these putative interactions. Substitution of residues serine^{204} (Ser^{204}) and Ser^{207} in helix 5, and Ser^{319} in helix 7 with alanine residues, affects agonist but not antagonist binding to the β-adrenergic receptor (Dixon et al., 1989; Strader et al., 1989a). Interestingly, substitution of Ser^{203} resulted in abnormally folded receptor protein, whereas replacement of the adjacent Ser^{204} residue did not (Strader et al., 1989a). Thus hydrogen bonding interactions involving hydroxyl amino acid side-chains, may be essential for maintainance of β-adrenergic receptor secondary or tertiary structure.

Two conserved phenylalanine residues at positions 289 and 290 in helix 6, and also tyrosine^{326} in helix 7, appear to be critical for agonist binding, possibly through hydrophobic interactions (Dixon et al.,

1989). In summary, the present data suggests that residues located in helices 5, 6 and 7 are involved in binding the catechol ring of agonists to the β-adrenergic receptor (Strader et al., 1989b). In addition, the ligand is anchored to the receptor by an ionic interaction between the carboxylate side-chain of Asp^{113} on helix 3 and the amino group of the ligand. These findings are consistent with the ligand binding domain being located within the hydrophobic transmembrane domain of the β-adrenergic receptor.

To corroborate findings from site-directed mutagenesis studies, photaffinity-labelling experiments were performed to determine sites of incorporation for various photoaffinity probes. Treatment of the β_2-adrenergic receptor with p-(bromoacetamido)benzyl-1-[^{125}I]iodocarazolol results in the labelling of a region of 14 amino acids located in helix 2 (Dohlman et al., 1988). Unfortunately the modified amino acid residue could not be determined. In a separate study, the sites of covalent labelling of purified turkey eryrthrocyte β_1-adrenergic receptor by the photoaffinity labels [^{125}I]iodocyanopindolol-diazirine (ICYPD) and [^{125}I]iodoazidobenzylpindolol (IABP) were determined (Wong et al., 1988). Both labels incorporate at two separate sites. One site is located at $tryptophan^{330}$ in the extracellular half of helix 7, whilst the other was less well defined being located in helices 3, 4 or 5 or in the first or second extracellular loop (Wong et al., 1988). This data suggests that the catecholamine binding site of the β-adrenergic receptor is formed by juxtaposed membrane spanning helices, distant from each other in the primary amino acid sequence.

The binding site for retinol in rhodopsin has been better defined by a combination of spectroscopic and biochemical studies (for review

see Findlay & Pappin, 1986). Retinol is covalently bound to a lysyl residue in helix 7 lying halfway through the lipid bilayer, a position homologous to Trp^{330} labelled with the photoaffinity labels ICYP and IABP. Therefore some homology in secondary and tertiary structure appears to exist between rhodopsin and β-adrenergic receptors.

The arrangement of the α-helices may be dictated by interactions of various amino acid side-chains, as well as the possible formation of disulphide bonds, shown to be essential for ligand binding in the rat adipocyte β-adrenergic receptor (Moxham & Malbon, 1985). The more hydrophobic residues of these α-helices may form a boundary with the plasma membrane, while the less hydrophobic amino acids project towards the interior of the protein. Juxtaposed helices have probably evolved in such a way as to minimise steric and electrostatic forces between each other. The precise orthogonal arrangement of the putative seven membrane-spanning helices in the β-adrenergic receptor is still unclear. They may be arranged in a circular orientation, with helices 1 and 7 being adjacent in the membrane. Alternatively, recent data from the study of chimeric β-adrenergic receptors suggests that the arrangement may be akin to the number 6, with helices 1 and 2 forming the tail (Frielle et al., 1988; Kobilka et al., 1988). Spectroscopic analysis of purified β-adrenergic receptor is probably required to solve this unknown.

1.3.2.5. Interactions with G proteins

Activation of adenylate cyclase by agonist-stimulated β-adrenergic receptor is mediated by the guanine nucleotide-binding protein, G_s (Gilman, 1984). Interaction of receptor and G_s presumably occurs at the cytoplasmic surface of the plasma membrane, where G_s is

located. Alignment of available amino acid sequences for G protein-linked receptors reveals striking homologies in the regions predicted to be located at the cytoplasmic surface of the plasma membrane (O'Dowd et al., 1988). These regions in turn, differ most in their C-terminal regions and their third putative intracellular loops (Dixon et al., 1987). The intracellular location of these structures has been confirmed by indirect immunofluorescence and a CHO cell line expressing a high level of β_2-adrenergic receptor (Wang et al., 1989).

Deletion of residues 239-272 in the third intracellular loop of β_2-adrenergic receptors results in the loss of adenylate cyclase coupling (Dixon et al., 1987). This region is therefore critical for the coupling of receptor to G_s. The lack of adenylate cyclase stimulation may be a result of elimination of a site on the receptor for G protein interaction, or distruption of the receptors secondary and/or tertiary structure. Further analysis of this region suggests that regions of the β-adrenergic receptor encompassing amino acids 222-229 and 258-270, are absolutely required for adenylate cyclase activation to occur (Strader et al., 1987a). This has subsequently been confirmed in studies using mutant receptors expressed in frog oocytes (O'Dowd et al., 1988). The central region of the third intracellular loop does not affect receptor-adenylate cyclase coupling (Dixon et al., 1987).

The regions of protein encompassing these important deletions are predicted to form amphipathic α-helices in solution (Cheung et al., 1989). Mastoparan, a 14-amino acid peptide toxin isolated from wasp venom, stimulates guanine nucleotide binding and hydrolysis by some G proteins (Higashijima et al., 1988, 1990). Mastoparan is predicted to form an amphipathic α-helix in solution, and interacts with the carboxyl

terminal of the α subunit of G_i (Weingarten et al., 1990). It is therefore possible that activation of G_s by the β-adrenergic receptor involves a similar interaction with amphipathic helices in the third intracellular loop, presumably exposed upon conformational changes induced by agonist binding. The analogous region in the α_2-adrenergic receptor which interacts with G_i, is also predicted to form an amphipathic α-helix in which positively charged amino acid residues are conserved with respect to the β-adrenergic receptor, but with different hydrophobic residues (Kobilka et al., 1987c).

Substitution of the amino terminal region of the third intracellular loop of the β-adrenergic receptor with the corresponding region of the α_2-adrenergic receptor does not alter the specificity of interaction with G proteins (O'Dowd et al., 1988), however its deletion removes G protein coupling (Dixon et al., 1989). These findings suggest that this region of the receptor is involved with G protein coupling, but that the specificity of a receptor for a particular G protein lies elsewhere in the primary sequence. Replacement of the entire third intracellular loop and adjacent helices (5 and 6) of the α_2-adrenergic receptor with the equivalent region of the β_2-adrenergic receptor, results in a small agonist-induced stimulation of adenylate cyclase by the chimeric receptor (Kobilka et al., 1988). Therefore some specificity of coupling to G_s and G_i appears to be located in this region.

Some single residue substitutions of the first and second intracellular loops result in the attenuation of adenylate cyclase stimulation by mutant β-adrenergic receptors (Kobilka et al., 1987b; O'Dowd et al., 1988; Fraser et al., 1988). In particular, substitution of the conserved proline at position 138 leads to significant impairment in

the ability of the mutant receptor to mediate isoproterenol stimulation of adenylate cyclase, but has no effect on agonist binding properties (O'Dowd et al., 1988). It is postulated that the unique physicochemical characteristics of proline (β bend) may be critical for proper alignment of other regions of the protein that participate more directly in receptor-G_s coupling. The second intracellular loop does not appear to confer specificity of G protein coupling on the receptor, as substitution of this loop by the analogous region of the M_1-muscarinic cholinergic receptor, results in full stimulation of adenylate cyclase (Dixon et al., 1989). Analysis of this region by deletion mutagenesis has been unsuccessful because of incorrect protein folding caused by the deletions (Dixon et al., 1987; Kobilka et al., 1987b).

Finally, regions of the carboxyl tail have been implicated in interactions between β-adrenergic receptors and G_s. The N-terminal segment of the cytoplasmic domain at the cytoplasmic end of helix 7 is critical for efficient coupling of receptors to G proteins (Higashijima et al., 1988; Dixon et al., 1989), and can account for some specificity of interaction (O'Dowd et al., 1988). Also substitution of a conserved cysteine residue (Cys^{341}) in the cytoplasmic tail, causes a marked impairment in the mutant receptor's ability to mediate isoproterenol stimulation of adenylate cyclase (O'Dowd et al., 1988). In rhodopsin, this cysteine residue has been shown to be involved in an intramolecular disulphide bridge (Al-Saleh et al., 1987), although this may be an experimental artefact since disulphide bridges rarely exist in intracellular proteins due to high concentrations of the reducing agent glutathione. Also in both rhodopsin (Ouchinnikov et al., 1988) and the human β_2-adrenergic receptor (O'Dowd et al., 1989), Cys^{341} is

palmitoylated, again suggesting that this residue is not part of a disulphide bridge in the native receptor. Mutation of Cys^{341} to Gly^{341} removes the site of thioesterification, and results in a non-palmitoylated mutant β-adrenergic receptor with drastically reduced ability to mediate isoproterenol-stimulation of adenylate cyclase (O'Dowd et al., 1989). Therefore modification of β-adrenergic receptor by palmitate may play a crucial role in the normal coupling of receptor to adenylate cyclase, perhaps via insertion of the palmitate moiety into the plasma membrane.

1.4. Regulation of β-adrenergic receptor number and expression

Many factors are thought to determine the β-adrenergic receptor complement of a cell. Perhaps the most widely investigated is β-adrenergic receptor desensitisation, which will be discussed later. Other studies have looked at different physiological states and non-catecholamine hormones to see if they alter and regulate β-adrenergic receptor densities, respectively.

1.4.1. Regulation in adipose tissue

Lactation modifies the responsiveness of adipocytes to catecholamines in rats (Vernon & Finley, 1986), sheep (Guesnet et al., 1987) and cattle (Jaster & Wegner, 1981). In cattle (Jaster & Wegner, 1981) and rats (Watt et al., 1990b), lactation produces an increase in the number of β-adrenergic receptors in adipocytes. Growth hormone has been implicated in these changes in both sheep (Watt et al., 1990a) and rats (Watt et al., 1990b). Fasting also increases the number of β-adrenergic receptors in rat adipocytes, with no change in receptor affinity (Giudicelli et al., 1982).

Some steroid hormones exert profound effects on adipose tissue

β-adrenergic receptor number (reviewed by Giudicelli et al., 1989). Adrenalectomy results in decreased lipolytic responses to catecholamines in rat adipocytes (Schonhofer et al., 1972; Fernandez & Saggerson, 1978), which is partly due to a decrease in β-adrenergic receptor number (Mazancourt et al., 1990). Dexamethasone has been shown to correct these changes in rats (Mazancourt et al., 1990) and have a permissive action on catecholamine-induced lipolysis (Lacasa et al., 1988). Estradiol has no effect on β-adrenergic receptors in hamster adipocytes (Pequery et al., 1986).

In the rat adipocyte, thyroid hormones do not alter β-adrenergic receptor number (Malbon et al., 1978). However, in human gluteal adipocytes, hyperthyroidism slightly increases β-adrenergic receptor number, whereas hypothyroidism results in a 50% reduction of β-receptors (Richelsen & Sorensen, 1987). Thyroid hormones also exert effects on β-adrenergic receptor density in many other tissues including heart, liver, lung, muscle and lymphocytes (Bilezikian & Loeb, 1983).

It therefore appears that a variety of physiological processes govern basal β-adrenergic receptor expression at the cell surface.

1.4.2. Differential regulation of β_1- and β_2-adrenergic receptors

In light of the fact that the genes encoding β-adrenergic receptor subtypes are distinct, there is the possibility that the genes may be differentially regulated. Indeed, differential regulation of β_1- and β_2-adrenergic receptors by glucocorticoids (Lai et al., 1982; Nakada et al., 1987) and sodium butyrate (Stadel et al., 1987) has been shown in 3T3-L1 preadipocytes. These effects are thought to result from activation of the β_2-adrenergic receptor gene by these agents. A similar phenomenon occurs in the central nervous system where the anti-

depressant desipiramine down-regulates β_1-adrenergic receptor in rat cerebral cortex (Beer et al., 1987). In the same study (±)-clenbuterol preferentially down-regulated β_2-adrenergic receptors. Differentiation of 3T3-L1 fibroblasts to adipocytes is associated with a 3-fold increase in β_2-adrenergic receptor mRNA levels, while β_1-adrenergic receptor mRNA levels increase up to day 2, then decline thereafter (Guest et al., 1990). This effect has also been shown in 3T3-F442A cells (Feve et al., 1990). Therefore differentiation alone promotes the up-regulation of the β_2-receptor transcripts and down-regulation of the β_1-receptor transcripts in this cell type. Differential down-regulation of β_1- and β_2-adrenergic receptor mRNAs is also observed in C6 glioma cells (Hough & Chuang, 1990). The mechanisms involved in this regulation are not known.

Glucocorticoids (e.g. dexamethasone) up-regulate the β_2-adrenergic receptor mRNA. β_2-Adrenergic receptor expression and mRNA levels increase in response to glucocorticoids (Collins et al., 1988; Hadcock & Malbon, 1988; Malbon & Hadcock, 1988). Glucocorticoid-response elements (GREs) located in the 5' region of the cloned β-adrenergic receptor sequence have been postulated to be the target site for the glucocorticoid response (Malbon & Hadcock, 1988). Nakada et al. (1989) identified 16 GREs in the mouse β_2-adrenergic receptor gene. When more β-receptor genes are isolated a better understanding of transcriptional regulation should be achieved.

1.4.3. β-Adrenergic receptor desensitisation

Desensitisation is an ubiquitous phemonenon whereby continuous exposure of cells to a stimulus results in the waning of the cellular response to that stimulus and, under some circumstances, to other stimuli as well. Prolonged exposure of β-adrenergic receptors to

agonists results in an attenuation of the responsiveness to agonist stimulation. β-Adrenergic receptor desensitisation has been the subject of numerous reviews (Sibley & Lefkowitz, 1985; Sibley et al., 1987; Benovic et al., 1988; Collins et al., 1990; Hausdorff et al., 1990). Two types of desensitisation have been defined: homologous and heterologous (Su et al., 1982). Homologous or 'agonist-dependent' desensitisation results in an attenuated response only to β-adrenergic receptor agonists. In contast, heterologous or 'agonist non-specific' desensitisation is characterised by a diminished responsiveness to additional hormones and occasionally to other activators of adenylate cyclase, including guanine nucleotides and fluoride ion. It is generally accepted that heterologous desensitisation begins after homologous desensitisation, and occurs within minutes to hours of agonist exposure.

As the mechanisms of β-adrenergic receptor desensitisation become better understood, three key processes have been identified: i) rapid uncoupling of β-adrenergic receptor from adenylate cyclase; ii) rapid sequestration of the receptor away from the cell surface; iii) down-regulation of the receptor resulting in a net decrease in cell receptor number.

Discussion of desensitisation will be divided into homologous and heterologous components, although interaction between the mechanisms associated with the two is not discounted.

1.4.3.1. Homologous desensitisation

Three main mechanisms have been implicated in this process; sequestration of receptor away from the cell surface, phosphorylation of the receptor by cAMP-dependent protein kinase (PKA), and phosphorylation by a specific β-adrenergic receptor kinase (βARK).

Within a few minutes of agonist exposure, β-adrenergic receptors are sequestered away from the cell surface into membrane-associated vesicles (Harden, 1983). Sequestration is a specialised form of endocytosis which does not involve the formation of endosomes and subsequent trafficking of any phagocytosed material to lysosomes. In addition, recycling of sequestered β-adrenergic receptors back to the plasma membrane has been observed (These vesicles can be separated from the plasma membrane by ultracentrifugation, and are devoid of G_s, G_i, or adenylate cyclase (Waldo et al., 1983). Sequestered β-adrenergic receptors are not accessible to hydrophilic ligands such as isoproterenol or the antagonist CGP 12177, but are labelled by the hydrophobic ligand [^{125}I]pindolol (Toews et al., 1984). Loss of β-adrenergic receptors from the cell surface is normally quantified by radioligand binding techniques. Radioligand binding can decline by 50-60% after exposure to agonists (Su et al., 1980). However, although radioligand binding provides a means by which the number and affinity of β-adrenergic receptors can be determined (Stiles et al., 1984), receptors with binding sites either occupied by agonist or inaccessible to the radioligand are not detected. By using antibodies to β-adrenergic receptors, several groups have found that receptor sequestration may be preceded by agonist-induced alteration(s) in the conformation of the receptor that destroys ligand binding (Guillet et al., 1985; Strader et al., 1987b; Zemcik & Strader, 1988). However, the β-adrenergic receptor complement, migration on SDS PAGE, and cellular location are largely unaffected by agonist stimulation, even though receptor binding of antagonist radioligands is markedly down-regulated (Wang et al., 1989a,b). It has been suggested that sequestration is a prerequisite for receptor

dephosphorylation (Sibley et al., 1986) or initiates long-term mechanisms of adaption to agonist exposure (Lohse et al., 1990a). The basic biochemical mechanisms involved in agonist-stimulated sequestration are unknown, but it is not triggered by receptor phosphorylation by PKA or βARK (Hausdorff et al., 1989; Lohse et al., 1990a).

Functional uncoupling of β-adrenergic receptor from its effector system, as part of desensitisation, occurs faster than sequestration in many cases (Waldo et al., 1983). A large body of evidence suggests that 70-80% of homologous desensitisation is caused by, or at least initiated by, phosphorylation of the receptor molecule (reviewed in Benovic et al., 1988; Lefkowitz et al., 1990). Two protein kinases have been implicated. Firstly, cAMP-dependent protein kinase (PKA), which becomes activated as a result of rises in intracellular cAMP levels in the course of β-adrenergic receptor activation (Sibley et al., 1984; Benovic et al., 1985). Secondly, the recently characterised cAMP-independent β-adrenergic receptor kinase (βARK) which phosphorylates only the agonist-occupied form of the β_2-adrenergic receptor (Benovic et al., 1986, 1987b). βARK was first identified in 1986 (Benovic et al., 1986), and later purified to >90% homogeneity from bovine cerebralcortex (Benovic et al., 1987b). On SDS PAGE, βARK migrates as a single 80 kDa polypeptide (Benovic et al., 1987b). The cloning of βARK identified two highly homologous clones (85% sequence homology), suggesting the presence of isoenzymes and possibly a family of receptor kinase genes (Benovic et al., 1989). The deduced sequence reveals that βARK has 689 amino acids, and contains a catalytic domain flanked by two other domains of approximately equal size.

Unlike phosphorylation by PKA, βARK activity is dependent on the

presence of β-arrestin, a 48 kDa protein (Benovic et al., 1987a). In the retina, an analogous protein called arrestin binds only to rhodopsin that has been phosphorylated by rhodopsin kinase, and thereby apparently sterically interupts signal transduction from rhodopsin to transducin (Wilden et al., 1986). A cDNA from a bovine brain library has been isolated which is 59% homologous to arrestin (Lohse et al., 1990b). Expression of the cDNA produces a protein, termed β-arrestin, that inhibits the function of βARK-phosphorylated β-adrenergic receptors by more than 75 percent, but not rhodopsin. It is proposed that β-arrestin acts in concert with βARK to effect homologous desensitisation of β-adrenergic receptors. βARK only phosphorylates agonist-occupied β-adrenergic receptors (Benovic et al., 1986), perhaps due to the unmasking of a βARK-phosphorylation site induced by ligand binding. The specificity of βARK is akin to that of rhodopsin kinase, a specific enzyme that phosphorylates rhodopsin in a light-dependent fashion, serving to uncouple rhodopsin from transducin, and hence desensitise signal transduction (Kuhn & Wilden, 1987). More recently, βARK has been shown to phosphorylate agonist-occupied α_2-adrenergic (Benovic et al., 1987c) and muscarinic cholinergic (Kwatra et al., 1989) receptors. βARK also phosphorylates light-activated rhodopsin (Benovic et al., 1987a). These results support the hypothesis that βARK may be of general importance in the regulation of receptors coupled to the modulation of adenylate cyclase, rather than being β-adrenergic receptor-specific.

Molecular cloning of several β-adrenergic receptor cDNAs and genes has identified two clear concensus sequences for phosphorylation by PKA, located in the putative third intracellular loop and in the N-terminal region of the predicted carboxyl tail of the β-adrenergic

receptor (Kobilka et al., 1987a; Frielle et al., 1987; Emorine et al., 1989). In the case of βARK, the important sites are located in the C-terminal end of the carboxyl tail, which is very serine- and threonine-rich (Dohlman et al., 1987b; Hausdorff et al., 1989). Deletion of one or more of these sites by site-directed mutagenesis, results in receptors with differing abilities to mediate changes in sensitivity and losses in maximum responsiveness (Hausdorff et al., 1989). In addition, low agonist concentrations preferentially induce phosphorylation at PKA sites and results in desensitisation. At higher agonist concentrations, phosphorylation occurs at both βARK and PKA sites, and 70-80% of maximal desensitisation occurs (Hausdorff et al., 1989). Interestingly, the highest levels of βARK mRNA expression are found in brain, spleen and heart, tissues which have high catecholamine levels i.e. with greatest levels of innervation (Benovic et al., 1989). This finding coupled to selective activation of βARK at high agonist concentrations is compatible with the notion that βARK is more abundant in tissues containg high numbers of nerve endings, which may produce high concentrations of noradrenaline.

In an elegant series of experiments, Lohse et al. (1990a) were able to examine the relative contributions of phosphorylation of β-adrenergic receptors by βARK and PKA, and receptor sequestration in homologous desensitisation using specific inhibitors. The polyanion heparin is a specific and effective inhibitor of βARK (Benovic et al., 1989), while the 31-amino acid PKA inhibitor peptide blocks phosphorylation by PKA (Scott et al., 1986). Sequestration was prevented by pretreatment of cells with Concanavalin A (Waldo et al., 1983). When individual pathways are blocked either the PKA or βARK mechanism alone

appear to result in 40-50% desensitisation, whereas sequestration alone causes 20-30% desensitisation. These findings suggest that the quantitative contributions of each mechanism are not additive, indicating some redundancy in signal transduction regulation. Also the results suggest that an agonist-dependent concentration gradient regulates the degree of desensitisation produced.

Despite the growing amount of information concerning the role of phosphorylation in β-adrenergic receptor desensitisation, little is known about the enzymatic basis for dephosphorylation of phosphorylated receptor. There is evidence that receptor dephosphorylation is catalysed by a phosphatase specifically associated with light-membrane vesicles (Sibley et al., 1986), proposed to contain sequestered β_2-adrenergic receptors (Stadel et al., 1983). More recently, latent phosphatase 2 was shown to specifically dephosphorylate β_2-adrenergic receptors (Yang et al., 1988). However purification and rigorous characterisation of the endogenous β_2-adrenergic receptor phosphatase will be required to demonstrate conclusively this potential role for latent phosphatase 2.

1.4.3.2. Heterologous desensitisation

Heterologous desensitisation of β-adrenergic receptors is not dependent on agonist-occupancy of the receptor ligand binding site, and primarily involves a functional uncoupling of receptor from G_s and adenylate cyclase (Sibley & Lefkowitz, 1985). This process is characterised by a marked reduction (down-regulation) in receptor number, and is demonstrable only after 4-12 h of agonist exposure. The estimated half-life of the β-adrenergic receptor in the plasma membrane is ~30 hours (Neve & Molinoff, 1986; Mahan & Insel, 1986). Considering this relatively long half-life, down-regulation could be achieved by a

decrease in receptor synthesis and/or an increase in receptor degradation.

1.4.3.2.1. Role of phosphorylation

Under appropriate conditions, cAMP-dependent heterologous desensitisation can be elicited (Sibley et al., 1987). The effect can be mimicked by incubation with dibutyryl-cAMP, 8-bromo-cAMP, and forskolin, and results in the down-regulation of both β-adrenergic (50% reduction) and prostaglandin E_1 receptors (Sibley et al., 1987; Bouvier et al., 1989). These and other results suggest a role for PKA (Sibley et al., 1984; Benovic et al., 1985), and possibly protein kinase C (Sibley et al., 1984; Kelleher et al., 1984), in down-regulation of receptors. They also demonstrated that cAMP alone can induce down-regulation in the absence of β-adrenergic agonist.

As noted earlier (section 1.4.3.1.), the β_2-adrenergic receptor contains two concensus sequences for PKA phosphorylation, and is a substrate for PKA. To examine the role of PKA phosphorylation in down-regulation, these two sites in the receptor have been disrupted by a site-directed mutagenesis approach. Deletion of one or both of these PKA phosphorylation sites results in mutant receptors with delayed cAMP-induced down-regulation (Bouvier et al., 1989). After several hours however, the mutant receptors undergo down-regulation at a rate which closely parallels that of wild-type receptors (Bouvier et al., 1989). It is clear then, that phosphorylation contributes to, but is not the major factor involved in, β-adrenergic receptor down-regulation. The initial delay in cAMP-induced down-regulation of mutant receptors, may be due to an alteration of phosphorylation (Collins et al., 1990). Interestingly, short-term exposure (30 min) of cells to adrenaline or dibutyryl-cAMP

stimulates the rate of β_2-adrenergic receptor gene transcription in DDT_1MF-2 cells (Collins et al., 1989).

There appears to be some requirement for the presence of G_s in down-regulation because β-adrenergic receptors in S49 lymphoma cells, lacking α_s, exhibit little agonist-induced down-regulation (Mahan et al., 1985). In addition, mutant receptors that are unable to couple to G_s also generally exhibit impaired ability to mediate agonist-induced down-regulation (Cheung et al., 1989). However, functional activation of adenylate cyclase is not an essential requirement (Mahan et al., 1985).

1.4.3.2.2. Regulation of β-adrenergic receptor messenger RNA (mRNA)

In contrast to short-term exposure (30 min), long-term exposure (>24 h) of cells to a β-adrenergic agonist results in a >50% reduction in β-adrenergic receptor mRNA (Hadcock et al., 1988; Collins et al., 1989). The down-regulation of β-adrenergic receptor mRNA displays a prominent cAMP-dependent component. Agents that increase intracellular cAMP via receptors or non-receptor pathways (such as forskolin and cholera toxin) stimulate a sharp decline in β-adrenergic receptor mRNA levels (Hadcock et al., 1988). The decline in mRNA levels appears to involve destabilistation of mRNA (agonist/cAMP induced), rather than a decrease in the rate of gene transcription (Hadcock et al., 1989a; Collins et al., 1989). It has been proposed that a second cAMP-independent signal transduction pathway also leads to a decline in mRNA levels (Hadcock et al., 1989b). This latter effect apparently requires the presence of G_s and PKA, but not the elevation of intracellular cAMP levels (Hadcock et al., 1989b). Recently, noradenaline has been shown to regulate the α_1-adrenergic receptor mRNA level in rabbit aortic smooth muscle cells, possibly involving destabilisation of the mRNA and a

co-factor whose transcription is induced by noradrenaline (Izzo, Jr, et al., 1990).

1.4.3.2.3. Role of endocytosis

A growing body of evidence suggests that down-regulation of β_2-adrenergic receptors may involve their endocytosis by way of clathrin-coated vesicles (CCVs). The internalisation of the β_2-adrenergic receptor is inhibited by concanavalin A (Wakshull et al., 1985), phenylarsine oxide (Hertel et al., 1985), and reduction of cellular ATP content, factors known to perturb internalisation by CCVs. In addition, distruption of the formation of clathrin-coated pits inhibits the internalisation of β_2-adrenergic receptors (Liao, 1990).

Valiquette et al. (1990) have proposed that two tyrosine residues (at positions 350 and 354) in the cytoplasmic tail of the human β_2-adrenergic receptor are involved in down-regulation. Mutation of these residues dramatically reduces the ability of the β_2-adrenergic receptor to undergo isoproterenol-induced down-regulation. This effect implicates CCVs since it is similar to observations indicating the involvement of tyrosine residues located in the cytoplasmic tails of several membrane-bound receptors in agonist-induced CCV-endocytosis (Valiquette et al., 1990, and references cited therein). In this same study, mutation of the tyrosine residues did not affect agonist-induced sequestration. There is evidence that sequestration of β_2-adrenergic receptors in human A431 cells involves non-coated vesicles (Raposo et al., 1989). Therefore down-regulation and sequestration may occur via independent pathways of internalisation.

1.5. Use of β-adrenergic agonists in livestock production

Drugs acting via adrenergic receptors are widely used therapeutic

agents. Perhaps the most well known example of this class of compounds are the so-called 'β-blockers', used extensively to treat cardiovascular complaints. Recently, several β-adrenergic agonists (β-agonists) have been identified that alter body composition in many species of animals. Some of these compounds, for example BRL 26830A (Arch et al., 1984) and LY 104119 (Yen et al., 1984) reduce body-fat deposition with little alteration of lean-body mass accumulation. Other β-agonists cause a 'repartitioning' of nutrients resulting in a reduction of adipose tissue and an increase in muscle growth (reviewed by Hanrahan et al., 1986; Yang & McElligott, 1989). These effects have very important implications for treatment of muscle wasting diseases (e.g. muscular dystrophy) in humans, and modification of carcass composition in the meat-producing industry.

β-Agonists currently under study include cimaterol (Jones et al., 1985; Hu et al., 1988; Eadara et al., 1989), clenbuterol (Baker et al., 1984; Ricks et al., 1984; Reeds et al., 1986), ractopamine (Anderson et al., 1987; Hausman et al., 1989), and L-644,969 (Convey et al., 1987; Kretchmar et al., 1989). Much of the early work to characterise the effects of β-agonists has been documented in agricultural animal studies. However, information concerning their effects in rats is available (Maltin et al., 1986a,b, 1987, 1989a; McElligott et al., 1987; Eadara et al., 1989; Hausman et al., 1989; MacLennan & Edwards, 1989).

1.5.1. Effects on skeletal muscle

β-Agonists are the most potent agents that can promote normal skeletal muscle growth (see Yang & McElligott, 1989). Current research is aimed at elucidating the mechanisms responsible for this effect, but early findings suggest roles for both direct and indirect actions on

muscle. One of the direct effects of β-agonists on muscle is stimulation of growth (Beerman et al., 1987; Claeys et al., 1989; MacLennan & Edwards, 1989; Maltin et al., 1989a). This has been attributed to an inhibition of protein degradation (McElligott et al., 1987; Bergen et al., 1989; Kretchmar et al., 1989), and increased protein synthesis (Emery et al., 1984; Helferich et al., 1988; Bergen et al., 1989). However, the effects on muscle protein metabolism are not definitive as yet, and therefore greater knowledge of the biochemical processes underlying these gross effects is needed.

The β-adrenergic receptor subtype on muscle has been shown to be predominantly β_2 (Apperly et al., 1976; Ligget et al., 1988). Evidence for β-adrenergic receptor-mediation of clenbuterol-induced muscle hypertrophy in rats has been presented (MacLennan & Edwards, 1989). Propranolol was found to attenuate clenbuterol effects on muscle growth thus implicating β-adrenergic receptors. This contrasts with work by Maltin and co-workers who found that propranolol addition to clenbuterol-supplemented diets did not attenuate hypertrophy in innervated (Maltin et al., 1987b) or denervated (Maltin et al., 1989b) soleus muscle, induced by clenbuterol. The latter findings are questionable since full antagonism by propranolol of the β-adrenergic effects of clenbuterol was not demonstrated. This direct effect is not suprising as clenbuterol shows high affinity towards both β_1- and β_2-adrenergic receptors, with selectivity towards β_2-adrenergic receptors (Cohen et al., 1982). However, infusion of clenbuterol causes a small increase in plasma noradrenaline, so that some effects of clenbuterol may be attributable to release of endogenous noradrenaline (Mersmann, 1989).

β-agonists interact with many endocrine systems and tissues in the body, and therefore indirect mechanisms may have an important role in β-agonist-induced hypertrophy. Possible indirect actions include: interaction with insulin or growth hormone; increased blood flow to muscle providing additional nutrients; increased availability of lipids for energy production to the muscle, resulting from increased adipose tissue lipolysis and concomitant elevation of plasma free fatty acid concentration (see Yang & McElligott, 1989 for full discussion).

In summary, more research is required to determine precisely the mode of action of β-agonists on muscle metabolism. In addition, the effects of other β-agonists must be characterised before a concensus sequence of events leading to muscle hypertrophy can be established.

1.5.2. Effects on adipose tissue

It is now quite clear that chronic β-agonist treatment decreases total carcass fat in meat-producing animals and laboratory rodents. Evidence exists which establishes a direct role for β-agonists in the control of lipolysis and lipogenesis in adipose tissue (Fain & Garcia-Sainz, 1983). The plasma concentration of free fatty acids increases in sheep (Beerman et al., 1986) and pigs (Mersmann, 1987; Mersmann et al., 1987, 1989) given β-agonists, suggesting increased lipid trafficking. However, as noted for effects on muscle, stimulation of noradrenaline release by β-agonists may have a role (Mersmann et al., 1989). In vitro, ractopamine (Merkel et al., 1987) and L-644,969 (Liu et al., 1988), but not clenbuterol (Merkel et al., 1987) increase lipolysis in pig adipose tissue. Stimulation of adipose tissue lipolysis in vitro is also evidenced with cimaterol in sheep (Hu et al., 1988) and rats (Eadara et al., 1989), clenbuterol in sheep (Thornton et al., 1985), and ractopamine

in rats (Hausman et al., 1989). Data relating to broilers is inconclusive (Yang & McElligott, 1989).

Effects of chronic β-agonist administration on lipogenesis in adipose tissue in vitro are ill-defined. In rats, cimaterol does not influence de novo fatty acid synthesis in either adipose tissue or liver (Eadara et al., 1989). Ractopamine on the other hand inhibits basal lipogenesis in rat adipocytes, and is more potent in the presence of low media concentrations of insulin (Hausman et al., 1989), Cimaterol feeding increases lipogenesis in sheep adipose tissue (although the effect is not statistically significant)(Hu et al., 1988), while clenbuterol inhibits lipogenic enzymes in cattle, showing a site-specific effect on adipose tissue (Smith et al., 1987). Also ractopamine has been shown to be anti-lipogenic in pig adipose tissue (Merkel et al., 1987; Liu et al., 1988). Suprisingly, cimaterol increased lipogenesis in lambs (Hu et al., 1987). Whether these inconsistences are due to species, adipose depot, β-agonist or experimental differences is not yet clear.

1.5.3. Novel β-agonists

β-Adrenergic receptors are present on almost every cell type, and regulate many physiological systems (see Table 1.1). Several novel β-agonists have been developed which exhibit selectivity towards adipose tissue (Arch et al., 1984; Wilson et al., 1984; Yen et al., 1984). Compounds that selectively stimulate brown adipocyte β-adrenergic receptors may become potent thermogenic anti-obesity drugs (Arch et al., 1984), while those specific for white adipose tissue are attractive repartitioning agents. The selectivity of these novel β-agonists appears to be a result of atypical β-adrenergic receptors being present on adipocytes (discussed in section 1.1.2.3). The advantage of using such

drugs as repartitioning agents is that their effects may be limited to adipose tissue, thus negating any concerns about cardiovascular side-effects. As more β-adrenergic receptor subtypes are identified pharmacologically and by direct gene isolation, tissue-specific effects of β-agonists in many tissues may become a therapeutic reality.

1.6. Objectives

The aim of this study was to characterise the β-adrenergic receptors present in sheep adipose tissues. Its purpose was to look for possible species and adipose tissue depot differences in the characteristics of adipose tissue β-adrenergic receptors, and to determine whether sheep adipose tissue possessed an atypical β_3-receptor, as observed in the rat. Achievement of these goals involved the purification of the β-adrenergic receptor from sheep adipose tissues, and subsequent investigation using radioligand binding and photoaffinity-labelling. The production of antibodies directed against the β-adrenergic receptor was also a key aim, as such antibodies would be useful for probing receptor structure and also for development of an immunological alternative to the use of β-agonists in livestock production.

Table 1.1. Physiological responses of adrenergic receptor subtypes

α_1-Adrenergic receptors

1. Smooth muscle contraction in blood vessels and genitourinary tract
2. Activation of glycogenolysis (liver)

α_2-Adrenergic receptors

1. Smooth muscle relaxation in genitourinary tract
2. Smooth muscle contraction in selected vascular beds
3. Inhibition of noradrenaline release from sympathetic nerve endings
4. Increased growth hormone secretion by the pituitary
5. Inhibition of lipolysis in adipose tissue (species specific)
6. Platelet aggregation (species specific)
7. Inhibition of insulin release by the pancreas
8. Stimulation of potassium and water secretion by salivary glands
9. Inhibition of renin release by juxtaglomerular cells in the kidney

β-Adrenergic receptors

1. Stimulation of rate and force of cardiac contraction
2. Stimulation of lipolysis
3. Stimulation of amylase secretion by salivary glands
4. Smooth muscle relaxation in bronchi, blood vessels, and genitourinary and gastrointestinal tracts
5. Facilitation of noradrenaline release
6. Increased glycogenolysis and gluconeogenesis in liver
7. Increased glycogenolysis in muscle
8. Increased insulin and glucagon secretion by pancreatic cells
9. Decreased growth hormone secretion by the pituitary
10. Stimulation of renin release by juxtaglomular cells in the kidney

Adapted from Lefkowitz et al. (1984).

Fig. 1.1. Adrenergic control of lipid metabolism in the adipocyte

β- and α_2-adrenergic receptor responses interact via their respective G proteins and adenylate cyclase, and modulate the intracellular cAMP concentration. Elevation of cAMP levels increases protein kinase A activity which phosphorylates both hormone-sensitive lipase (stimulatory) and acetyl CoA carboxylase (inhibitory). The net result is an increase in the rate of lipolysis and a reduction in de novo fatty acid synthesis. H_s denotes β-agonist, H_i α_2-agonist, β-$\overline{AR}$ β-adrenergic receptor, α_2-$\overline{AR}$ α_2-adrenergic receptor, α_s $G_{s\alpha}$ subunit, α_i $G_{i\alpha}$ subunit, β & γ G protein subunits, AC adenylate cyclase, ATP adenosine triphosphate, PPi pyrophosphate, GTP guanosine triphosphate.

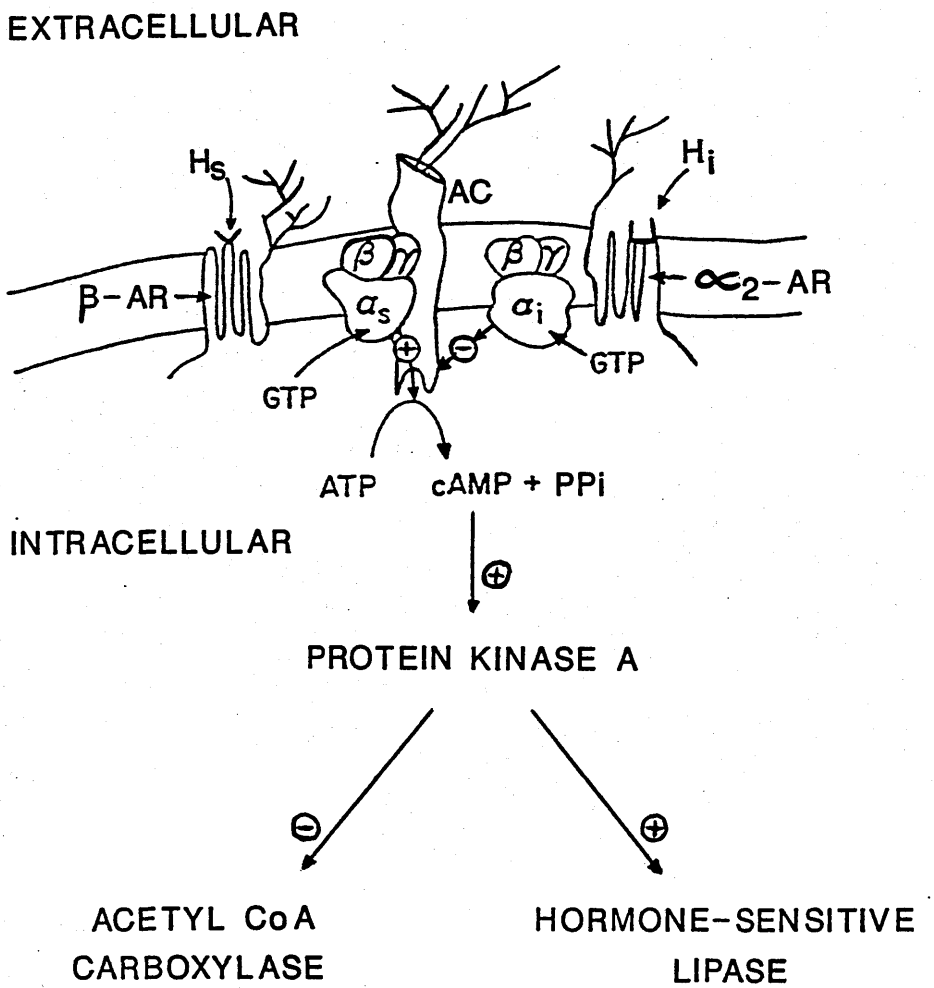
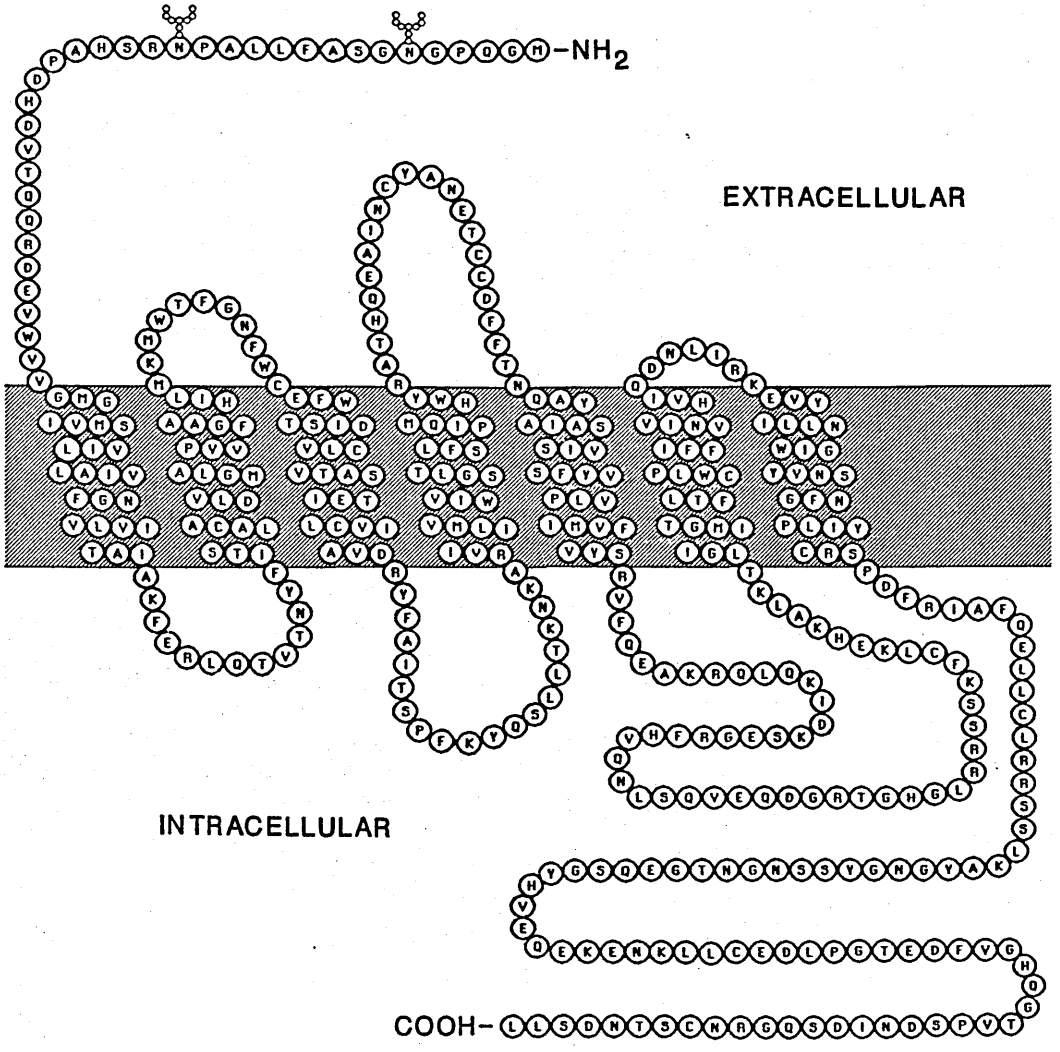


Fig. 1.2. Model illustrating the amino acid sequence and presumptive membrane architecture of the human β_2-adrenergic receptor

The amino acid sequence was determined from a cDNA encoding the human β_2-adrenergic receptor. The arrangement of the polypetide chain was predicted using hydropathicity analysis. Taken from Kobilka et al. (1987).



CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO - MATERIALS AND METHODS

2.1. Reagents

CGP 20712A was generously given by Drs Schroter and Schleibli of CIBA-GEIGY A.G. (Basel, Switzerland). ICI 118,551 was a gift from ICI Pharmaceuticals (Alderley Park, Macclesfield, Cheshire, U.K.). Polyclonal antiserum CM 13 was a generous gift from Dr Craig Malbon (State University of New York, Stony Brook, New York, U.S.A.). Monoclonal antibody BRK2 was a gift from Professor A. D. Strosberg (Pasteur Institute, Paris, France). All radiochemicals were purchased from Amersham International plc (Amersham, Bucks, U.K.). 1,4-Butanediol diglycydylether was purchased from Aldrich (Gillingham, Dorset, U.K.). Tissue culture reagents were purchased from Northumbria Biologicals Ltd (Cramlington, Northumberland, U.K.). All other reagents were obtained from Sigma Chemical Company (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.).

2.2. Animals

2.2.1. Sheep

Sheep were all Finn x Dorset Horn cross-breds. The animals were given hay and water *ad libitum* plus 400 g/day of a cereal mix, for at least 4 weeks before slaughter. Both wether lambs (6-9 months old) and 3-4-year old ewes were used. Lactating ewes received 1400 g/day of a cereal mix, possessed 2-3 lambs, and were used 18 days *post partum*. Sheep were anaesthetised with an intrajugular injection of 20-30 ml of Sagatal (May & Baker, Dagenham, Essex, U.K.), given by Dr R. G. Vernon. The animals were exsanguinated when unconscious and adipose tissue removed as quickly as possible.

2.2.2. Rats

Wistar female virgin rats (160-180 g) from A. Tuck & Son (Rayleigh, Essex, U.K.) were given Labsure irradiated CRM diet (Labsure, Poole, Dorset, U.K.) and water ad libitum. Rats were killed by cervical dislocation and adipose tissue removed as quickly as possible.

2.3. Preparation of membrane fractions

2.3.1. Preparation of sheep adipocyte membranes

Adipose tissue (20-80 g) was removed from the required fat-depot and transported to the laboratory in 0.9% (w/v) saline at 39°C. Adipocytes were prepared by collagenase digestion using the method of Rodbell (1964). Adipose tissue (about 20 g) was minced in a petri-dish using scissors, and digested in sealed plastic flasks containing 10 ml of gassed (95% O_2, 5% CO_2) Krebs Ringer bicarbonate (1.2 mM $CaCl_2$) buffer, containing 25 mM Hepes (pH 7.3), 2 mM Na acetate, 0.3 mg/ml D-glucose, 1.5 mg/ml collagenase (Type II (Cat. No. C 6885), Sigma) and 40 mg/ml BSA (dialysed and fatty acid free), for 1 h at 37°C in a shaking water bath. The digest was passed through a plastic strainer, and the floating adipocytes washed three times with 10 ml of gassed (95% O_2, 5% CO_2) Krebs Ringer bicarbonate buffer, containing 25 mM Hepes (pH 7.3), 2 mM Na acetate, 0.3 mg/ml D-glucose, 10 mg/ml BSA at 37°C.

Adipocytes were lysed by addition of 10 ml of 10 mM Tris-HCl (pH 7.4), 20 mM EDTA, 0.2 M sucrose containing 0.1 mg/ml bacitracin, 17.5 μg/ml benzamidine HCl, 5 μg/ml soybean trypsin inhibitor, 0.2 mM PMSF at 37°C. The suspension was vortexed for 2 min, transferred to a 50 ml plastic centrifuge tube, and quickly centrifuged at 36,900xg (SS34 rotor; Sorvall RC-5B, DuPont, U.S.A.) for 20 min at 37°C. The fat and infranatant were poured off and the tubes plunged into ice. The pellet

was gently resuspended in 5 ml of ice-cold 10 mM Tris-HCl (pH 7.4), 90 mM NaCl using a glass homogeniser. Pellets from other digestions were pooled at this stage and centrifuged at 1000xg (Minifuge T, Hereaus) for 10 min at 4°C, in order to remove residual cell debris and connective tissue. The supernatant was recovered and recentrifuged at 36,900xg (SS34 rotor; Sorvall RC-5B) for 20 min at 4°C. The resulting membrane pellet was resuspended in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl and stored in liquid nitrogen.

2.3.2. Preparation of sheep adipose tissue membranes

Adipose tissue (1 kg) was collected from the omental fat-depot and placed in 0.9% (w/v) saline at 39°C. 100 g aliquots were mixed with 200 ml of 10 mM Tris-HCl (pH 7.4), 20 mM EDTA, 0.2 M sucrose containing 0.1 mg/ml bacitracin, 17.5 μg/ml benzamidine HCl, 5 μg/ml soybean trypsin inhibitor, 0.2 mM PMSF at 40°C, and homogenised in a warm blender (Waring, U.S.A.) at high speed for 35 sec. The homogenate was quickly centrifuged at 2,800xg (Minifuge T, Hereaus) for 5 min at 40°C. The sample was cooled on ice until the fat solidified, and the infranatant removed and centrifuged at 40,000xg (6 x 100 ml rotor; Prepspin 50, MSE) for 20 min at 4°C. The pellet was resuspended in 10 ml of ice-cold 10 mM Tris-HCl (pH 7.4), 90 mM NaCl using six strokes of a large-bore glass homogeniser fitted with a Teflon pestle. The suspension was centrifuged at 1000xg (Minifuge T, Hereaus) for 10 min at 4°C. The supernatant was recovered and recentrifuged at 36,900xg (SS34 rotor; Sorvall RC-5B) for 20 min at 4°C. The resulting membrane pellet was resuspended in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, and stored in liquid nitrogen.

2.4. Preparation of rat adipocyte membranes

Rat adipose tissue was removed and transported to the laboratory

in 0.9% (w/v) saline at 37°C. Adipocyte membranes were prepared as described previously for sheep adipose tissue (section 2.3.1), except that 10 g of adipose tissue was placed in each digestion flask and sodium acetate was omitted from the digestion buffer.

2.5. Assay of β-adrenergic receptors

2.5.1. Assay of β-adrenergic receptors in membranes

β-Adrenergic receptors in membranes were assayed using the method of Malbon et al. (1978), with some modifications. Membranes (100 µg protein) were incubated with 10 nM [^{3}H](-)-dihydroalprenolol ([^{3}H]DHA), 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM ascorbate in a total volume of 200 µl, for 10 min at 37°C. The reaction was quenched by addition of 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and the incubation mixture filtered rapidly through a single 2.5 cm Whatman GF/C filter disc. The filter was washed twice with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and added to 10 ml of scintillation fluid (Emulsifier Safe, Packard). The radioactivity trapped on the filters was counted using a LKB 1215 Rackbeta liquid scintillation counter. Non-specific binding was defined as the radioligand binding remaining after incubation in the presence of 100 µM (-)-isoproterenol.

When [^{125}I]-iodocyanopindolol was used instead of [^{3}H]DHA, a radioligand concentration of 75 pM was used, and samples were incubated for 60 min at room temperature. Also the amount of membranes was reduced to 15 µg protein/assay tube.

2.5.2. Assay of soluble β-adrenergic receptors

Solubilised β-adrenergic receptors were assayed using the two methods of Vauquelin et al. (1979) with modifications.

2.5.2.1. Gel filtration method

The soluble sample (100 µg protein) was incubated with 10 nM [^{3}H]DHA in a buffer composed of 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.1 mM ascorbate for 15 min at 30°C, in a total volume of 250 µl. Following incubation, the sample was cooled on ice for 5 min and bound ligand separated from free by gel filtration on a single 3.5 ml Sephadex G50 column (Figure 2.1). The column was equilibrated at 4°C with 3ml of 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.05% (w/v) digitonin, immediately prior to sample loading. When the sample had entered the gel, 1 ml of equilibration buffer was passed through the column and any eluate dicarded. Receptor-ligand complexes were eluted directly into a scintillation vial by further addition of 1.4 ml of buffer. Scintillation fluid (10 ml : Emulsifier Safe, Packard) was added and the radioactivity quantified using a LKB scintillation counter. Non-specific binding was defined as the radioligand binding remaining in the presence of 100 µM (-)-isoproterenol.

2.5.2.2. Polyethylene glycol (PEG) precipitation method

The soluble sample (100 µg protein) was incubated with 10 nM [^{3}H]DHA for 15 min at 30°C as in section 2.5.2.1 above. The sample was cooled on ice for 1 min, and 40 µl of sheep immunoglobulin (6 mg/ml) and 290 ul of ice-cold 16% (w/v) PEG 6000 (in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl) added. The sample was vortexed, and incubated on ice for a further 10 min. The sample was filtered through a single 2.5 cm Whatman GF/F filter and washed twice with 5 ml of ice-cold 8% (w/v) PEG 6000, 10 mM Tris-HCl (pH 7.4), 90 mM NaCl. The radioactivity was determined as above. Non-specific binding was defined as the radioligand binding remaining in the presence of 100 µM (-)-

isoproterenol.

When [^{125}I]-iodocyanopindolol was used instead of [^{3}H]DHA, a radioligand concentration of 75 pM was used, and samples were incubated for 60 min at room temperature. Also the amount of membranes was reduced to 15 µg protein/assay tube.

2.6. Preparation of digitonin solutions

Digitonin (99% pure; BDH Limited, Poole, England) was prepared as a 5% (w/v) solution in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl by boiling gently for 10 min. After cooling to 4°C, the solution was centrifuged at 1000xg for 10 min, filtered through a 0.2 µm syringe filter (Flow laboratories), and stored at 4°C.

2.7. Solubilisation of sheep adipose tissue membranes

Adipose tissue membranes were solubilised using a modification of the method of Cubero and Malbon (1984). Isolated membranes were pelleted by centrifugation at 36,900xg (SS34 rotor; Sorvall RC-5B) for 15 min at 4°C. The membrane pellet was resuspended in ice-cold 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.2 mM PMSF, 1% (w/v) digitonin, using six strokes of a glass homogeniser, and stirred gently for 1.5 h at 4°C. The final membrane protein concentration was 2 mg/ml. The sample was centrifuged at 36,900xg (SS34 rotor; Sorvall RC-5B) for 20 min at 4°C, to remove insoluble material. The supernatant was removed and the concentration of digitonin reduced to 0.5% (w/v) by addition of 10 mM Tris-HCl (pH 7.4), 90 mM NaCl. Solubilised plasma membranes were stored in liquid nitrogen, and used within 1 week of preparation.

2.8. Affinity chromatography of β-adrenergic receptors

2.8.1. Synthesis of Sepharose 4B-alprenolol affinity gel

(-)-Alprenolol was linked to sodium thiosulphate-derivatised

Sepharose 4B as described by Caron et al. (1979), with the modifications used by Shorr et al. (1981). Also (-)-alprenolol d-tartrate was used instead of the hydrochloride salt for the synthesis.

2.8.2. Purification of β-adrenergic receptors on Sepharose 4B-alprenolol gel

Affinity chromatography was performed essentially as described by Caron et al. (1979) with some modifications. A Sepharose 4B-alprenolol affinity column (Omnifit), containing 10 ml of gel was used for the affinity purification. The gel was washed free of azide with 20 ml of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, and equilibrated with 20 ml of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 0.2% (w/v) digitonin at room temperature. Digitonin-solubilised membranes were loaded onto the column at room temperature, at a flow-rate of 12 ml/h. The column was equilibrated to 4°C (20 min) and washed with 40 ml of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 0.05% (w/v) digitonin at 4°C, at the increased flow-rate of 20 ml/h. Following washing, the column was placed at room temperature for 20 min. The bound β-adrenergic receptors were subsequently eluted with 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 0.05% (w/v) digitonin, 1 mM (-)-isoproterenol at the same flow-rate. Aliquots of column fractions (0.5 ml) were desalted by gel filtration (section 2.9), then assayed for the presence of β-adrenergic receptors, using the gel filtration method (section 2.5.2.1). β-Adrenergic receptor containing fractions were stored in liquid nitrogen.

The affinity column was regenerated with 20 ml of 2 M NaCl, 50 ml of water, and saturated with 0.02% (w/v) sodium azide for storage at 4°C.

2.9. Removal of (-)-isoproterenol from partially purified β-adrenergic receptor preparations

Separation of free (-)-isoproterenol and protein was achieved by gel filtration at 4°C. A 10 ml plastic pipette (Costar) was taken and filled with approximately 10 ml of Sephadex G50 (gel bed dimensions - 20 x 0.8 cm). V_o for the column was determined using Blue Dextran (0.2 mg/ml) and found to be 4.2 ml (Figure 2.2). The column was equilibrated at 4°C with 10 ml of ice-cold 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.05% (w/v) digitonin. The soluble sample was loaded carefully onto the top of the gel bed using a Pasteur pipette, and the column eluate collected. The sample was run through the column using more of the same buffer. The first 4.2 ml of eluate was discarded (this volume represents the void volume of the column), and the β-adrenergic receptors collected in the next 2.2 ml, and stored in liquid nitrogen.

2.10. Photoaffinity-labelling of β-adrenergic receptors

2.10.1. Photoaffinity-labelling of β-adrenergic receptors in adipose tissue membranes

Photoaffinity-labelling of β-adrenergic receptors with (±)-3-[^{125}I]iodocyanopindolol-diazirine ([^{125}I]ICYPD) was performed as described by Burgermeister et al. (1983), with some modifications (see Figure 2.3 for the chemical structure of [^{125}I]ICYPD). Adipose tissue membranes (600 µg protein) were suspended in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1 mM ascorbic acid containing 1 nM [^{125}I]ICYPD in a total volume of 600 µl, under dim red light in a dark room. The sample was incubated for 1 h at 30°C in the dark. Following incubation, the sample was transferred to a 50 mm plastic petri-dish, and the volume increased to 6 ml by addition of N_2-saturated ice-cold 50 mM Tris-HCl (pH 7.4), 5 mM

EDTA. The dish was photolysed on ice for 1 h with long wave U.V. light (366 nm), using an U.V. lamp (2 x 8 W tubes, Allen) at a distance of 8 cm. Samples were centrifuged immediately at 20,000 rpm (SS34 rotor; Sorvall RC5B) for 10 min at 4°C. The membrane pellet was washed once with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, resuspended in the required buffer and stored at -20°C. Non-specific photoaffinity labelling was determined by preparing an identical preparation in the presence of 100 µM (-)-isoproterenol.

Photoaffinity-labelled preparations were not boiled prior to gel electrophoresis, according to the method of Laemmli (1970), as this results in the formation of high molecular weight aggregates (Shorr et al. 1982, 1985).

2.10.2. Photoaffinity-labelling of soluble β-adrenergic receptors

Photoaffinity-labelling was performed according to Burgermeister et al. (1983) with some changes. β-Adrenergic receptors were partially purified by affinity chromatography. Partially purified receptors were incubated with 1 nM [^{125}I]ICYPD in 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1 mM ascorbate in a total volume of 250 µl, for 2-3 h at room temperature. The sample was loaded onto a 3.5 ml Sephadex G50 column (gel bed dimensions: 12 x 0.6 cm) prequilibrated at 4°C with 3 ml of ice-cold 5 mM Tris-HCl (pH 7.4), 0.05% (w/v) digitonin. When the sample had entered the gel, 1 ml of equilbration buffer was run through the column and the eluate discarded (represents the column void volume). The β-adrenergic receptors were eluted with a further 1.4 ml of the same buffer and diluted to a volume of 3 ml with N_2-saturated ice-cold 5 mM Tris-HCl (pH 7.4), and placed in an open 50 mm petri-dish on ice. The sample was photolysed for 30 min using an U.V. lamp (2 x 8 W bulbs:

Allen) at a distance of 8 cm. The photoaffinity-labelled β-adrenergic receptors were concentrated by lyophilisation or using a Centricon 10 micro-concentrator (Amicon).

2.11. Electrophoretic techniques

2.11.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS PAGE was performed according to Laemmli (1970) with some modifications. Samples for electrophoresis were solubilised in SDS sample buffer (25 mM Tris-HCl (pH 6.5), 8% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue), and incubated for 1 h at room temperature. Electrophoresis was performed in a Protean II (Bio-Rad) electrophoresis apparatus, equipped with 20 x 16 cm plates. Samples were resolved using a 2 cm, 4% stacking gel and a 10% resolving gel (for gel buffers and mixtures, see Hames, B. D., 1981). Gels were run at 120 V for the stacking gel, and 200 V through the resolving gel until the dye front was 1 cm from the bottom of the gel. Gels were stained using either 0.1% (w/v) Coomassie blue R250, 40% (v/v) methanol, 10% (v/v) acetic acid, or with a silver stain kit (Bio-Rad) according to manufacturers instructions. For autoradiography, gels were stained and equilibrated in 7% (v/v) acetic acid, 1% (v/v) glycerol for 1 h at room temperature. Gels were dried on a Bio-Rad gel dryer at 80°C, and exposed to Fuji X-ray film in an autoradiography cassette with intensifying screens at -70°C.

Molecular weights of proteins were calculated from a calibration curve constructed from the mobilities of proteins contained in a MW-SDS-6H molecular weight marker kit (Sigma). A calibration curve is shown in Figure 2.4. Marker proteins were: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); bovine serum albumin (66 kDa); ovalbumin

(45 kDa); carbonic anhydrase (29 kDa).

2.11.2. Isoelectric focussing (IEF)

IEF was performed using the method of O'Farrell (1975), with ampholyte pH ranges of 3-10 and 5-8. Samples were loaded at the anode end of the gel. IEF was performed overnight at 1 mA/gel (V_{max} - 800 V) at room temperature. Gels were stained with Coomassie blue and sliced for counting, or equilibrated and run in a second dimension.

Iso-electric points of proteins were calculated from a calibration curve constructed from the positions of marker proteins with known pI. A calibration curve is shown in Figure 2.5. Proteins used: bovine serum albumin (pI=4.9); carbonic anhydrase (pI=6.1); haemoglobin (pI=7.5).

2.11.3. Two-dimensional polyacrylamide gel electrophoresis

Two-dimensional PAGE was performed using the method of O'Farrell (1975), with some minor modifications.

Samples were subjected to isoelectric focussing (IEF) as described in section 2.11.2. The IEF gels were equilibrated for 15 min in 50 ml of SDS equilibration buffer (62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue). Gels were either frozen and stored at -20°C or run immediately in a second dimension.

Second dimension gel electrophoresis was performed according to Laemmli (1970). The second dimension was run in 16 x 16 cm plates using a 1.5 cm, 2.5% stacking gel and a 10% resolving gel. The IEF gel was sealed on top of the SDS gel using warm 1% (w/v) agarose (low gelling temperature) in 62.5 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS. Electrophoresis was performed at 80 V through the stacking gel, and

200 V through the resolving gel. Gels were stained and autoradiographed as decribed for SDS PAGE.

2.11.4. Two dimensional benzyldimethyl-n-hexadecylammonium chloride (16-BAC)/SDS PAGE

Two dimensional electrophoresis was performed essentially as described by Macfarlane (1989), with modifications. The first dimension (16-BAC) was run in 1.5 mm thick slab gels using 20 x 16 cm plates (Protean II, Bio-Rad). Electrophoresis was performed towards the cathode at 30 mA/gel overnight (about 16 h) at room temperature. Following electrophoresis, gels were either stained and autoradiographed as for SDS PAGE, or individual gel lanes cut out and run in a second dimension using SDS PAGE. Strips (about 6 mm) were cut from the 16-BAC gel and equilibrated (without previous drying) for 1 h in 1.5 M Tris-HCl (pH 8.3), 4% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue. The gel slice was sealed on top of an SDS polyacrylamide gel (2 cm 4% stacking gel, 10% resolving gel) in 20 x 16 cm plates (Protean II, Bio-Rad) using 1% (w/v) low gelling temperature agarose in 62.5 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS. Electrophoresis was performed towards the anode at 60 mA/gel at room temperature. Gels were stained and autoradiographed as for SDS PAGE.

2.12. Production of antibodies against the β-adrenergic receptor

2.12.1. Immunisation protocol

Individual six-week-old female BALB/c mice (A. Tuck & Son, Rayleigh, Essex, U.K.) were immunised with three different immunogens: Partially purified β-adrenergic receptors (1 pmol) from sheep adipose tissue membranes (mice 1-3); NaOH-treated sheep adipose tissue membranes (50 μg protein: mouse 4); 58 KDa proteins (50 μg protein)

electroeluted from SDS PAGE gels of sheep adipose tissue membranes (mice 5-8). Suspended immunogens were emulsified in twice their volume of Freund's adjuvant (1 part complete : 1 part incomplete), and mice received intraperitoneal injections (300 µl) of the resulting emulsions. Animals were boosted (3 times) every 4 weeks with the same amounts of immunogen in Freund's incomplete adjuvant (1 part immunogen : 2 parts adjuvant), until a positive immune response was achieved. Three days prior to fusion, mouse 2 was boosted intraperitoneally with 2 pmol of partially purified β-adrenergic receptor in PBS.

2.12.2. Medium for fusion

Myeloma medium: RPMI 1640 medium, 10% (v/v) FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine.

HAT medium: Myeloma medium, 20% (v/v) FCS, 0.1 mM hypoxanthine, 0.4 µM aminopterin, 16 µM thymidine.

HT medium: Myeloma medium, 20% (v/v) FCS, 0.1 mM hypoxanthine, 16 µM thymidine.

2.12.3. Fusion protocol

Mouse spleen cells were fused with non-secreting mouse myeloma P3 X63-Ag 8.653 cells (Kearney et al., 1979) according to the method of Galfre & Milstein (1981). Briefly, 1.2 x 10^8 spleen cells and 1.7 x 10^8 myeloma cells in the logarithmic phase of growth were mixed and pelleted at 1000 rpm for 10min. The cells in the pellet were fused by addition of 0.8 ml prewarmed (37°C) 40% (w/v) PEG 1550 (in RPMI 1640 medium) using a 1 ml syringe and 23g needle, slowly over 1 min. The cells were allowed to stand for 1 min, and 1 ml of serum-free RPMI 1640 medium added slowly over 1 min, followed by 20 ml of myeloma medium over 5 min. Cells were spun at 1000 rpm for 10 min and resuspended gently in

15 ml of myeloma medium, 20% (v/v) FCS, 4% (v/v) hybridoma growth factor. The cell suspension was split into 3 x 5 ml aliquots, each receiving a different plating treatment into 96-well plates (Linbro, Flow Laboratories). The rationale behind this was to investigate the effect of HAT medium on the efficiency of hybridoma production, by staggering the time at which cells were exposed to HAT medium post-fusion.

Tray 1: 50 µl/well of cell suspension into wells containing 50 µl of myeloma medium, 20% (v/v) FCS.

Tray 2: 5 ml of 2 x HT medium (20% (v/v) FCS) and incubated for 3 h at 37°C.Cells were spun at 1000 rpm for 10 min and resuspended in 10ml of HAT medium, 2% (v/v) hybridoma growth factor. The cells were plated at 100 µl/well.

Tray 3: 50 µl/well of cell suspension into wells containing 50 µl/well of 2 x HAT medium (20% (v/v) FCS).

The following day, 50 µl of medium was removed from each well of tray 1 and replaced with 50 µl of 2 x HAT medium (20% (v/v) FCS). On day 6 after fusion, cells were fed with 100 µl/well HAT medium. Three half-replacements with HAT medium were carried out during the next two weeks. Aminopterin was omitted from the medium during expansion of positive clones into 24-well plates. On days 14 and 18 after the fusion, supernatants were assayed for antibodies against the β-adrenergic receptor using an enzyme immunoassay (EIA).

2.12.4 Enzyme immunoassay (EIA)

Hybridoma supernatants were initially screened for binding to sheep adipose tissue membranes, and sheep erythrocyte ghosts. Sheep erythrocyte ghosts were used as a negative contol in this assay for antibodies against the β-adrenergic receptor, since they had previously

been shown to be devoid of measurable β-adrenergic receptors. Sheep adipose tissue membranes and sheep erythrocyte ghosts were adsorbed onto 96-well polystyrene plates overnight at room temperature, at a protein concentration of 1 μg/well in PBS. The plates were washed three times with PBS, 0.05% (v/v) Tween 20 (PBS Tween), and blocked with 1% (w/v) BSA in PBS for 1 h at room temperature. Hybridoma supernatants (diluted 1:2.5 in RPMI 1640 medium) were transferred to the plates and incubated for 2 h at room temperature. After three washings with PBS Tween, 100 μl of goat anti-mouse IgG alkaline phosphatase conjugate (diluted 1:1000) in PBS Tween was added and incubated for 1.5 h at room temperature. The plates were washed three times with PBS Tween and 200 μl of substrate (0.1% (w/v) 4-nitrophenyl phosphate, 0.1 M glycine, 10 mM $ZnCl_2$, 10 mM $MgCl_2$) added, and the absorbance recorded at 405 nm in a Titertek Twinreader. The assay was performed in duplicate. Antisera or hybridoma supernatants were classed as containing antibodies against the β-adrenergic receptor when the absorbance change against sheep adipose tissue membranes (both plates) were significantly higher than those for sheep erythrocyte ghost-coated wells (both plates). During the screening of hybridomas, the background absorbance for each plate was defined as the mean absorbance reading for 10 wells devoid of hybridomas.

2.12.5. Expansion of positive hybridomas

Wells deemed to possess antibodies against the β-adrenergic receptor by EIA were expanded into a 24-well plate. The contents of these positive wells were removed from the 96-well plates and transferred to a well of a 24-well plate (Linbro, Flow) containing 1 ml of HT medium and 10^7 mouse thymocytes as a feeder layer. After 3 d the cells were fed with a further 1 ml of HT medium and screened for

antibodies against the β-adrenergic receptor after 1 week.

2.13. Competition of [^{125}I]ICYP binding to sheep adipose tissue membranes by hybridoma culture supernatants.

Sheep adipose tissue membranes (15 μg protein) were incubated with 100 μl of hybridoma culture supernatant in 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM ascorbate in a total volume of 200 μl, for 1 h at 37°C. Following incubation, [^{125}I]ICYP was added to give a concentration of 75 pM, and the tubes incubated for 1 h at room temperature. The reaction was quenched by addition of 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and the mixture filtered rapidly on a single 2.5 cm Whatman GF/C filter. The filter was washed twice with 5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$. [^{125}I]ICYP bound to the filters was quantified using a LKB Gamma counter.

2.14. Immunoprecipitation of soluble sheep adipose tissue membrane β-adrenergic receptors by hybridoma culture supernatants.

Digitonin-solubilised sheep adipose tissue membranes (30 μg protein) were incubated with 100 μl of hybridoma culture supernatant in 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 0.1 mM ascorbate in a total volume of 250 μl, and incubated overnight at 4°C. Precipitation of the first antibody was achieved by addition of either 100 μl of agarose-coupled goat anti-mouse IgG (Sigma) or 40 μl of sheep anti-mouse IgG (SAPU). Tubes were vortexed and incubated for 2 h at room temperature, and subsequently centrifuged at 3000 r.p.m. for 30 min at room temperature. 200 μl of supernatant was removed and the number of β-adrenergic receptors remaining quantified using 75 pM [^{125}I]ICYP and PEG precipitation, as described previously (section 2.5.2.2). Non-specific binding was determined using 100 μM (-)-isoproterenol.

2.15. Immunoprecipitation of soluble photoaffinity-labelled sheep adipose tissue membrane β-adrenergic receptors by hybridoma culture supernatants

Sheep adipose tissue membrane β-adrenergic receptors were photoaffinity-labelled with [^{125}I]ICYPD (section 2.10.1), and subsequently solubilised with 1% (w/v) digitonin (section 2.7), as described previously. Solubilised photoaffinity-labelled sheep adipose tissue membranes (120 µg protein) were incubated with 200 µl of hybridoma culture supernatant overnight at 4°C. 150 µl of SAC-CEL (Anti-mouse/rat) was added, and the tubes vortexed and left at room temperature for 30 min. Samples were centrifuged at 1000 xg for 10 min at room temperature, and the supernatant decanted. [^{125}I]ICYPD in the pellet was quantified using a LKB Gamma counter. 150 µl of SDS sample buffer (25 mM Tris-HCl (pH 6.5), 8% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue was added to each sample, and the tubes vortexed and left at room temperature for 1 h. Samples were centrifuged at 1000 xg for 5 min to pellet the SAC-CEL beads. The SDS sample buffer was removed and subjected to SDS PAGE and autoradiography, as described previously (section 2.11.1).

2.16. Immunoblotting technique

Proteins were subjected to SDS PAGE as described, using a Mini Protean II (Bio-Rad) gel apparatus, and a running voltage of 150 V. Transfer of proteins to nitrocellulose membrane (Gelman Sciences; BioTrace NT, 0.45 µm) was performed according to Towbin et al. (1979), using a Bio-Rad Mini Transblot cell. Efficient transfer was achieved at 100 V for 1h in 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol. The nitrocellulose was rinsed once in TBS (5 mM Tris-HCl (pH 7.6), 0.9% (w/v)

NaCl), and saturated with TBS, 1% (w/v) dried skimmed milk (Marvel) for 1 h at room temperature. The nitrocellulose was placed in a 16-channel clamp (Biometra) and diluted antisera in TBS, 1% (w/v) Marvel pipetted into separate channels and incubated overnight at room temperature. The first antibody was flushed from the clamp with TBS, and the nitrocellulose transferred to a shallow tray and rinsed three times with TBS, 1% (w/v) Marvel (5 min/rinse). Fresh TBS, 1% (w/v) Marvel containing horseradish peroxidase-labelled second antibody (1:100; SAPU) was added and incubated for 2 h at room temperature with occasional shaking. After extensive washing with TBS, binding of second antibody was revealed using 4-chloro-1-napthol/H_2O_2 substrate.

2.17. Protein determination

Protein was measured by the method of Bradford (1976) using the Bio-rad protein assay (Cat. No. 500-0006). BSA (Fraction V) was used as a protein standard (1-5 μg standard curve). Assays were performed in 96-well microtitre plates, and read using a Titertek Multiskan at 600 nm.

In samples containing digitonin, the final concentration of detergent in each well was kept below 0.1% (w/v), so that the interference of digitonin with the protein assay was minimal (see Figure 2.6).

2.18. Analysis of data

Results are expressed as mean +/- S.E.M. Radioligand competition curves were analysed using a four-parameter logistic equation (ALLFIT; De Lean et al., 1978). Analysis of IC_{50} values from competition curves obtained for lactating and non-lactating sheep was performed using analysis of variance utilising the GENSTAT computer program.

Statistical analysis of paired or unpaired observations was performed using a Student's t-test.

Fig. 2.1. Separation of soluble membrane protein (β-adrenergic receptor) from free [^{3}H]DHA

Digitonin-solubilised sheep adipocyte membranes (100 μg protein) were incubated with 10 nM [^{3}H]DHA for 10 min at 30°C, in 10 mM Tris-HCl (pH 7.4), 90 mM NaCl, 0.1 mM ascorbate, in a total volume of 300 μl. The sample was loaded onto a pre-equilibrated column (5 ml pipette, Costar) filled with 3.5 ml of Sephadex G50 (gel bed dimensions - 12 x 0.6 cm). Elution was achieved with 10 mM Tris-HCL (pH 7.4), 90 mM NaCl, 0.05% (w/v) digitonin, and 0.2 ml fractions collected for protein (open cicles) and [^{3}H]DHA (closed circles) measurement. The void volume (V_o) for the column was 1.75 ml, determined using Blue Dextran 2000.

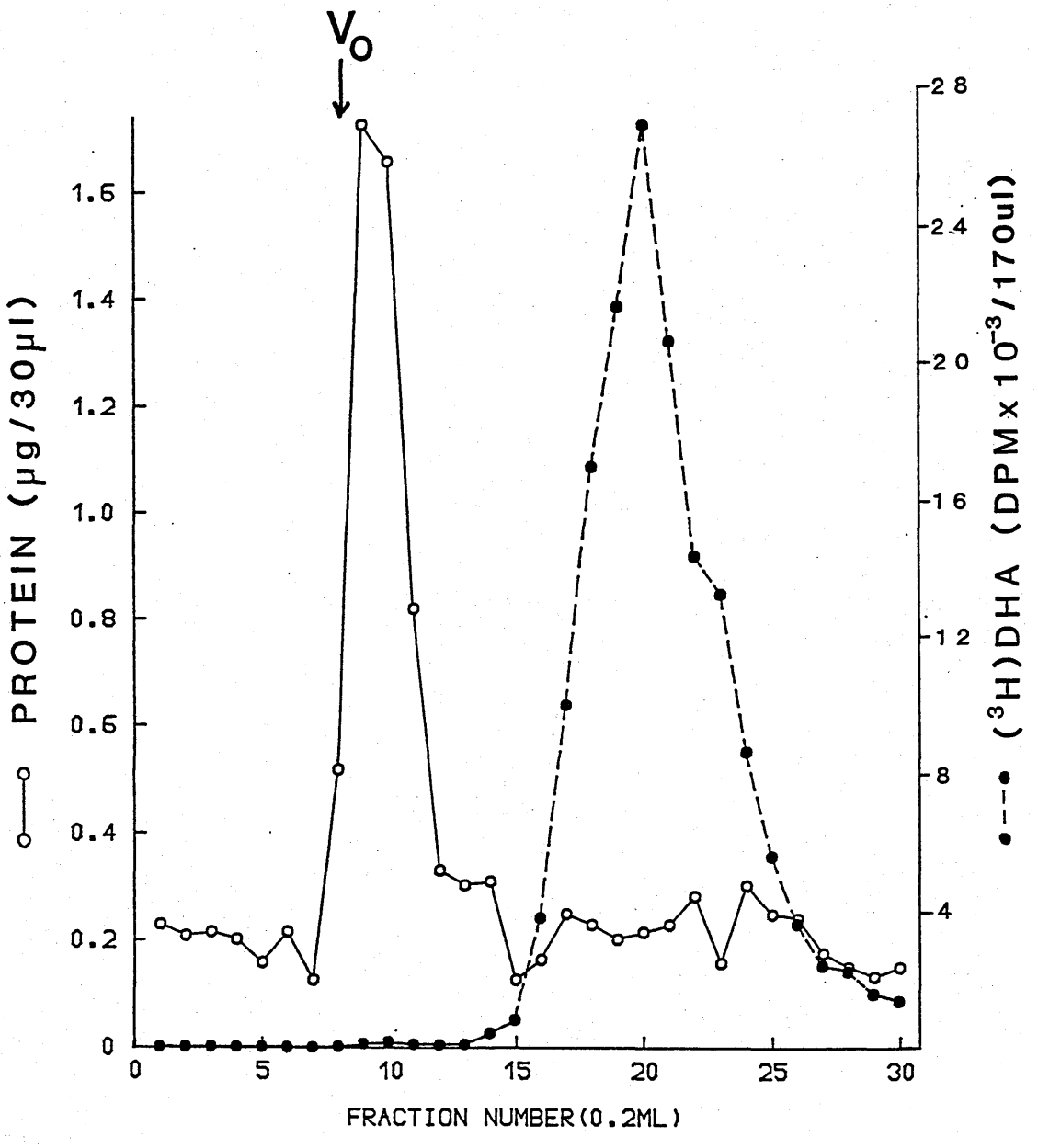

V_O
PROTEIN (μg/30μl)
(^{3}H)DHA (DPM x 10^{-3}/170ul)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
4
8
12
16
20
24
28
0
5
10
15
20
25
30
FRACTION NUMBER(0.2ML)

Fig. 2.2. Determination of the void volume (Vo) for 0.8 x 12 cm Sephadex G50 column

A 10 ml plastic pipette (Costar) was blocked with glass wool, and filled with Sephadex G50 to give a gel bed with dimensions of 0.8 x 20 cm. Next, 400 µl of Blue Dextran 2000 (0.2 mg/ml) mixed with 3 drops of glycerol was loaded onto the column, and eluted with distilled water at room temperature. The eluate was collected, and 200 µl samples were read at 540 nm using a Titertek Twinreader. Vo was found to be 4.2 ml (Maximum absorbance for Blue Dextran at 540 nm).

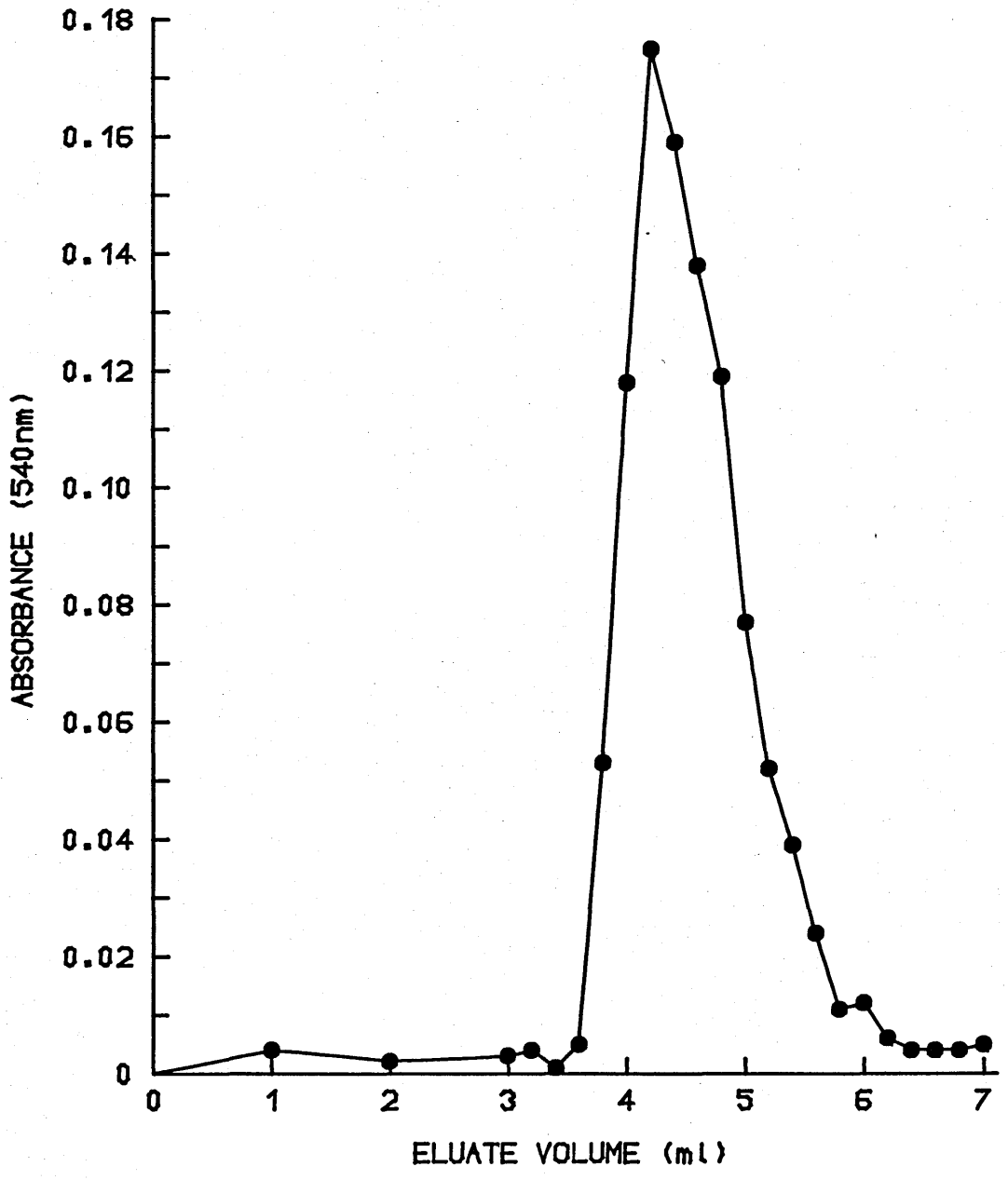

0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
ABSORBANCE (540nm)
0
1
2
3
4
5
6
7
ELUATE VOLUME (ml)

Fig. 2.3. Chemical structure of (±)-3-[^{125}I]iodocyanopindolol diazirine

[^{125}I]ICYPD consists of iodocyanopindolol linked to a photoreactive moiety, which absorbs ultra-violet at 366 nm.

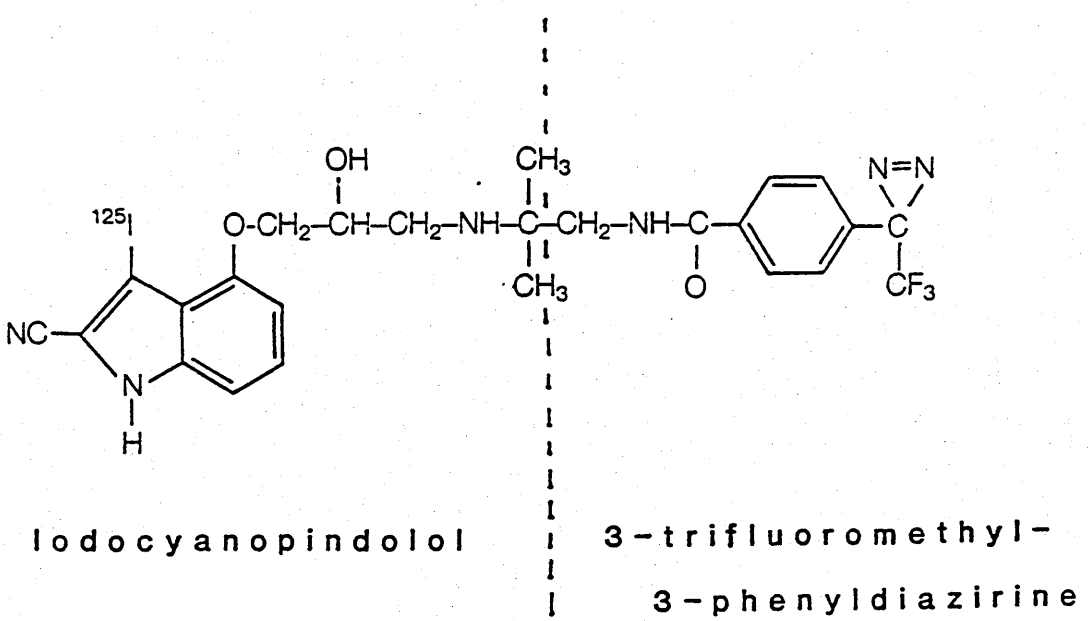


Fig. 2.4. Calibration curve for determination of molecular weights for proteins by SDS PAGE

Proteins from the MW-SDS-6H (Sigma) molecular weight marker kit were subjected to SDS PAGE, using a 10% resolving gel. Proteins were visualised with Coomassie blue stain, and their relative mobilities (Rf) calculated. Proteins used: myosin (205 kDa); β-galactosidase (116 kDa); phosphorylase b (97.4 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa); carbonic anhydrase (29 kDa).

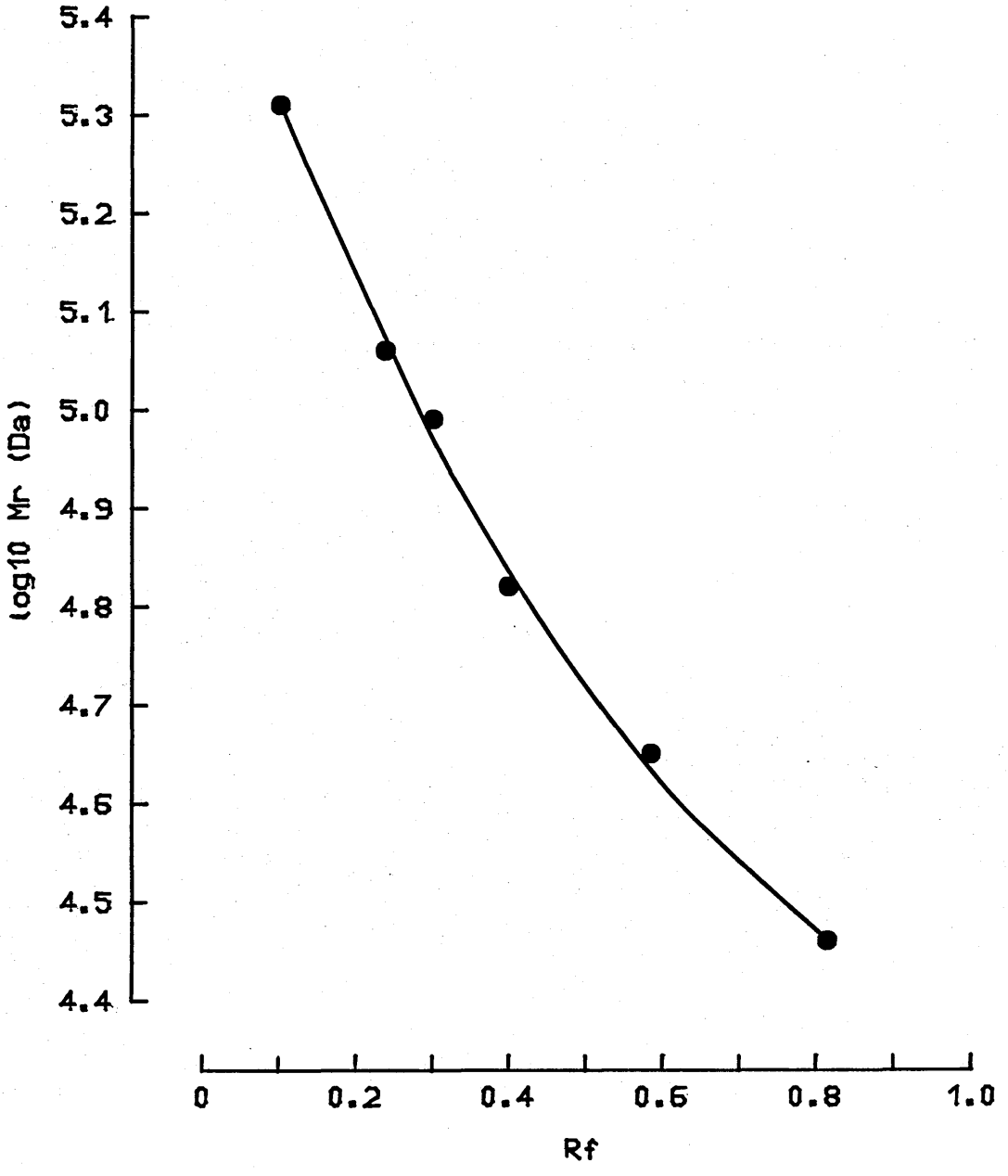

5.4
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
log10 Mr (Da)
0
0.2
0.4
0.6
0.8
1.0
Rf

Fig. 2.5. Calibration curve for the calculation of isoelectric points (pI) of proteins following IEF

Proteins with known isoelectric points were subjected to IEF using an ampholyte pH gradient of 3-10, and localised with Coomassie blue staining. The mobility (Rf) of the proteins was calculated relative to the anode (Rf=0) and the cathode (Rf=1) ends of the gel. Proteins used: bovine serum albumin (pI=4.9); carbonic anhydrase (pI=6.1); haemoglobin (pI=7.5).

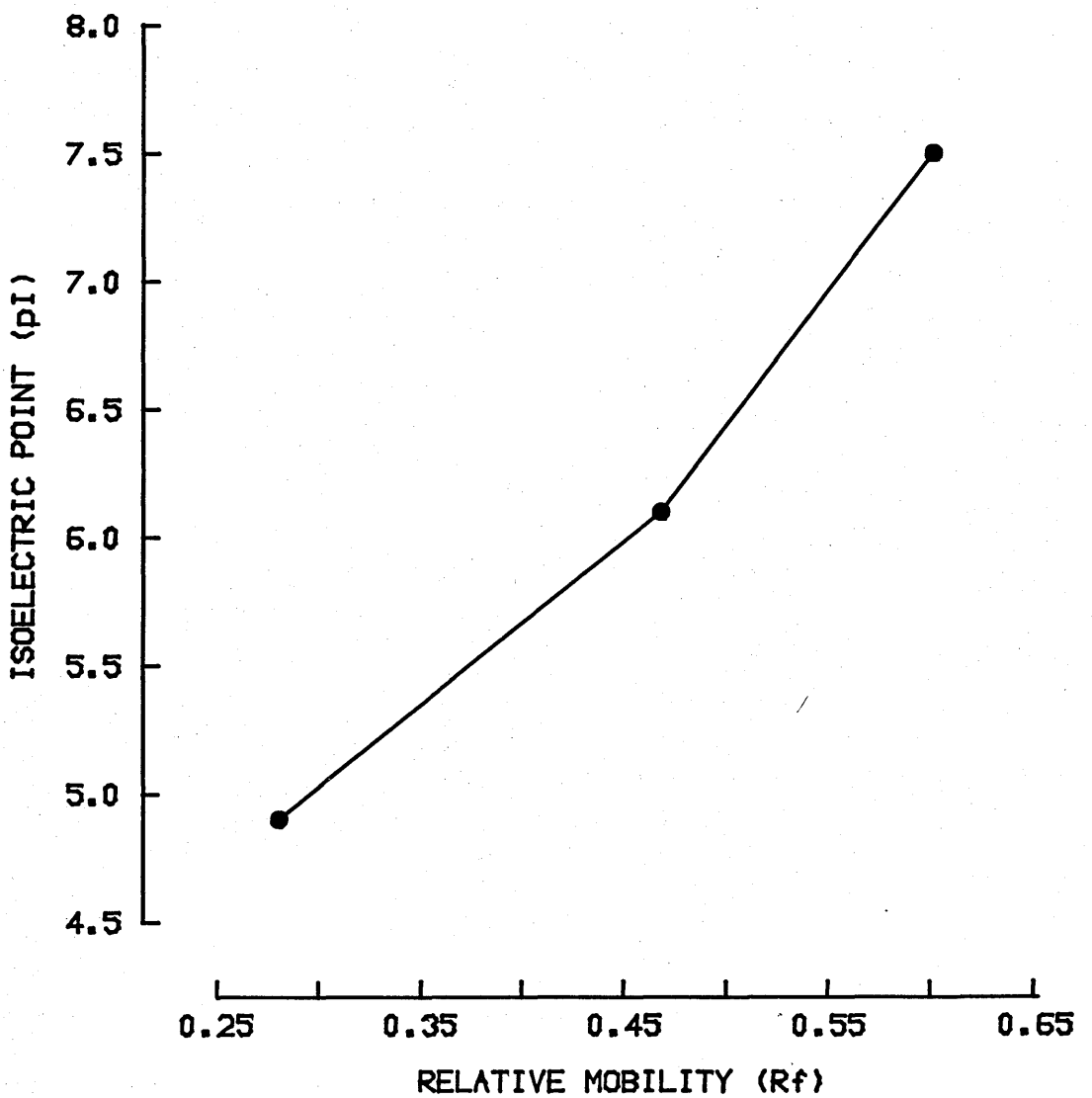

8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
ISOELECTRIC POINT (pI)
0.25
0.35
0.45
0.55
0.65
RELATIVE MOBILITY (Rf)

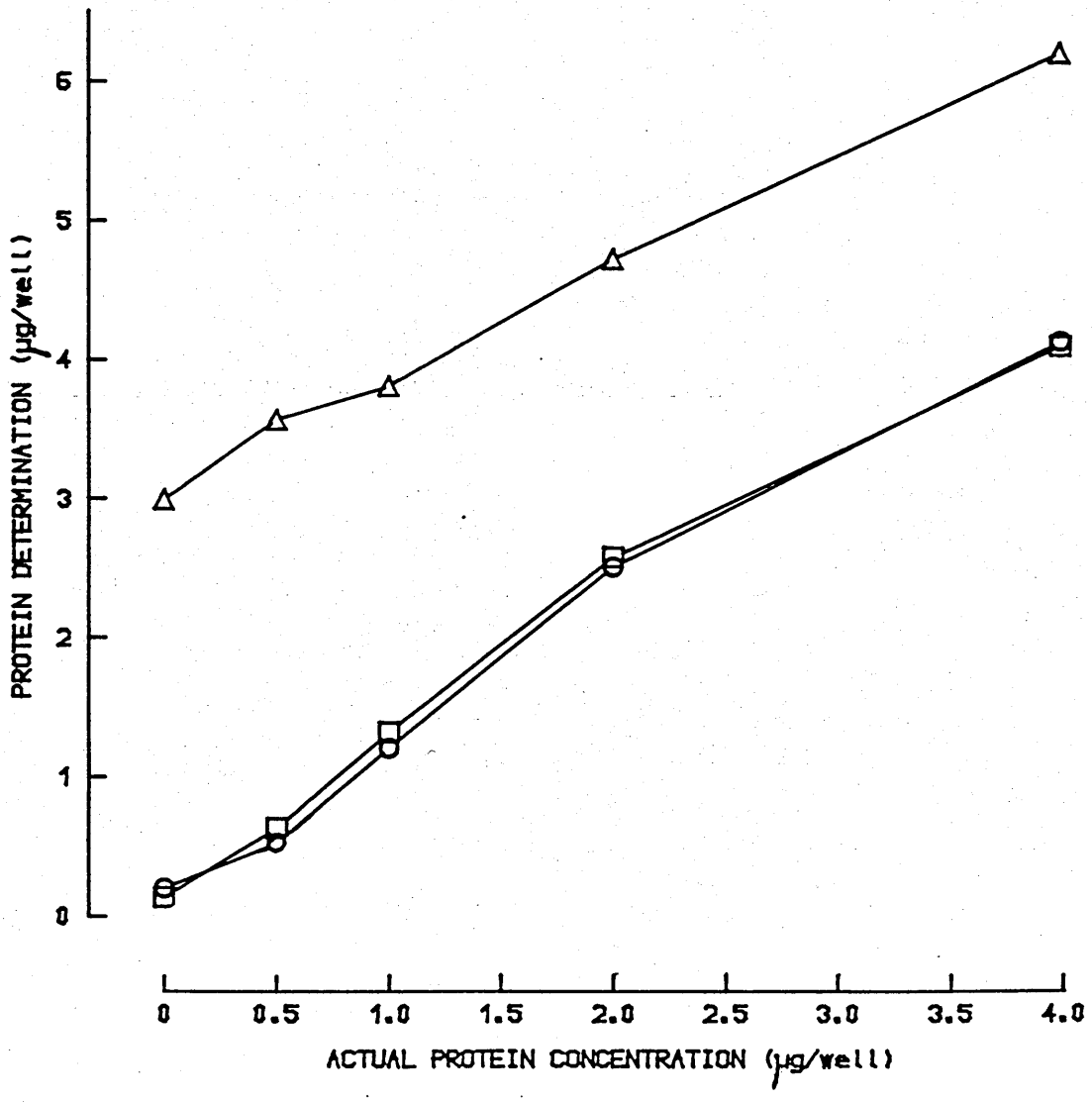
Fig. 2.6. The effect of various levels of digitonin on the Bradford protein assay

BSA was quantified at concentrations of 0.1, 0.5, 1.0, 2.0 and 4.0 µg/50 µl (well), in the presence of 0%, 0.1% and 0.5% (w/v) digitonin, using a Bio-Rad protein assay kit, according to the manufacturers instructions. The determined values are plotted against the actual protein concentrations in each well. Each point represents the mean of triplicate readings.

o----o 0.0% (w/v) digitonin

□----□ 0.1% (w/v) digitonin

Δ----Δ 0.5% (w/v) digitonin


PROTEIN DETERMINATION (µg/well)
0
1
2
3
4
5
6
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
ACTUAL PROTEIN CONCENTRATION (µg/well)

CHAPTER THREE

PURIFICATION OF THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

CHAPTER THREE - PURIFICATION OF THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

3.1. Introduction

In light of the key role which β-adrenergic receptors play in adipose tissue metabolism, it was decided to attempt the purification of the receptor(s) from adipose tissue, with a view to increasing our knowledge of their physical characteristics and obtaining enough partially-purified receptor for the production of antibodies against the β-adrenergic receptor. The approach used involved affinity chromatography of β-adrenergic receptor to gain an initial purification. The success of this process was monitored using SDS PAGE and radioligand binding. An alternative method using 16-BAC gel electrophoresis was also studied, because of its claimed high protein loading capacity and resolution, plus its previous use for purification of protein antigens for the production of monoclonal antibodies.

3.2. Experimental procedure

Details of all methods used for the purification of β-adrenergic receptors are contained in Chapter 2. Briefly, sheep adipose tissue membranes were isolated from omental adipose tissue (unless otherwise mentioned). Subcutaneous adipose tissue was used for the preparation of adipocyte membranes. Adipose tissue was obtained from either wether lambs, non-lactating ewes, or lactating ewes (as indicated). Membranes were solubilised with the plant glycoside digitonin, and the resulting preparation passed through a Sepharose 4B-alprenolol affinity column. Affinity column eluates were analysed using radioligand binding assays and photoaffinity-labelled with [^{125}I] iodocyanopindolol-diazirine ([^{125}I]ICYPD).

3.3. Results

3.3.1. Preparation of sheep adipose tissue membranes

Initial studies during this work were performed using sheep subcutaneous adipocyte plasma membranes, whose preparation involved collagenase digestion (section 2.3.1). These membranes proved to be a good source of β-adrenergic receptors, typically containing 150 fmol/mg protein of receptors, determined using 10 nM [^{3}H]DHA. However, the use of collagenase limited the amount of adipose tissue which could be processed at any one time (typically 80 g), and yielded on average about 10 mg of membrane protein. It was therefore decided to use a larger-scale purification method using whole adipose tissue (section 2.3.2), in order to isolate the large quantities of membranes required for β-adrenergic receptor purification. This method has a high capacity (>1 kg adipose tissue), and involves no addition of exogenous proteases (as contained in collagenase preparations) which might degrade β-adrenergic receptors. This method also gave a good yield (although about half that obtained using collagenase digestion) of membranes (30-50 mg/kg adipose tissue), with high specific activities of β-adrenergic receptors, usually 200-400 fmol/mg protein (Table 4.4 and Figure 4.11). However one disadvantage of the method is that contamination of the adipocyte plasma membrane preparation with stroma-vascular membranes was unavoidable.

3.3.2. Solubilisation of sheep adipose tissue membranes

Sheep adipose tissue membranes were solubilised using the plant glycoside digitonin. Typically, 35 mg of sheep adipose tissue membrane protein were solubilised with a protein yield of 60-65%. Solubilisation times of 1.5 h gave better protein yields compared to 1 h, and it was also very important to homogenise the sheep adipose tissue membranes

thoroughly with the digitonin solution. Trials with 0.3% and 1.5% digitonin solutions did not increase the yield or specific activity of β-adrenergic receptors in soluble preparations, in contrast to their beneficial usage during the purification of hamster lung β_2-adrenergic receptor by J. L. Benovic (personal communication). The yield of β-adrenergic receptors was not determined, but the ligand binding activity of receptors seemed to be lower in solubilised preparations than in membranes (see Tables 3.1 & 3.2).

3.3.3. Affinity chromatography of β-adrenergic receptors

Synthesis of the Sepharose 4B-alprenolol affinity resin was achieved as described in Chapter 2. Some clumping of the gel occurred during the synthesis, and was removed by passing the suspension through a 10 ml plastic syringe. Use of a temperature of 40°C for the final coupling of alprenolol to the gel matrix reduced aggregation.

β-Adrenergic receptors from sheep (wether) omental adipose tissue membranes were solubilised in digitonin, and subjected to affinty chromatography on a 10 ml Sepharose 4B-alprenolol column. During loading of the preparation, greater than 95% of the protein passed through the column (Table 3.1 and Figure 3.1). This is rather a high value for protein retention on an affinity column matrix, and reflects the ion-exchange properties of the covalently-bound alprenolol. Washing the column with 0.5 M NaCl did result in greater elution of protein, but decreased the yield of β-adrenergic receptors. The amount of protein contained in fractions 11-14 (see Table 3.1) which were pooled for monoclonal antibody production, represents 0.6% of the protein loaded onto the column. For the third purification, this figure dropped to 0.1% (Table 3.2). Bound receptors were eluted with a front of 1 mM (-)-

isoproterenol; the peak of receptor binding was detected in fraction 11 (Table 3.1. and Figure 3.2). Fractions of eluted receptors were subjected to gel filtration prior to assay, to remove the (-)-isoproterenol. The peak fraction contained 16.4 pmol/mg of β-adrenergic receptors, representing a purification of 105 fold. The yield of β-adrenergic receptors was 43%.

The results described represent the first purification performed. In subsequent purifications, the level of purification increased markedly to >300 fold with a 65% yield (1.828 pmol) of receptors (Table 3.2).

3.3.4. Studies using photoaffinity-labelled β-adrenergic receptors

3.3.4.1. Photoaffinity-labelling of β-adrenergic receptors

β-Adrenergic receptors were photoaffinity-labelled with [^{125}I] iodocyanopindolol-diazirine ([^{125}I]ICYPD), which can be labelled to a high specific activity (~2000 Ci/mmol). Use of 1 nM [^{125}I]ICYPD for the initial equilibration step meant that saturating concentrations of the ligand were present (see Figure 4.3 for [^{125}I]ICYP), if one assumes similar binding characteristics for [^{125}I]ICYPD and [^{125}I]ICYP. Quantification of bound [^{125}I]ICYPD following photoaffinity-labelling revealed that β-adrenergic receptors were labelled to almost the same extent as with [^{125}I]ICYP (results not shown). However, when samples were subjected to SDS PAGE (Figure 3.3), most of the counts loaded migrated with the dye front. This indicated that only a few percent of [^{125}I]ICYPD counts were covalently bound to receptor. Two more washings of the photoaffinity-labelled preparations did not reduce the number of non-covalently bound counts. Fewer counts were located at the dye front when photoaffinity-labelling was performed in the presence of 10 μM (-)-alprenolol (Figure 3.3). This was presumably due to the blocking of non-

β-adrenergic receptor binding sites (non-specific) by alprenolol.

3.3.4.2. SDS PAGE

Membrane-bound and partially purified β-adrenergic receptors from sheep (wether) subcutaneous adipocytes were photoaffinity-labelled with [^{125}I]ICYPD and subjected to SDS PAGE. According to the method of Laemmli (1970), samples are boiled for a few minutes prior to electrophoresis. However, boiling of photoaffinity-labelled samples prior to SDS PAGE, resulted in the formation of high molecular weight aggregates (Figure 3.4, lanes 1 and 2). Only a specifically-labelled 30 kDa protein migrated into the resolving gel (Figure 3.4, lane 1). When samples were not boiled, specifically-labelled proteins with molecular weights of 58, 42.5 and 30 kDa were seen (Figure 3.4, lane 3). Non-specific labelling was restricted to a 45.8 kDa protein (Figure 3.4, lane 4). Also, the pattern of labelling was found to be the same for omental and subcutaneous adipose tissue depots, and for adipose tissue from wethers and ewes (c.f. Figures 3.4 & 3.6). The broad banding on the autoradiographs is characteristic of glycoproteins.

SDS PAGE of partially-purified photoaffinity-labelled β-adrenergic receptors from sheep (wether) subcutaneous adipocytes, revealed a single 58 kDa labelled protein (Figure 3.5, lane 1). This labelling was inhibited by 10 μM (-)-alprenolol (Figure 3.5, lane 2). Also no increase in apparent molecular weight occurred when samples contained high concentrations of the disulphide bridge reducing agents β-mercaptoethanol and dithiothreitol, or the alkylating agent N-ethylmaleimide (Figure 3.5, lanes 3-5, respectively).

Photoaffinity-labelling of rat adipocyte β-adrenergic receptors, prepared from abdominal adipose tissue, revealed specific labelling of a

single 48 kDa band (Figure 3.6). Non-specific labelling was much higher than for sheep adipocyte membranes run on the same gel.

3.3.4.3. Isoelectric focussing (IEF) and 2D PAGE

IEF of photoaffinity-labelled β-adrenergic receptors from sheep (wether) subcutaneous adipocyte membranes revealed a single labelled peak (Figure 3.7a), not present when photoaffinity-labelling was performed in the presence of 10 μM (-)-alprenolol (Figure 3.7b). On an ampholyte pH range of 3-10, the peak was at pH 6.15 (Figure 3.8a), and at pH 6.25 on the narrower pH range of 5-8, with a shoulder on its acidic side, indicating the possible presence of several receptor isoforms (Figure 3.8b). As with SDS PAGE, most of the [^{125}I]ICYPD loaded was non-covalently bound and remained at the acidic end of the gel.

Partially-purified photoaffinity-labelled β-adrenergic receptors from sheep (wether) subcutaneous adipocytes gave different results. On a pH range of 3-10, the peak at pH 6.25 was seen, and also an additional broader peak at pH 4.85 (Figure 3.9a). On a pH range of 5-8, only a shoulder at the acidic end of the gel was observed (Figure 3.9b).

To determine whether the broad acidic peak on the pH 3-10 gel (Figure 3.9a) represented β-adrenergic receptor, the separated proteins were run in a second dimension using SDS PAGE. After a 20 day exposure period, nine labelled proteins were seen, all having a molecular weight of 58 kDa (Figure 3.10). The autoradiograph was scanned with a Bio-Rad video densitometer to give the relative proportions of these labelled species (Figure 3.11). Peaks 6 and 7 represent the major isoforms. No photoaffinity-labelled proteins were observed when labelling was performed in the presence of 100 μM (-)-isoproterenol (results not shown).

3.3.4.4. 16-BAC PAGE

16-BAC PAGE is an electrophoretic technique akin to SDS PAGE, which utilises the cationic detergent 16-BAC (SDS is anionic). It was used because it can accomodate higher protein loadings than SDS PAGE, and can also be used preparatively in a two-dimensional mode. 16-BAC PAGE of photoaffinity-labelled β-adrenergic receptors from sheep (lactating ewe) adipose tissue and subsequent autoradiography, revealed a broad protein band about halfway down the gel (Figure 3.12). This band was displaced by 100 μM (-)-isoproterenol. No [^{125}I]ICYPD was evident at the dye front. However when 1 mg of sheep (lactating ewe) adipose tissue membrane protein was used, and the gel sliced and counted, well-defined peaks were obtained with [^{125}I]ICYPD appearing to reside at both ends of the gel (Figure 3.13).

Proteins resolved using 16-BAC PAGE were subjected to electrophoresis in a second dimension using SDS PAGE. Two-dimensional separation of sheep (lactating ewe) adipose tissue membranes resolved the proteins along a diagonal line, indicating an overiding effect of molecular weight on the resolving process (Figure 3.14). Using photoaffinity-labelled β-adrenergic receptors from sheep (lactating ewe) adipose tissue membranes, it was possible to identify several specifically labelled proteins (Figures 3.15 & 3.16). The resolution was much poorer than that obtained using IEF as the first dimensional separation. In a separate experiment, a gel slice containing the major peak of photoaffinity-labelling (slices 26-28), was cut from a 16-BAC PAGE gel of photoaffinity-labelled β-adrenergic receptors from sheep (lactating ewe) adipose tissue membrane β-adrenergic receptors, and run in a second dimension (Figure 3.17a). Slicing and counting of the SDS

PAGE gel resulted in a single peak (Figure 3.17b). Similar results were obtained when the irradiation time during photoaffinity-labelling was 10 min (data not shown).

3.4. Discussion

Preparation of sheep omental adipose tissue membranes from whole adipose tissue was useful for purification of the β-adrenergic receptor. Although digestion of adipose tissue with collagenase was gave a good yield of adipocyte membranes, it had limited capacity. Radioligand binding data and SDS PAGE of photoaffinity-labelled β-adrenergic receptors also indicated that use of collagenase causes degradation of some receptors. Collagenase preparations are known to contain trypsin and clostripain, which have been shown to degrade adipocyte-specific cell surface antigens (Al-Jafari et al., 1986). Also as the duration of collagenase digestion increases, so does the damage to cell surface antigens (Al-Jafari et al., 1986). It is therefore understandable why membranes not exposed to collagenase digestion contain more intact β-adrenergic receptors as found with preparations of sheep adipose tissue membranes.

A critical step in the purification of a membrane bound protein is its extraction or solubilisation from the phospholipid bilayer. There is a wide variety of detergents available for solubilising membrane proteins, including non-ionic detergents such as Lubrol PX, Triton X-100, octyl glucoside and digitonin, and ionic detergents such as sodium cholate, CHAPS and SDS (for review see Hjelmeland & Chrambach, 1984). From the above list of detergents, only digitonin is capable of solubilising β-adrenergic receptors in an active state i.e. a state which binds adrenergic ligands (Caron & Lefkowitz, 1976). As an alternative to

digitonin, some workers have used dodecyl-β-maltoside with similar results (Tota & Strader, 1990). Solubilisation of sheep adipose tissue membranes in 1% digitonin for 1.5 h was found to be optimal. The protein yield of 65% is comparable to that achieved for rat adipocyte plasma membranes (Cubero & Malbon, 1984).

Affinity chromatography is a useful step for the purification of the β-adrenergic receptor, and can provide a 1000 fold purification and receptor recoveries of 50% (Vauquelin et al., 1979; Graziano et al, 1985; Cubero & Malbon, 1984). The Sepharose 4B-alprenolol affinity resin described by Caron et al., 1979 was used in light of its extensive use in many laboratories (Shorr et al., 1982; Cubero & Malbon, 1984; Bahouth & Malbon, 1987). The 304 fold purification achieved for β-adrenergic receptors from sheep omental adipose tissue is comparable to that obtained for dog lung (Homcy et al., 1983) and frog erythrocytes (Caron et al., 1979; Shorr et al., 1981). The Sepharose 4B-alprenolol affinity column increased in efficiency during successive purifications. The higher specific activities of β-adrenergic receptor which resulted may be explained by a decrease in non-specific binding of protein to the column. However this does not explain the increase in receptor yield.

The partially-purified preparations obtained were of sufficient purity for limited studies on receptor characteristics i.e. photoaffinity-labelling, SDS PAGE and IEF, and contained enough β-adrenergic receptor protein to attempt the production of monoclonal antibodies (Chapter 5). 1-2 pmol of β-adrenergic receptor has been used for immunisations during the production of monoclonal antibodies directed against the turkey erythrocyte β_1-adrenergic receptor (Couraud et al., 1985; Fraser & Venter, 1980), human A431 β_2-adrenergic receptor (Kaveri et al., 1987),

and calf β_2-adrenergic receptor (Fraser & Venter, 1980). Therefore two affinity purifications provided sufficient β-adrenergic receptors for the immunisation of three animals (Chapter 5). SDS PAGE of partially-purified β-adrenergic receptor preparations indicated the presence of large variety of non-β-adrenergic receptor proteins, and hence they were unsuitable for the production of polyclonal antisera. Despite using about 1 kg of sheep adipose tissue, the amount of membranes (30-50 mg protein) obtained was insufficient to allow recycling through a second affinity column, as utilised by other researchers to increase the purity of the β-adrenergic receptor preparations (Graziano et al., 1985; Shorr et al., 1981; Homcy et al., 1983). Also HPLC (Shorr et al., 1981, 1985; Cubero & Malbon, 1984; Graziano et al., 1985) and hydrophobic chromatography (Bahouth & Malbon, 1987) were unsuitable for the same reason.

Photoaffinity-labelling has proven to be a valuable tool in the biochemical characterisation of the β-adrenergic receptor. Photoaffinity probes possess high specific activities and high affinity (Kd $<10^{-9}$) for the β-adrenergic receptor. In this study, photoaffinity-labelling was used to determine the molecular size of the receptor in membrane preparations, and to follow the affinity purification. Photoaffinity-labelling of β-adrenergic receptors from both subcutaneous and omental sheep adipose tissue membranes with [^{125}I]ICYPD, specifically labelled a protein with a molecular weight of 58 kDa on SDS PAGE. Minor proteins at molecular weights of 42.5 kDa and 30 kDa were also present, and may arise from proteolytic degradation of the 58 kDa protein. Heterogeneity resulting from endogenous proteolytic activity varies among species and tissues, and can be limited with protease inhibitors and metal-ion

chelators such as EDTA and EGTA (Benovic et al., 1983; Strosberg et al., 1984; Strulovici et al., 1984). Boiling of samples in SDS sample buffer prior to SDS PAGE, results in the formation of high molecular weight aggregates, a phenomenon which has been described previously for turkey erythrocyte β_1-adrenergic receptors (Shorr et al, 1982). It is unclear whether this aggregation is a property of native β-adrenergic receptor or crosslinking of the photoaffinity probe. The problem was overcome by denaturing samples at room temperature for 1 h.

Photoaffinity-labelling of the β-adrenergic receptors of rat adipocytes labelled a protein with a molecular weight of 48 kDa. This contrasts with the molecular weight determined for purified radiodinated rat adipocyte β-adrenergic receptors of 67 kDa (Cubero & Malbon, 1984). In addition, this receptor has been shown to migrate on SDS PAGE as a 54 kDa protein under non-reducing conditions, but as a protein of 67 kDa in the presence of high concentrations of the disulphide bridge reducing agents β-mercaptoethanol (10% (v/v)) or dithiothreitol (10 mM) (Moxham & Malbon, 1985). This effect is also seen with S49 mouse lymphoma cells which possess a homogeneous population of β_2-adrenergic receptors (Moxham et al., 1986), and human β_1- and β_2-adrenergic receptors (Bahouth & Malbon, 1987). Radio-iodination is not the cause of this effect since identical results have been obtained using unlabelled purified receptor and immunoblotting techniques (Moxham et al., 1986). This finding calls into question prior speculation that a minor 55 kDa protein observed in purified (Cubero & Malbon, 1984) and photoaffinity-labelled (Stiles et al., 1984b) preparations of β-adrenergic receptor, respectively, represents a proteolytic fragment of the 67 kDa form of the receptor. An equally plausible explanation for the appearance of the

55 kDa form is that of incomplete reduction of intramolecular disulphide bridges in the receptor prior to SDS PAGE. The reasons for the apparent lack of a 67 kDa photoaffinity-labelled protein in this study are unclear, but it might reflect instability of this β-adrenergic receptor under these conditions, or degradation by proteases contained in the collagenase used during adipocyte membrane preparation. Also, the low apparent molecular weight is unlikely to be a result of incomplete reduction of intramolecular disulphide bridges in the β-adrenergic receptor, as high concentrations of β-mercaptoethanol (10% (v/v)) were used.

The frog erythrocyte β_2-adrenergic receptor has a molecular weight of 58 kDa, as identified by photoaffinity-labelling and photoaffinity crosslinking (Lavin et al., 1981, 1982), as well as by purification methods (Shorr et al., 1981). It migrates as a broad band centred around molecular weight 58 kDa on SDS PAGE (Shorr et al., 1981). The reasons for this apparent micro-heterogeneity, also seen with sheep adipose tissue β-adrenergic receptors in this study are not clear, but it may relate to heterogeneity of carbohydrate moieties present on the glycoprotein receptor. [^{125}I]ICYPD-labelling of other mammalian β-adrenergic receptors tends to result in labelling of proteins of molecular weight 62-67 kDa, whereas with avian β-adrenergic receptors e.g. those of turkey, pigeon and duck, two proteins are labelled having molecular weights of 38-40 kDa and 45-50 kDa (for review see Lefkowitz et al., 1983). The sheep adipose tissue β-adrenergic receptor therefore appears to have a molecular weight between these two groups.

After affinity purification of β-adrenergic receptors from sheep subcutaneous adipocytes, only a 58 kDa protein was photoaffinity-

labelled, suggesting that this protein represents the active form of the receptor, since it is the only species able to bind to the antagonist alprenolol on the affinity matrix. The apparent molecular weight of membrane-bound or partially-purified, photoaffinity-labelled β-adrenergic receptors from sheep (wether) subcutaneous adipocytes was unaffected by addition of high concentrations of β-mercaptoethanol (10% (v/v)). It is therefore possible that this β-adrenergic receptor does not contain the disulphide bridges clearly present in other β-adrenergic receptors, including that of rat adipocytes. However, photoaffinity-labelling may stabilise the three-dimensional topography of the β-adrenergic receptor, and render it insensitive to unfolding after disulphide bridge reduction, due to auxillary crosslinking by the photoaffinity probe (Moxham & Malbon, 1985).

The pI of photoaffinity-labelled β-adrenergic receptors from sheep (wether) subcutaneous adipocytes was 6.15-6.25. In contast, IEF of photoaffinity-labelled partially-purified β-adrenergic receptor preparations showed heterogeneity in the form of nine distinct labelled proteins of more acidic pI. This has not been reported for any other β-adrenergic receptor. The isoelectric point of the β_1-adrenergic receptor of turkey erythrocytes has been reported to be 5.5 (Charlton et al., 1980), 5.26 (Fraser, 1984), and 6.0 (Shorr et al., 1982). The rat adipocyte β-adrenergic receptor has a pI of 5.1 (Moxham et al., 1986). The values for β_2-adrenergic receptors have been reported as 3.72 (Soiefer & Venter, 1980) in dog lung, 4.18 in the calf lung (Soiefer & Venter, 1980), and 5.1 in rat liver (Moxham et al., 1986). In most of the observations cited above, peaks of isoelectric-focussed labelled β-adrenergic receptor were broad, again indicating micro-heterogeneity (see

Moxham et al., 1986; Shorr et al., 1981; Venter & Fraser, 1981). The shift in pI observed upon purification of the β-adrenergic receptor from sheep subcutaneous adipocytes may be due to additional protein-protein interaction during IEF, or a function of differences in glycoproteins attached to the receptor. The pI of membrane-bound receptor is higher than any quoted in the literature, and is closest to that of β_1-adrenergic receptor.

With a view to purifying the β-adrenergic receptor by preparative PAGE, sheep adipose tissue membranes were analysed using the benzyldimethyl-n-hexadecylammonium chloride (16-BAC) gel electrophoresis system, described by MacFarlane (1983). This uses the cationic detergent 16-BAC for protein solubilisation, and consists of a discontinuous polyacrylamide gel system operating at pH 4.0-1.5. This particular method was studied since it has both high resolving power and high loading capacity (approximately 100 mg protein). Using a two dimensional system involving SDS PAGE, proteins have been purified from cell homogenates in sufficient amounts for NH_2-terminal sequencing and production of specific polyclonal antisera (MacFarlane, 1989).

16-BAC PAGE of photoaffinity-labelled β-adrenergic receptors from sheep omental adipose tissue produced an indistinct banding pattern with autoradiography. However, when individual gel lanes were sliced and the [^{125}I]ICYPD counted, clearly defined peaks of labelled proteins were obtained. The distribution of labelled proteins was similar to that seen with SDS PAGE. Analysis of sheep adipose tissue membrane proteins using two dimensional 16-BAC/SDS PAGE (MacFarlane, 1989) gave good separation, with the proteins distributed about a diagonal line. This indicates that the separation in both dimensions is related to the molecular weight of

the protein. This decreases the potential resolution, compared to that obtainable with IEF as the first dimension, since separation is based solely on charge (O'Farrell, 1975).

An identical separation using photoaffinity-labelled membrane-bound β-adrenergic receptor revealed separation of several specifically-labelled proteins, but the resolution was poor compared to that obtained using IEF as the first dimensional separation. Therefore it was decided to locate the major specifically-labelled protein in the 16-BAC gel by slicing a 'marker' lane. A horizontal slice containing the major and highest molecular weight protein was cut from the remainder of the gel, and run down an SDS PAGE gel. Using fresh [^{125}I]ICYPD photoaffinity probe, a single labelled protein on the SDS PAGE gel was obtained. It therefore appeared that this method was suitable for preparative isolation of β-adrenergic receptor from sheep adipose tissue, since receptor from 30 mg of sheep adipose tissue membrane protein could be 'concentrated' into the final SDS PAGE gel. However, quantification of β-adrenergic receptor proved to be impossible. As with SDS PAGE, most of the [^{125}I]ICYPD counts loaded were non-covalently bound, confounding any correlation between recovery of [^{125}I]ICYPD and β-adrenergic receptor. This is most likely attributable to the high affinity of [^{125}I]ICYPD for β-adrenergic receptors (K_d 30-50 pM). Indeed it has been possible to subject both [^{125}I]ICYP-labelled turkey β_1-adrenergic receptors (Shorr et al., 1982) and [^{125}I]hydroxybenzylpindolol-labelled frog β_2-adrenergic receptors (Shorr et al., 1981) to IEF, even though both ligands bind non-covalently. TCA and acetone precipitation were employed to reduce non-covalently bound counts, but without success. With no quantification of

β-adrenergic receptor possible, it was decided to use affinity chromatography for the purification of receptor.

Table 3.1. Affinity chromatography of the β-adrenergic receptors of sheep adipose tissue: analysis of column fractions

Digitonin-solubilised β-adrenergic receptors (22.3 mg protein) prepared from sheep (wether) omental adipose tissue were subjected to affinity chromatography on a 10 ml Sepharose 4B-alprenolol column. (-)-Isoproterenol was added after fraction 8 to elute the β-adrenergic receptors. SSATM - solubilised sheep adipose tissue membranes. N/D - none detected.

Fraction	Volume (ml)	Protein (mg/ml)	[^{3}H]DHA binding (fmol/mg protein)	[^{3}H]DHA binding (fmol)
SSATM	35.3	0.632	155.4	3465.4
1	10	0.039	36.43	14.21
2	10	0.670	34.24	229.41
3	10	0.723	28.87	208.73
4	10	0.713	21.74	155.01
5	10	0.183	31.97	58.50
6	10	0.012	N/D	N/D
7	10	N/D	N/D	N/D
8	6	N/D	N/D	N/D
9	4	0.0088	N/D	N/D
10	4	0.0082	N/D	N/D
11	4	0.0116	16362.7	759.2
12	4	0.0097	8528.6	330.9
13	4	0.0063	6224.5	156.9
14	4	0.0045	4922.0	88.6
15	4	0.0041	3601.1	59.1
16	4	0.0036	2289.0	33.0
17	4	0.0031	3186.0	39.5
18	4	0.0027	2487.6	26.9

Table 3.2. Affinity chromatography of the β-adrenergic receptors of sheep adipose tissue: analysis of column fractions from third purification

Digitonin-solubilised β-adrenergic receptors (24.7 mg protein) from sheep (wether) omental adipose tissue were subjected to affinity chromatography on a Sepharose 4B-alprenolol column. The data represents the third purification performed using a newly-synthesised 10 ml column. The yield of β-adrenergic receptors was 65% and the purification achieved was 304 fold. Only the initial preparation and fractions 11-14 were assayed. SSATM - solubilised sheep adipose tissue membranes

Fraction	Volume (ml)	Protein (mg/ml)	[^{3}H]DHA binding (fmol/mg protein)	[^{3}H]DHA binding (fmol)
SSATM	34.26	0.721	114.6	2831.4
11	4.0	0.0021	34858.6	812.2
12	4.0	0.0017	24598.4	509.2
13	4.0	0.0017	17571.7	363.7
14	4.0	0.0013	8008.0	143.3

Fig. 3.1. Affinity chromatography of the β-adrenergic receptors of sheep adipose tissue: Elution profile of protein

Digitonin-solubilised β-adrenergic receptors (22.3 mg membrane protein) prepared from sheep (wether) omental adipose tissue were subjected to affinity chromatography using a 10 ml Sepharose 4B-alprenolol column. Fractions 1-4 (10 ml) - eluate during sample loading: fractions 5-8 (10 ml) - eluate during column washing: fractions 9-18 (4 ml) - eluate during elution of β-adrenergic receptors with 1 mM (-)-isoproterenol (values in parentheses indictate volume of each fraction). This data is tabulated in Table 3.1. Protein retention on the column was <5% of that loaded.

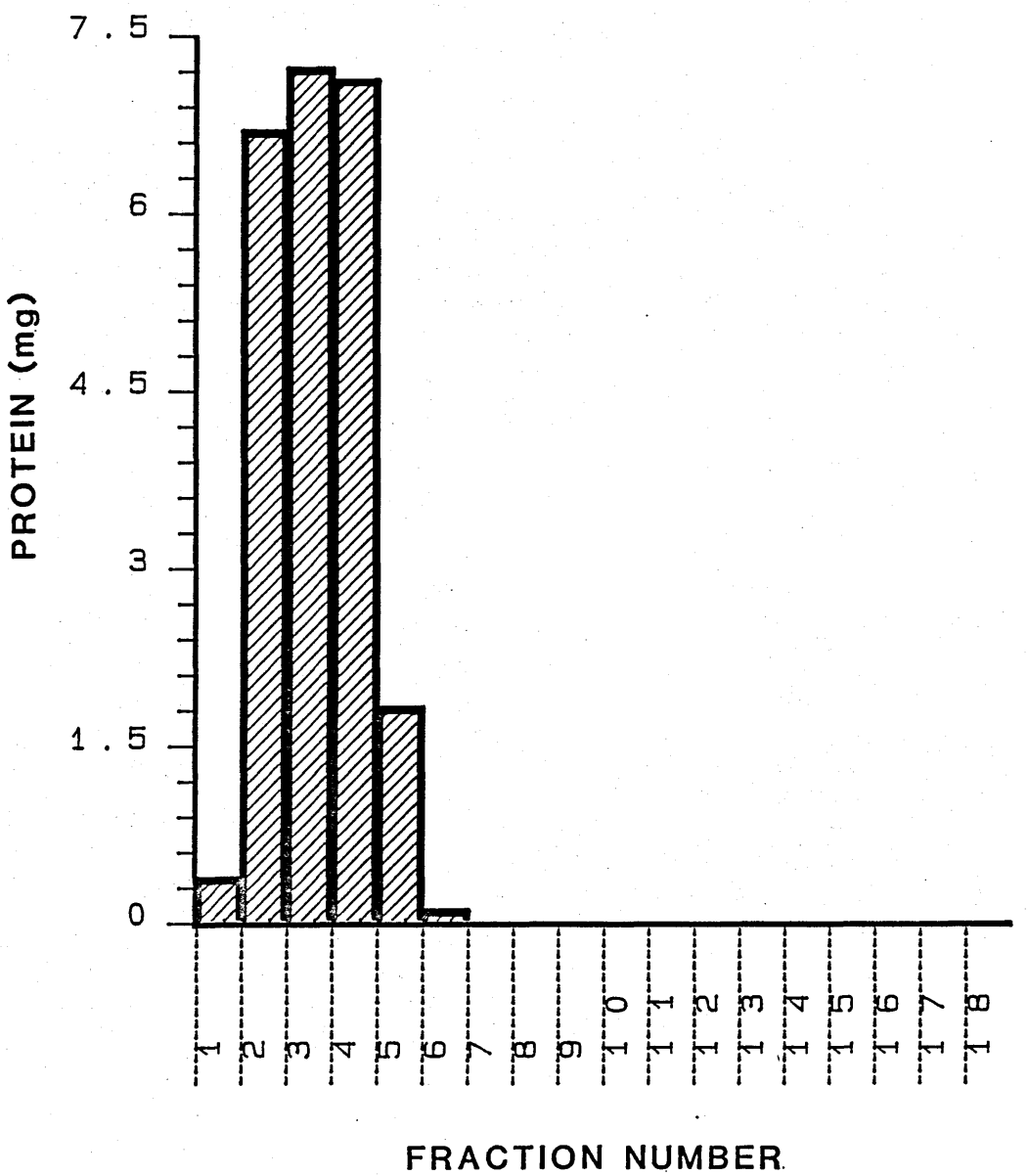

7.5
6
4.5
3
1.5
0
PROTEIN (mg)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
FRACTION NUMBER

Fig. 3.2. Affinity chromatography of the β-adrenergic receptors of sheep adipose tissue: Elution profile of β-adrenergic receptors

Digitonin-solubilised β-adrenergic receptors (22.3 mg membrane protein) prepared from sheep (wether) omental adipose tissue were subjected to affinity chromatography using a 10 ml Sepharose 4B-alprenolol column. Fractions 1-4 (10 ml) - eluate during sample loading: fractions 5-8 (10 ml) - eluate during column washing: fractions 9-18 (4 ml) - eluate during elution of β-adrenergic receptors with 1 mM (-)-isoproterenol (values in parenthesis indictate volume of each fraction). β-Adrenergic receptors in each fraction were quantified with [^{3}H]DHA. Fractions 9-18 were desalted prior to assay, in order to remove the (-)-isoproterenol used to elute the β-adrenergic receptors. This data is tabulated in Table 3.1. The yield of β-adrenergic receptors was 43% and the purification achieved was 105 fold. Approximately 95% of the protein loaded passed directly through the column.

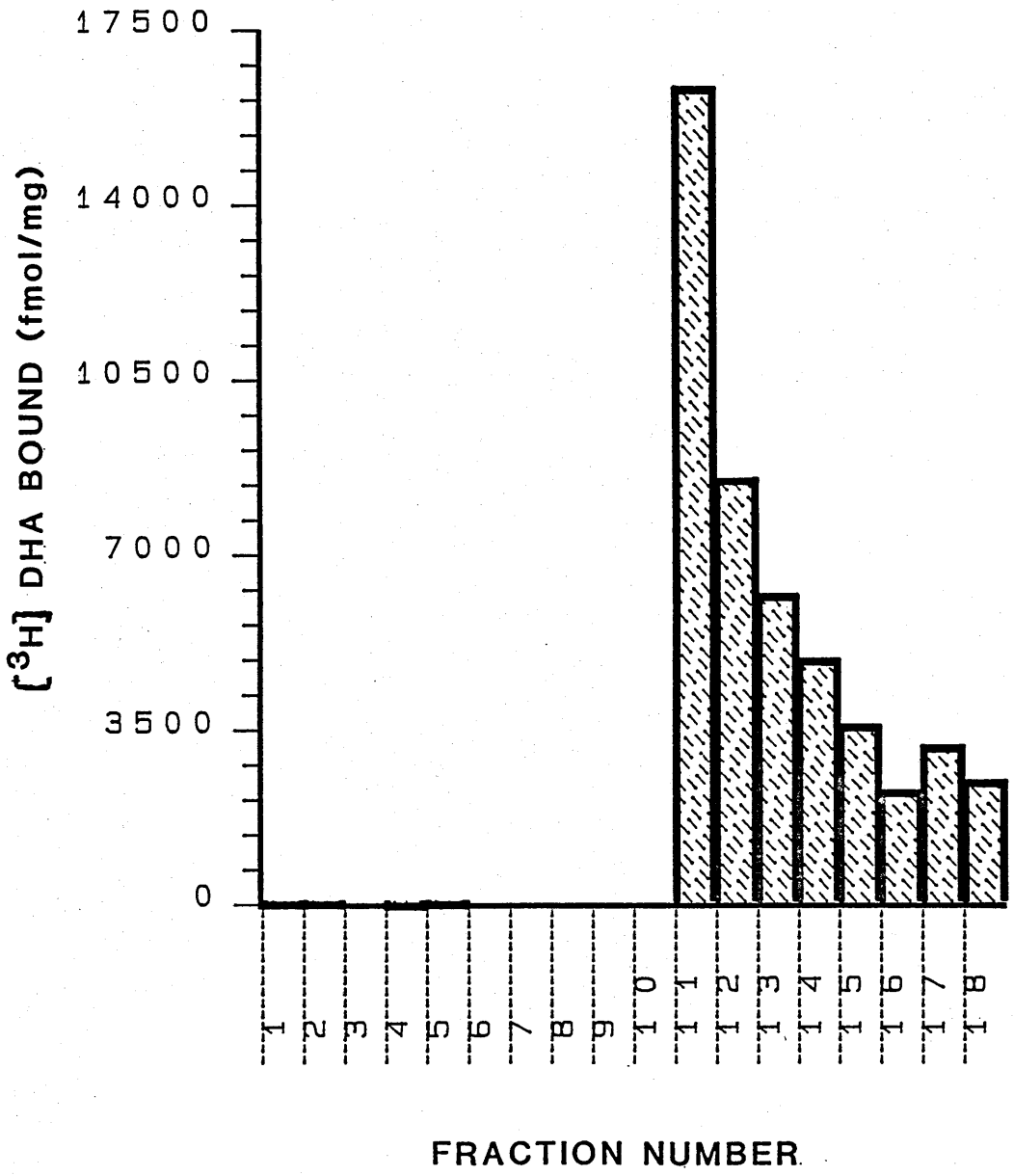

17500
14000
10500
7000
3500
0
[3H] DHA BOUND (fmol/mg)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
FRACTION NUMBER

Fig. 3.3. SDS PAGE of photoaffinity-labelled β-adrenergic receptors of sheep adipocyte membranes

Adipocyte β-adrenergic receptors (335 μg protein) prepared from sheep (wether) subcutaneous adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD in the absence (●----●) or presence (o----o) of 10 μM (-)-alprenolol, and subjected to SDS PAGE. The gel was stained, dried, subjected to autoradiography, and finally sliced (2 mm slices) and counted. [^{125}I]ICYPD radioactivity in samples prior to SDS PAGE was 83,000 cpm and 37,500 cpm in the absence and presence of 10 μM (-)-alprenolol, respectively. Slice No. 1 represents the top of the resolving gel.

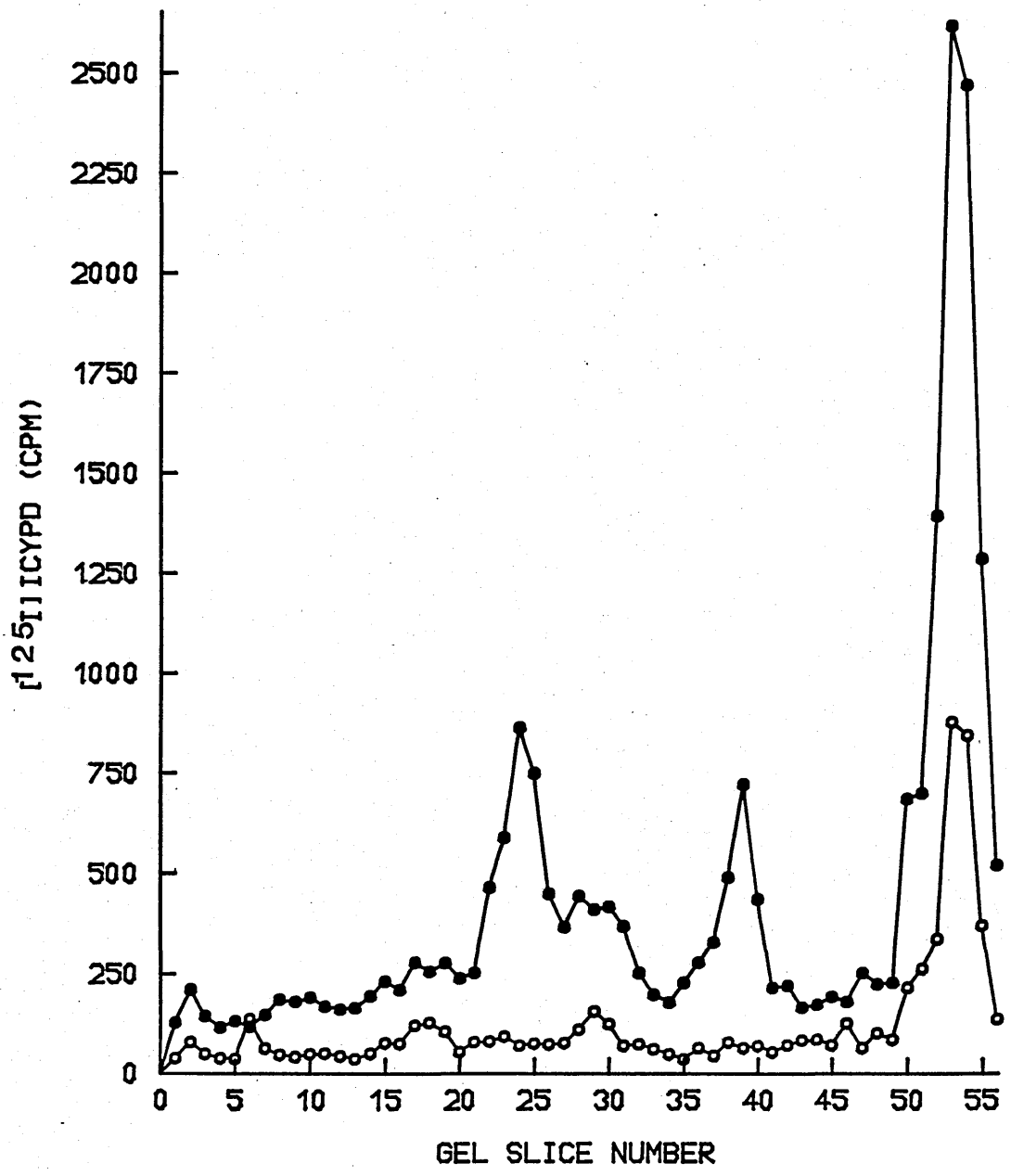

[125I]ICYPD (CPM)
2500
2250
2000
1750
1500
1250
1000
750
500
250
0
0
5
10
15
20
25
30
35
40
45
50
55
GEL SLICE NUMBER

Fig. 3.4. Autoradiograph of SDS PAGE of photoaffinity-labelled β-adrenergic receptors of sheep adipocyte membranes

Adipocyte β-adrenergic receptors (335 μg protein) prepared from sheep (wether) subcutaneous adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD and subjected to SDS PAGE. Non-specific photoaffinity-labelling was determined in the presence of 10 μM (-)-alprenolol (Lanes 2 and 4). Two samples were boiled for 3 min prior to electrophoresis (Lanes 1 and 2). The exposure time for the autoradiograph was 42.5 h. The arrow indicates the direction of electrophoresis. Molecular weights for markers are given in kDa.

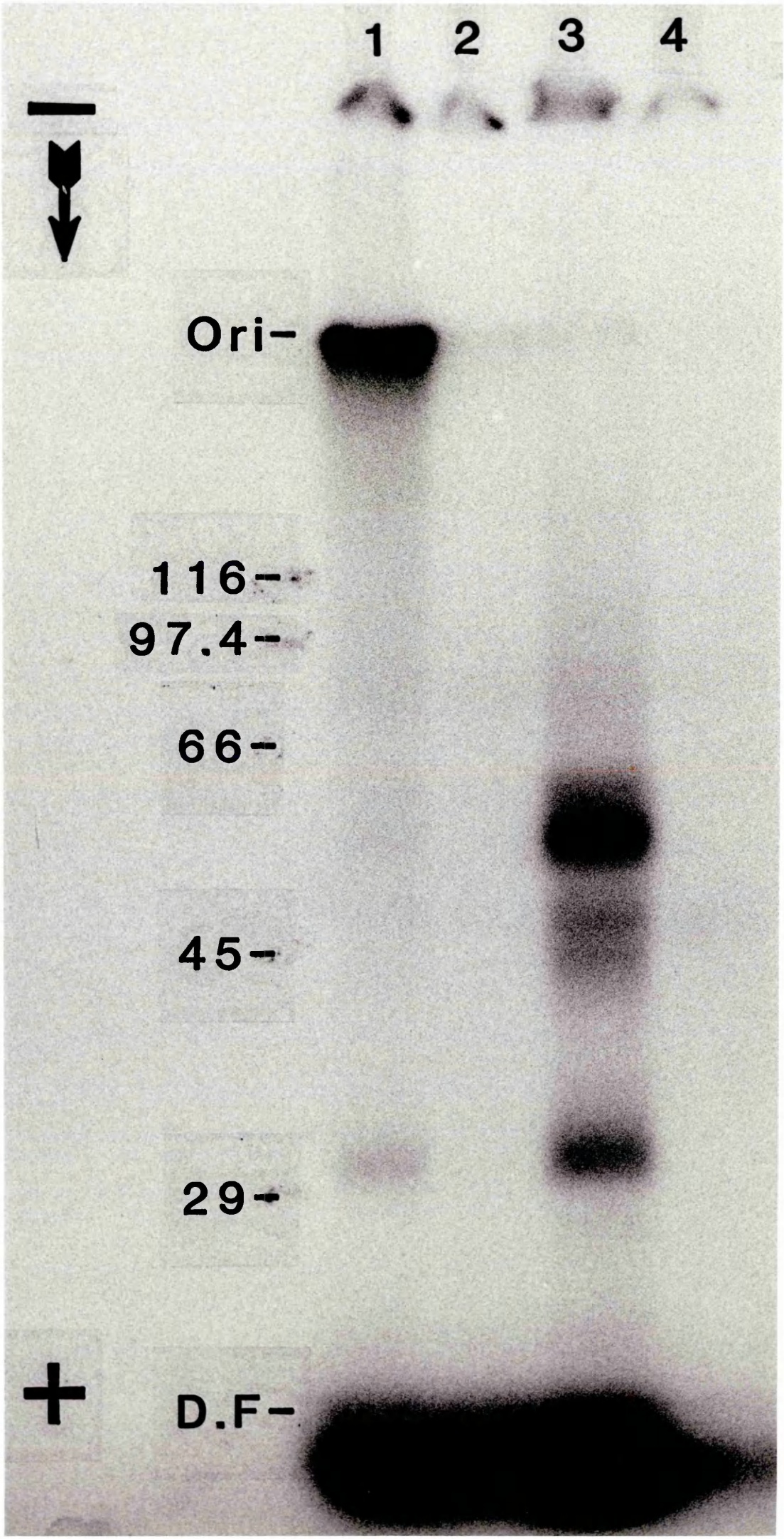

1
2
3
4
Ori-
116-
97.4-
66-
45-
29-
D.F-

Fig. 3.5. Autoradiograph of SDS PAGE of photoaffinity-labelled partially purified β-adrenergic receptors of sheep adipocyte membranes

Partially purified adipocyte β-adrenergic receptors prepared from sheep (wether) subcutaneous adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD, lyophilised and subjected to SDS PAGE. Non-specific photoaffinity-labelling was determined in the presence of 10 μM (-)-alprenolol (Lane 2). Additions were made to some samples prior to electrophoresis: Lanes 1 and 2 - none; lane 3 - 10% (v/v) β-mercaptoethanol; lane 4 - 10 mM dithiothreitol; lane 5 - 10 mM N-ethylmaleimide. The exposure time for the autoradiograph was 7 days. The arrow indicates the direction of electrophoresis. Molecular weights for markers are given in kDa.

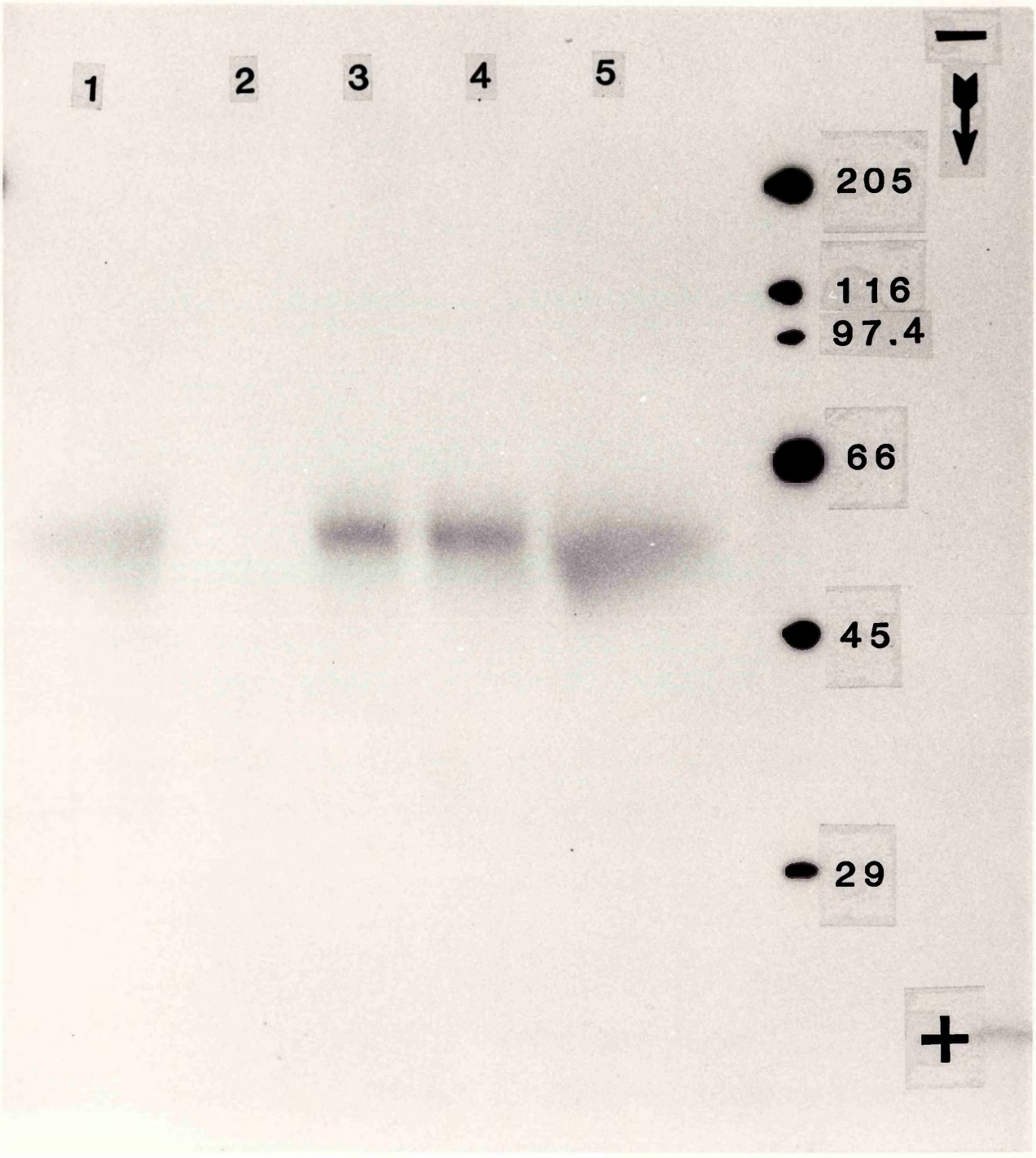

1
2
3
4
5
205
116
97.4
66
45
29
−
+

Fig. 3.6. Autoradiograph of SDS PAGE of photoaffinity-labelled β-adrenergic receptors of sheep and rat adipose tissue

β-Adrenergic receptors (200 μg membrane protein) from sheep (lactating ewe) omental adipose tissue and rat abdominal adipocyte membranes were photoaffinity-labelled with [^{125}I]ICYPD and subjected to SDS PAGE. Non-specific photoaffinity-labelling was determined in the presence of 100 μM (-)-isoproterenol (Lanes 2 and 4). Lanes 1 and 2 - sheep adipose tissue membranes. Lanes 3 and 4 - rat adipocyte membranes. The exposure time for the autoradiograph was 7 days. The arrow indicates the direction of electrophoresis. Molecular weights for markers are given in kDa.

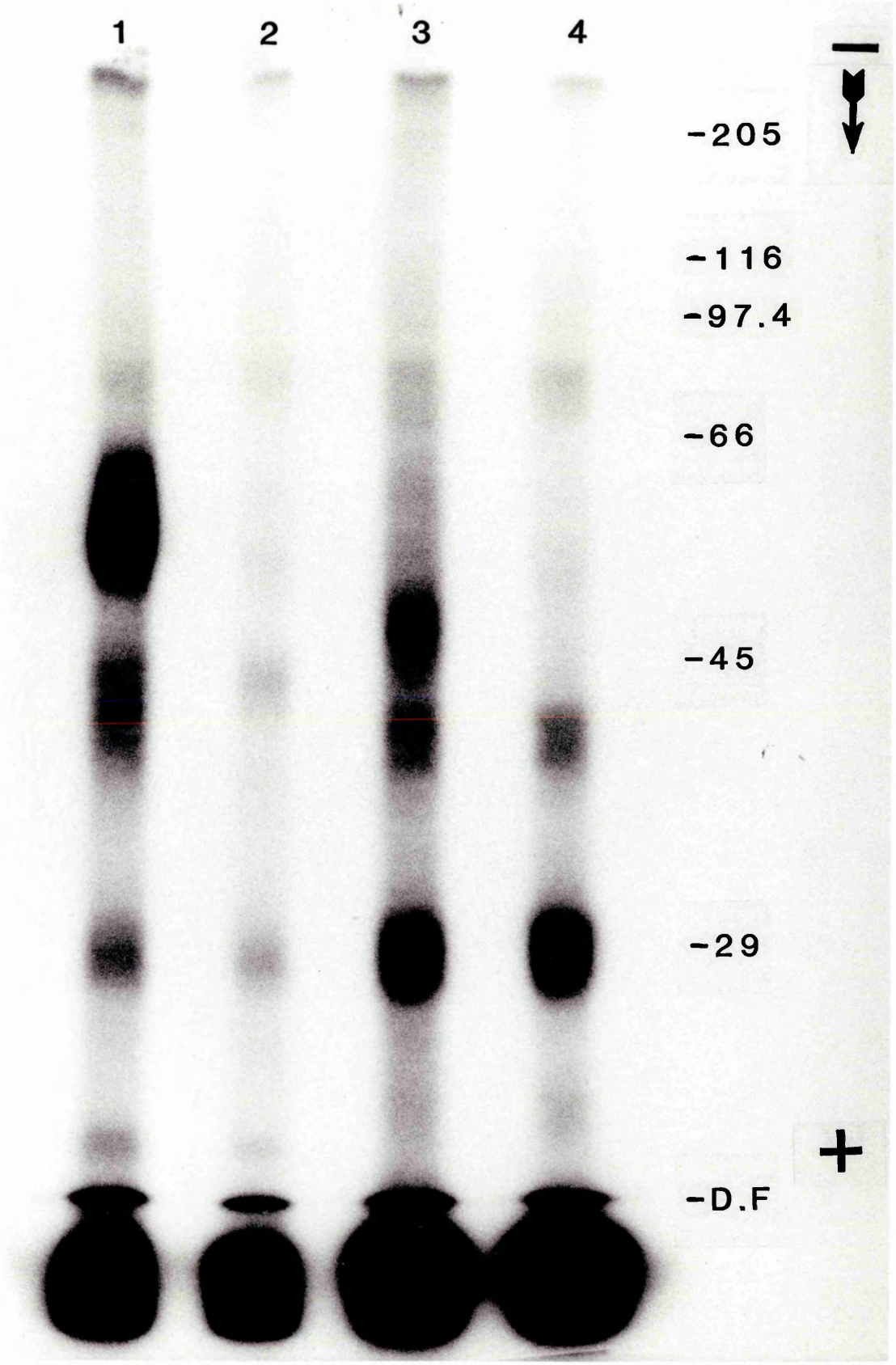
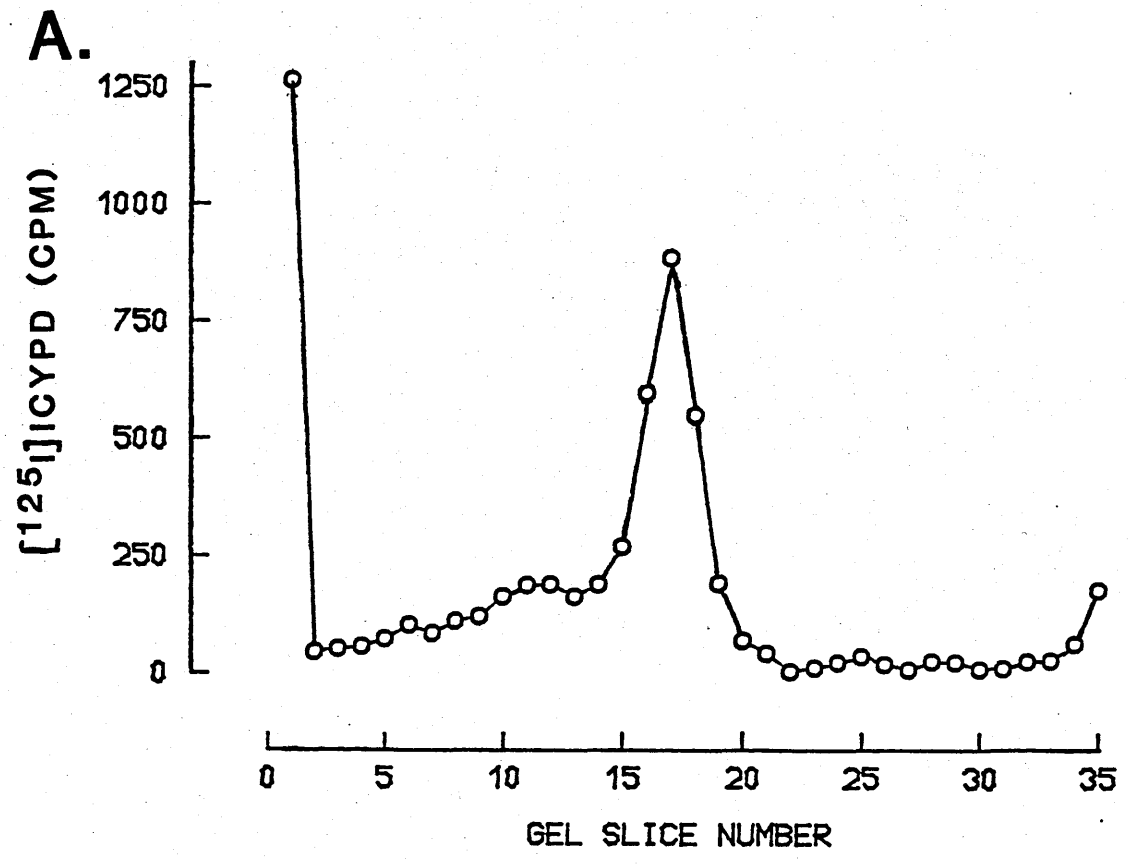

1
2
3
4
-205
-116
-97.4
-66
-45
-29
-D.F

Fig. 3.7. Isoelectric focussing of sheep adipocyte β-adrenergic receptors photoaffinity-labelled in the presence or absence of 10 μM (-)-alprenolol

Adipocyte β-adrenergic receptors (100 μg membrane protein) from sheep (wether) subcutaneous adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD, in the absence (A) or presence (B) of 10 μM (-)-alprenolol, and subjected to IEF (ampholyte pH range - 3-10). Gels were sliced (2 mm slices) and counted. Slice No. 1 represents the acidic (anode) end of the IEF gel.


A.
[125I]ICYPD (CPM)
1250
1000
750
500
250
0
0
5
10
15
20
25
30
35
GEL SLICE NUMBER

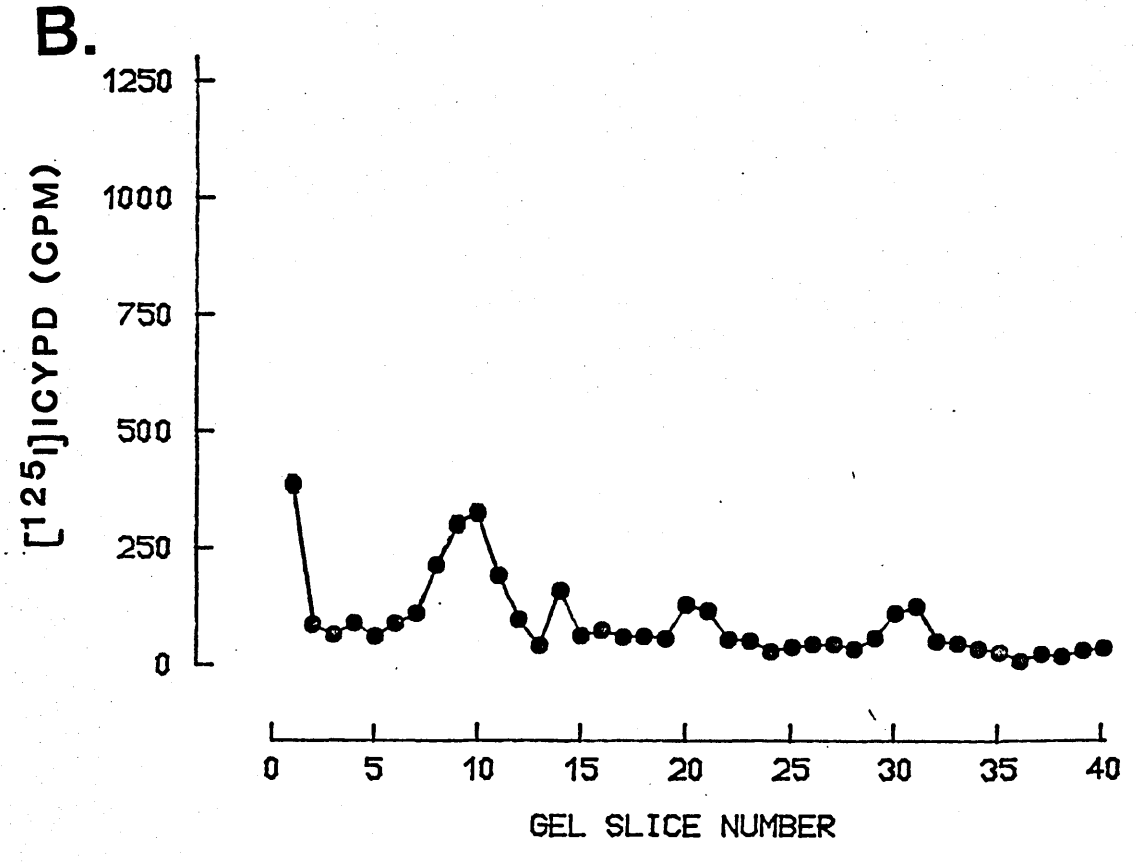
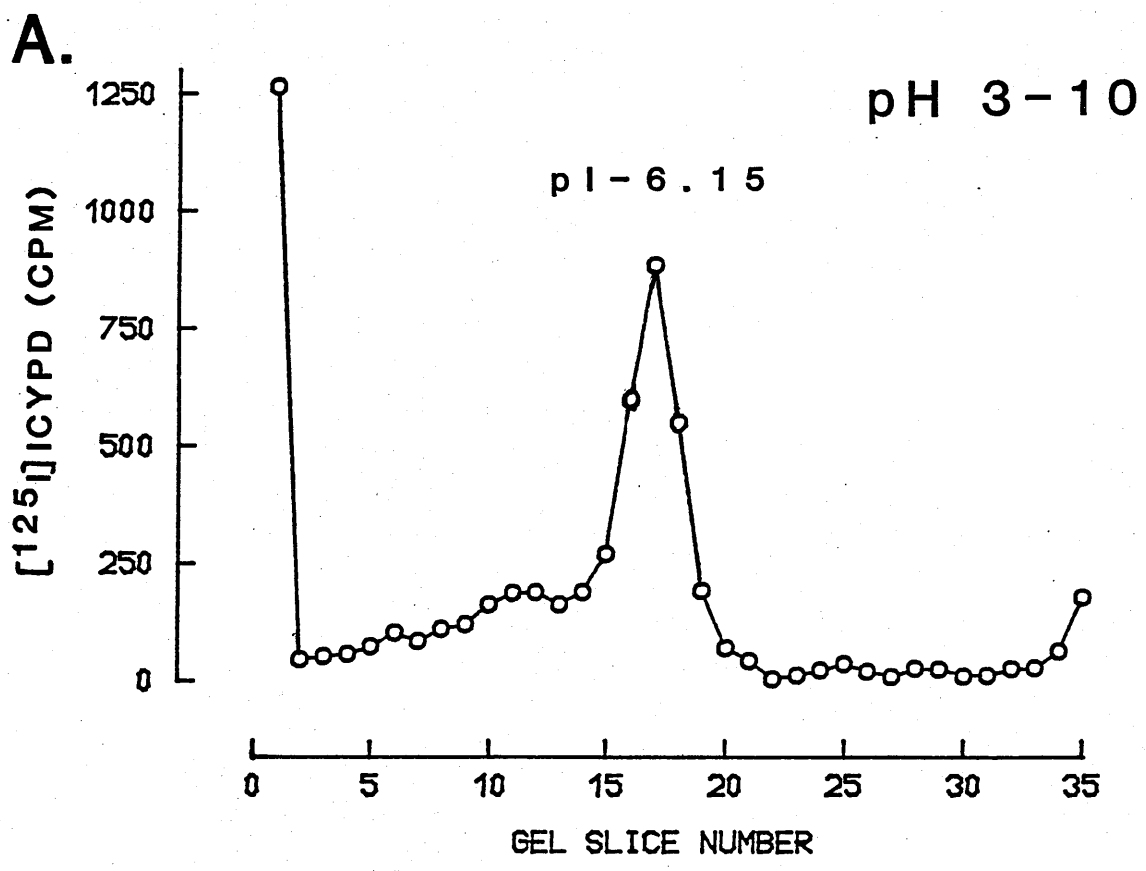

B.
[125I]ICYPD (CPM)
1250
1000
750
500
250
0
0
5
10
15
20
25
30
35
40
GEL SLICE NUMBER

Fig. 3.8. Isoelectric focussing of photoaffinity-labelled β-adrenergic receptors of sheep adipocytes

Adipocyte β-adrenergic receptors (100 μg membrane protein) from sheep (wether) subcutaneous adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD and subjected to IEF. Gels were sliced (2 mm slices), and counted for localisation of [^{125}I]ICYPD. In A, ampholyte pH range = 3-10; 100 μg protein. In B, ampholyte pH range = 5-8; 50 μg protein. Slice No. 1 represents the acidic (anode) end of the IEF gel.


A.
pH 3-10
pI-6.15
[125I]ICYPD (CPM)
1250
1000
750
500
250
0
0
5
10
15
20
25
30
35
GEL SLICE NUMBER

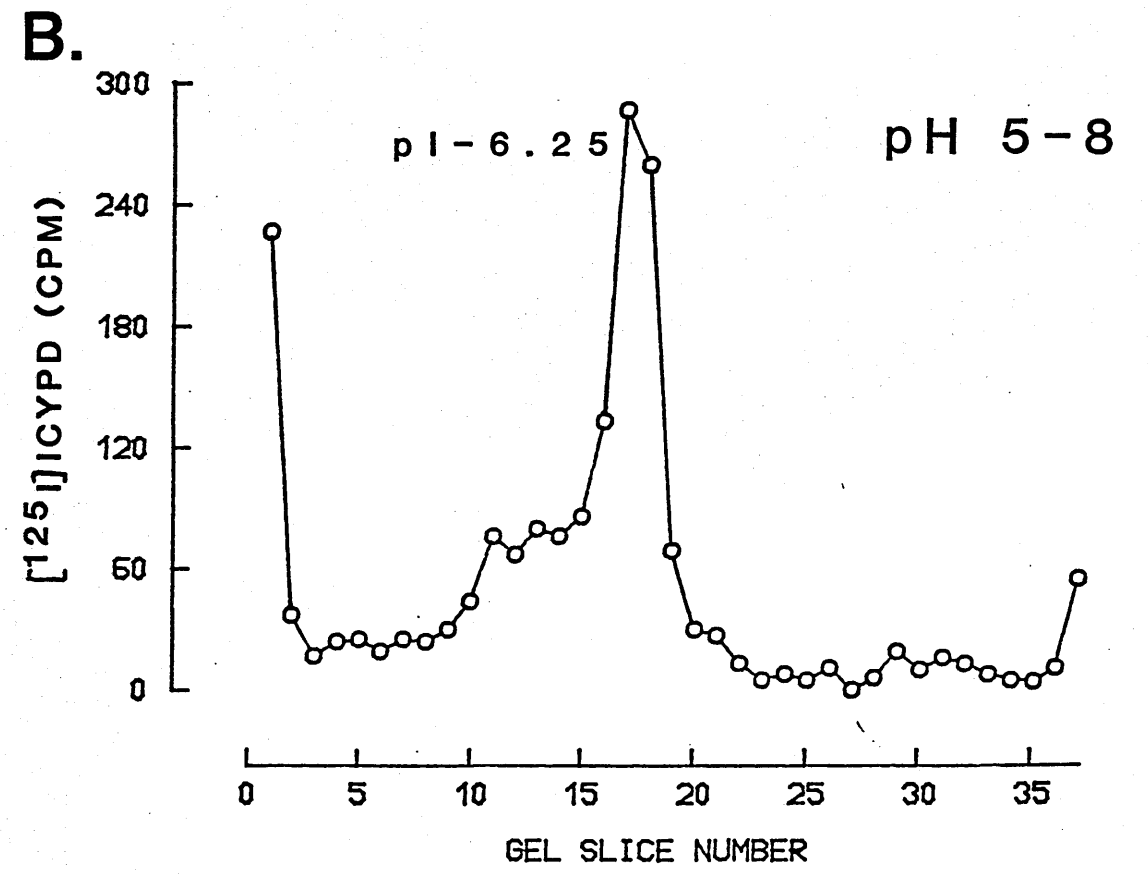

B.
pH 5-8
pI-6.25
[125I]ICYPD (CPM)
300
240
180
120
60
0
0
5
10
15
20
25
30
35
GEL SLICE NUMBER

Fig. 3.9. Isoelectric focussing of photoaffinity-labelled partially purified β-adrenergic receptors of sheep adipocytes

β-Adrenergic receptors were partially purified from sheep (wether) subcutaneous adipocytes by affinity chromatography, and photoaffinity-labelled with [^{125}I]ICYPD. The samples were lyophilised and subjected to IEF. Gels were sliced (2 mm slices), and counted. In A, ampholyte pH range = 3-10; In B, ampholyte pH range = 5-8. Slice No. 1 represents the acidic (anode) end of the IEF gel.

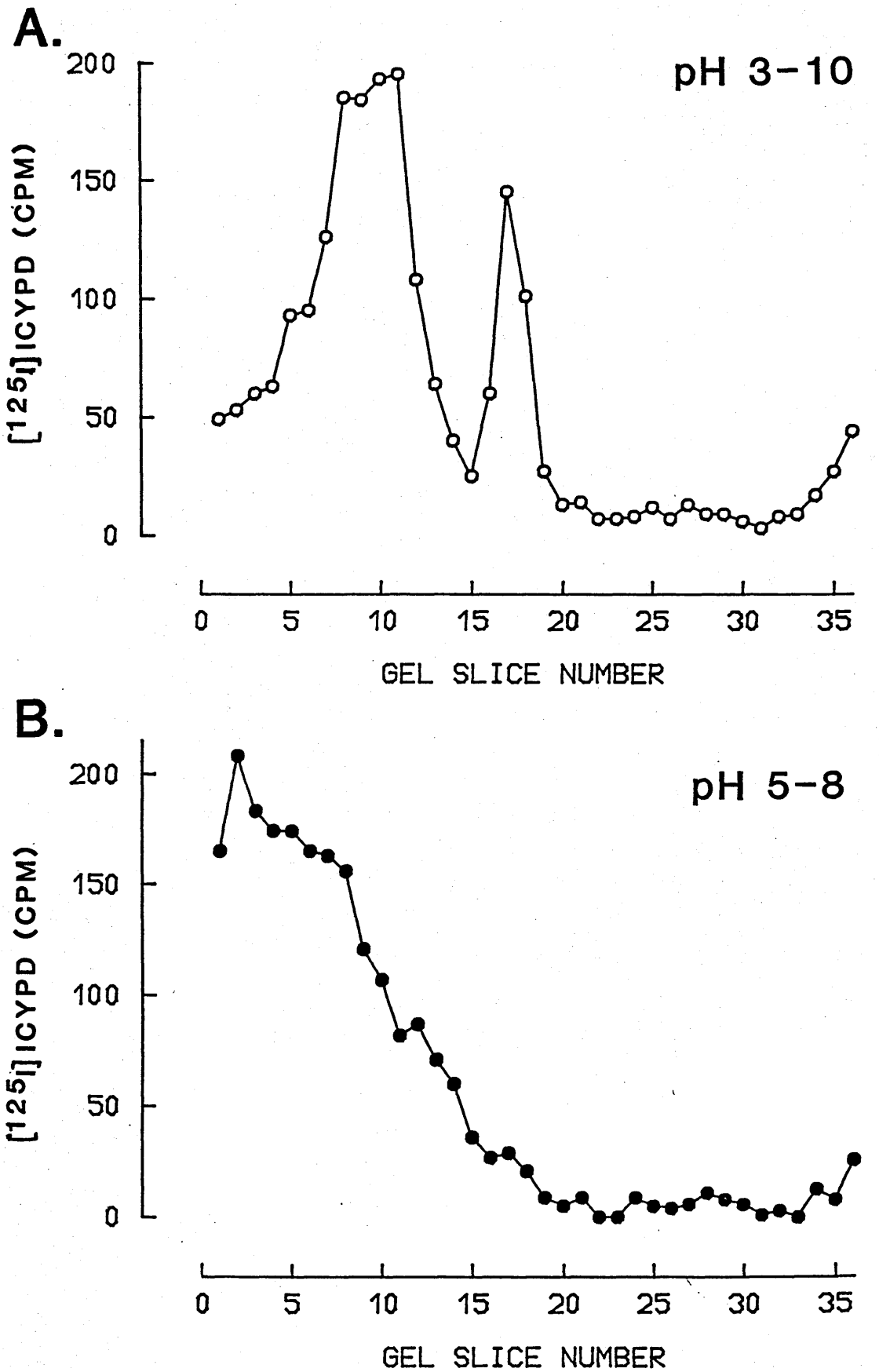

A.
pH 3-10
[125I]ICYPD (CPM)
200
150
100
50
0
0
5
10
15
20
25
30
35
GEL SLICE NUMBER
B.
pH 5-8
[125I]ICYPD (CPM)
200
150
100
50
0
0
5
10
15
20
25
30
35
GEL SLICE NUMBER

Fig. 3.10. Autoradiograph of two-dimensional PAGE of photoaffinity-labelled partially purified β-adrenergic receptors of sheep adipocytes

1st dimension - IEF. 2nd dimension - SDS PAGE. Partially purified β-adrenergic receptors prepared from sheep (wether) subcutaneous adipocytes were photoaffinity-labelled with [^{125}I]ICYPD. The sample was resolved by IEF using a pH gradient of 3-10, and subsequently separated in a second dimension by SDS PAGE. The exposure time for the autoradiograph was 20 days. The arrows indicate the direction of electrophoresis for each dimension. Molecular weights for markers are given in kDa.

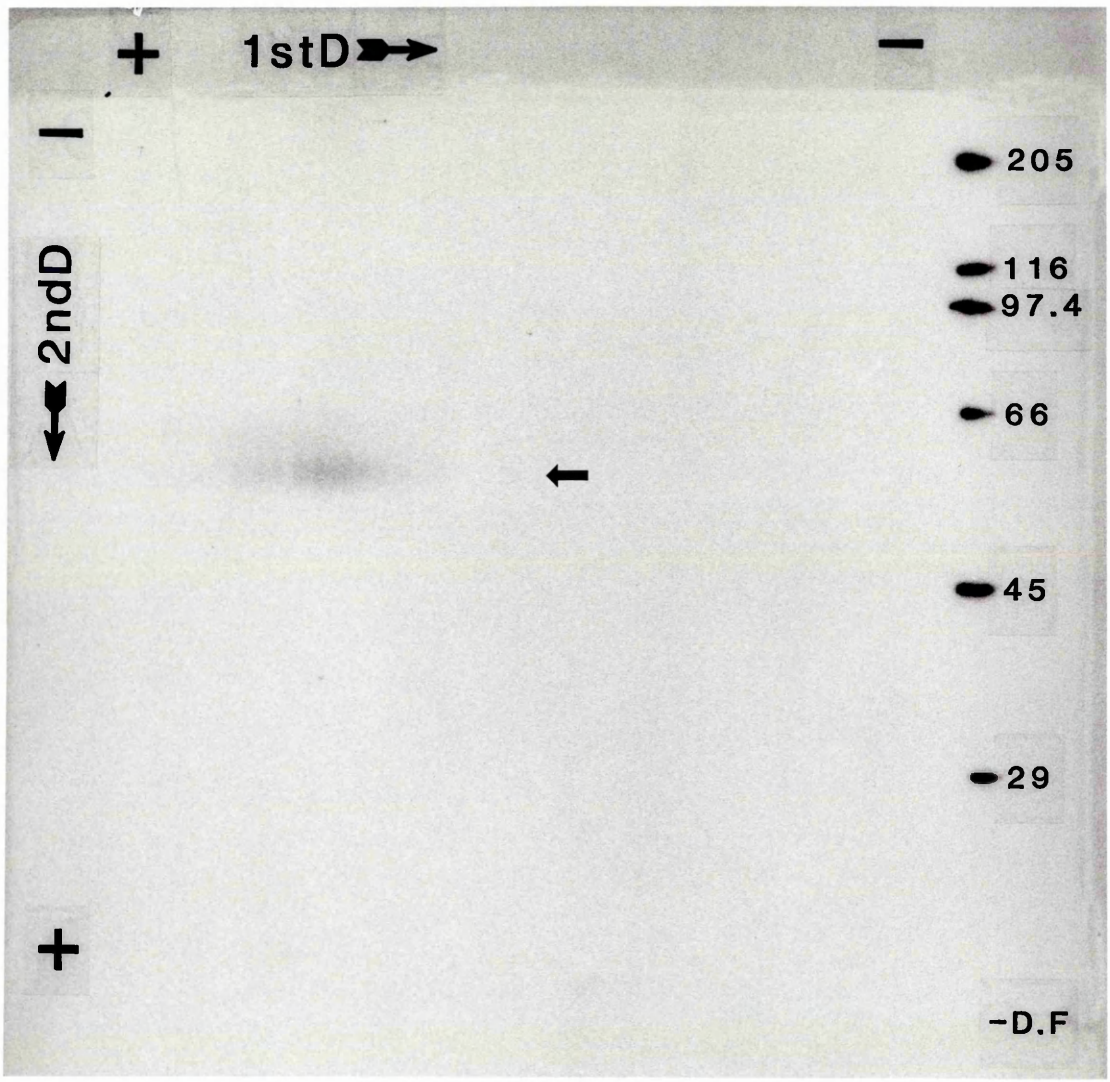
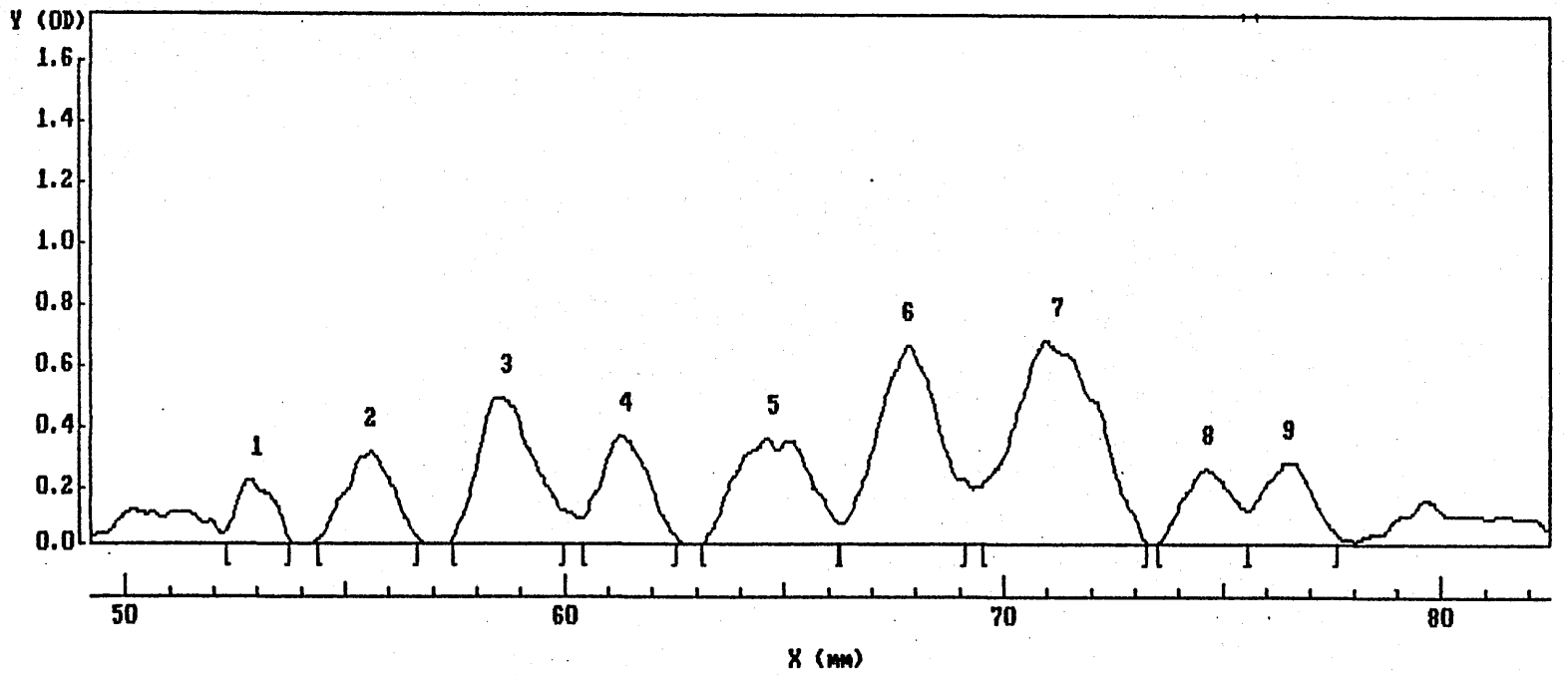

+
1stD
−
−
2ndD
205
116
97.4
66
45
29
+
-D.F

Fig. 3.11. Densitometric scan of the autoradiograph showing 2D-PAGE of photoaffinity-labelled partially-purified β-adrenergic receptors of sheep adipocytes

The autoradiograph shown in Figure 3.10 was scanned using a Bio-Rad video densitometer, parallel to the direction of SDS PAGE at an estimated molecular weight of 58 kDa. The proteins labelled with [^{125}I]ICYPD at the acidic end of the gel were seen as nine distinct peaks. Below the scan is a table of parameters, calculated for each peak.



PEAK No.	POSITION mm	mm FROM ORIGIN	RELATIVE MOBILITY	HEIGHT O.D.	WIDTH mm	AREA OD.mm	REL. AREA % TOTAL	% SELECTED
1	53.0	3.8	0.11	0.210	0.97	0.205	3.10	3.52
2	55.6	6.4	0.19	0.310	1.27	0.393	5.93	6.75
3	58.8	9.6	0.29	0.480	1.51	0.728	10.98	12.49
4	61.4	12.2	0.37	0.360	1.27	0.461	6.95	7.91
5	64.8	15.6	0.47	0.350	2.02	0.712	10.74	12.22
6	67.9	18.7	0.56	0.660	1.66	1.095	16.53	18.79
7	71.3	22.1	0.66	0.670	2.28	1.529	23.07	26.23
8	74.8	25.6	0.77	0.260	1.27	0.332	5.01	5.70
9	76.6	27.4	0.82	0.270	1.37	0.372	5.62	6.39

Fig. 3.12. Autoradiograph of 16-BAC PAGE of photoaffinity-labelled β-adrenergic receptors of sheep adipose tissue

β-Adrenergic receptors from sheep (lactating ewe) omental adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD and subjected to 16-BAC PAGE. Non-specific photoaffinity-labelling was determined in the presence of 100 μM (-)-isoproterenol (Lanes 3 and 4). Protein loadings were; lanes 1 and 3 - 200 μg membrane protein, lanes 2 and 4 - 100 μg membrane protein. The exposure time for the autoradiograph was 14 days. The arrow indicates the direction of electrophoresis.

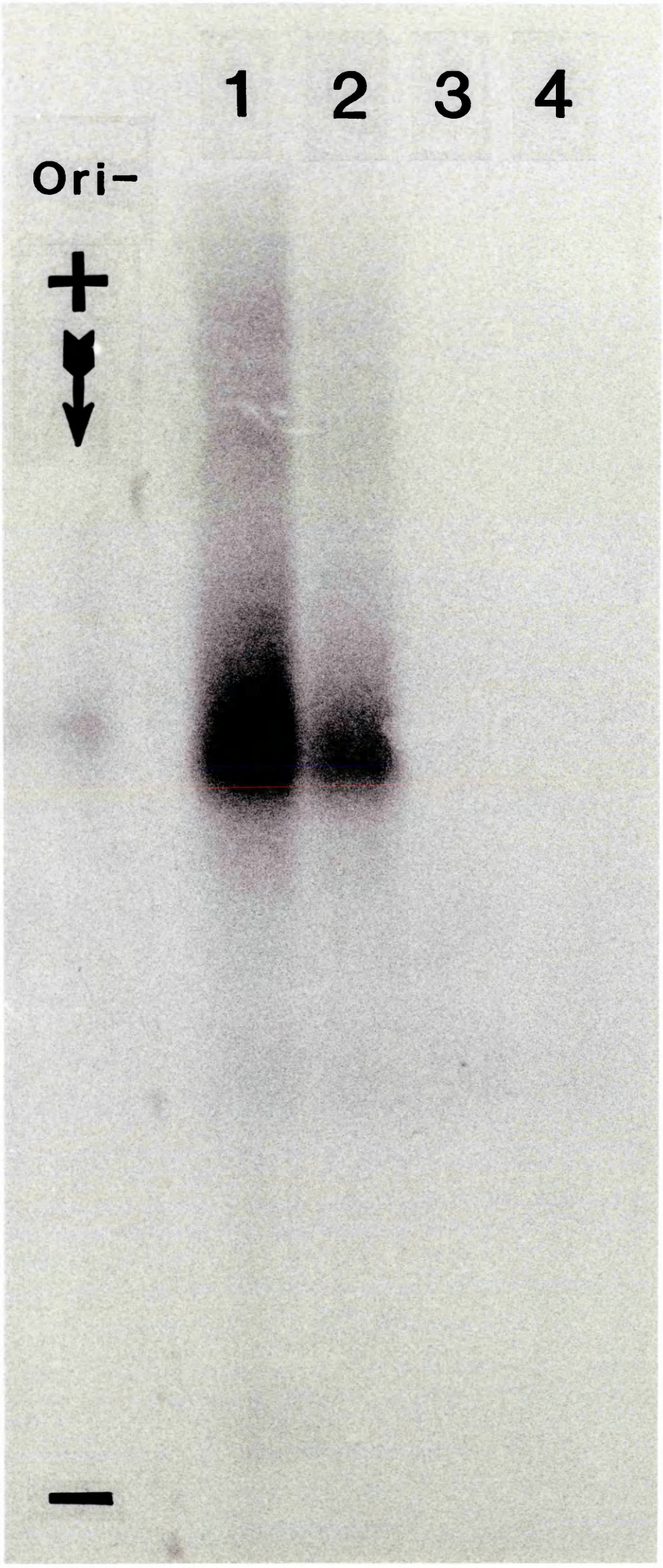

1 2 3 4
Ori-
+

Fig. 3.13. 16-BAC PAGE of photoaffinity-labelled β-adrenergic receptors of sheep adipose tissue

β-Adrenergic receptors (1 mg membrane protein) from sheep (lactating ewe) omental adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD and subjected to 16-BAC PAGE overnight at 30 mA. The gel was stained with Coomassie blue, sliced (2 mm slices) and counted. Slice No. 1 represents the top of the resolving gel.

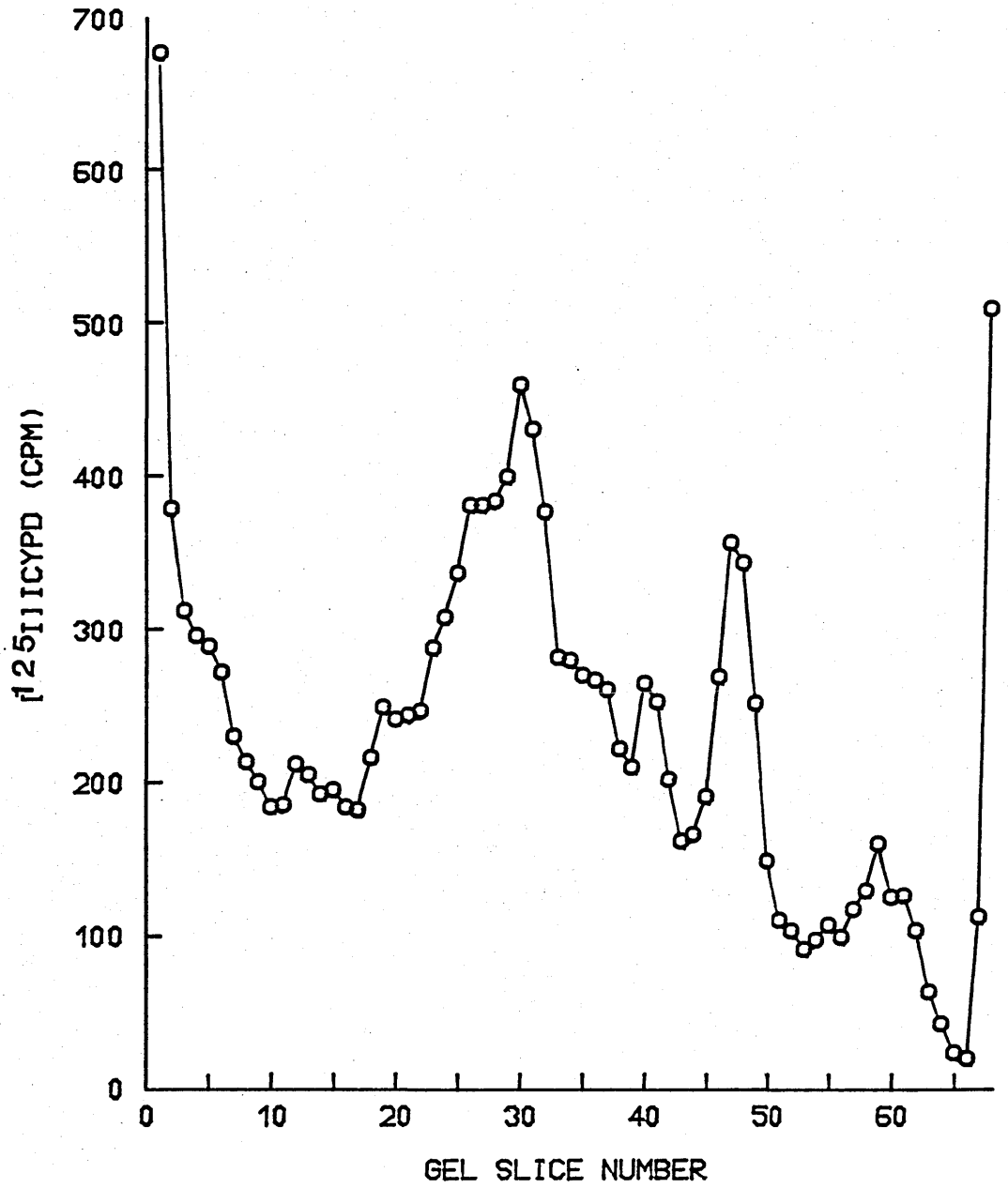

700
600
500
400
300
200
100
0
[125I]ICYPD (CPM)
0
10
20
30
40
50
60
GEL SLICE NUMBER

Fig. 3.14. Two dimensional 16-BAC/SDS PAGE of sheep adipose tissue membrane proteins

1st dimension - 16-BAC PAGE. 2nd dimension - SDS PAGE. Sheep (lactating ewe) omental adipose tissue membrane proteins (100 µg protein) were separated by 16-BAC PAGE, and further resolved in a second dimension using SDS-PAGE. Proteins were localised by Coomassie blue staining. The arrows indicate the direction of electrophoresis in each dimension.

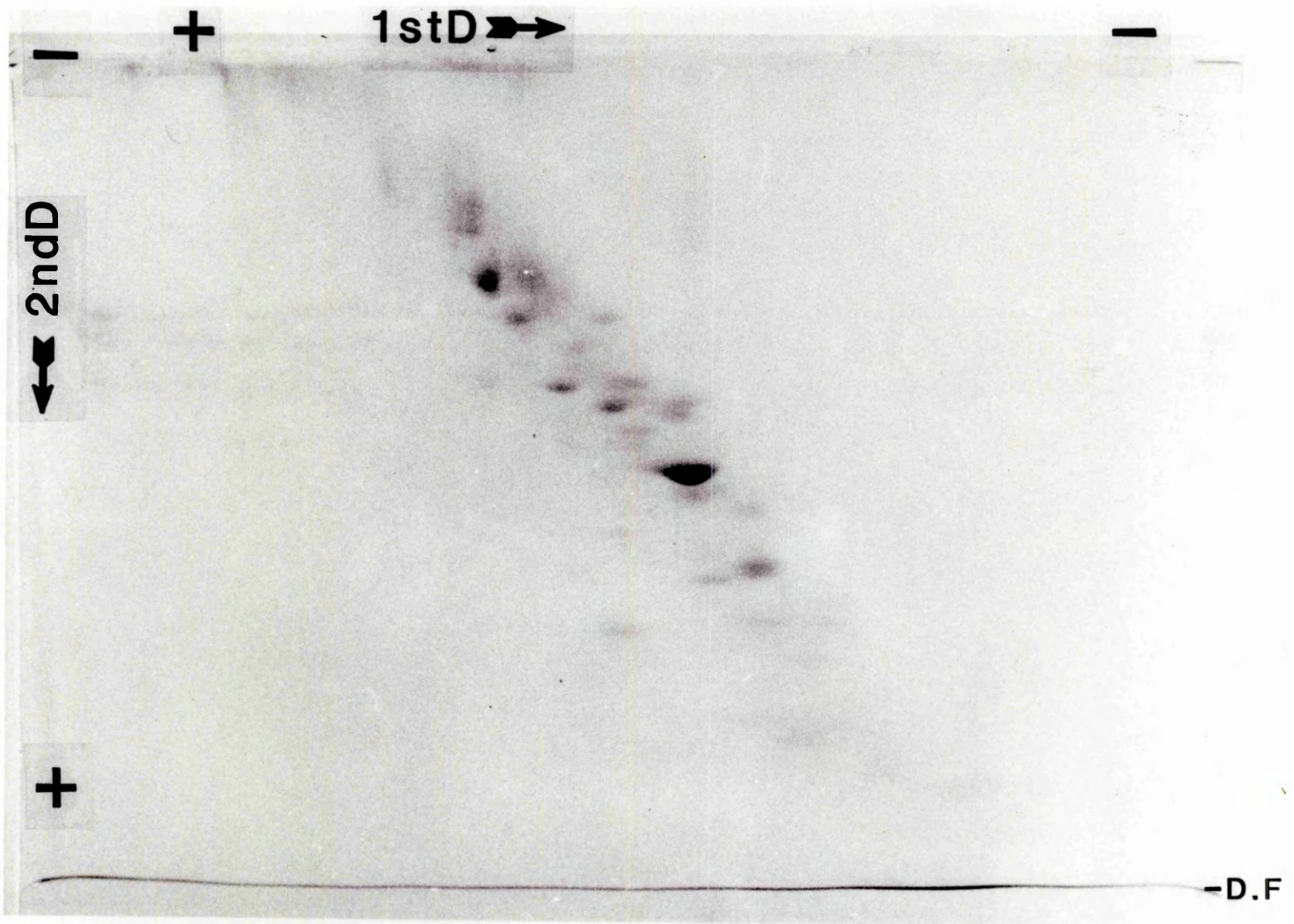

1stD
2ndD
D.F

Fig. 3.15. Autoradiograph of two dimensional 16-BAC/SDS PAGE of photoaffinity-labelled β-adrenergic receptors of sheep adipose tissue

1st dimension - 16-BAC PAGE. 2nd dimension - SDS PAGE. β-Adrenergic receptors (400 μg membrane protein) from sheep (lactating ewe) omental adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD and subjected to 16-BAC PAGE. Samples were further resolved in a second dimension using SDS PAGE. The exposure time for the autoradiograph was 3 weeks. The arrows indicate the direction of electrophoresis in each dimension.

+
1stD
−
−
2ndD
+

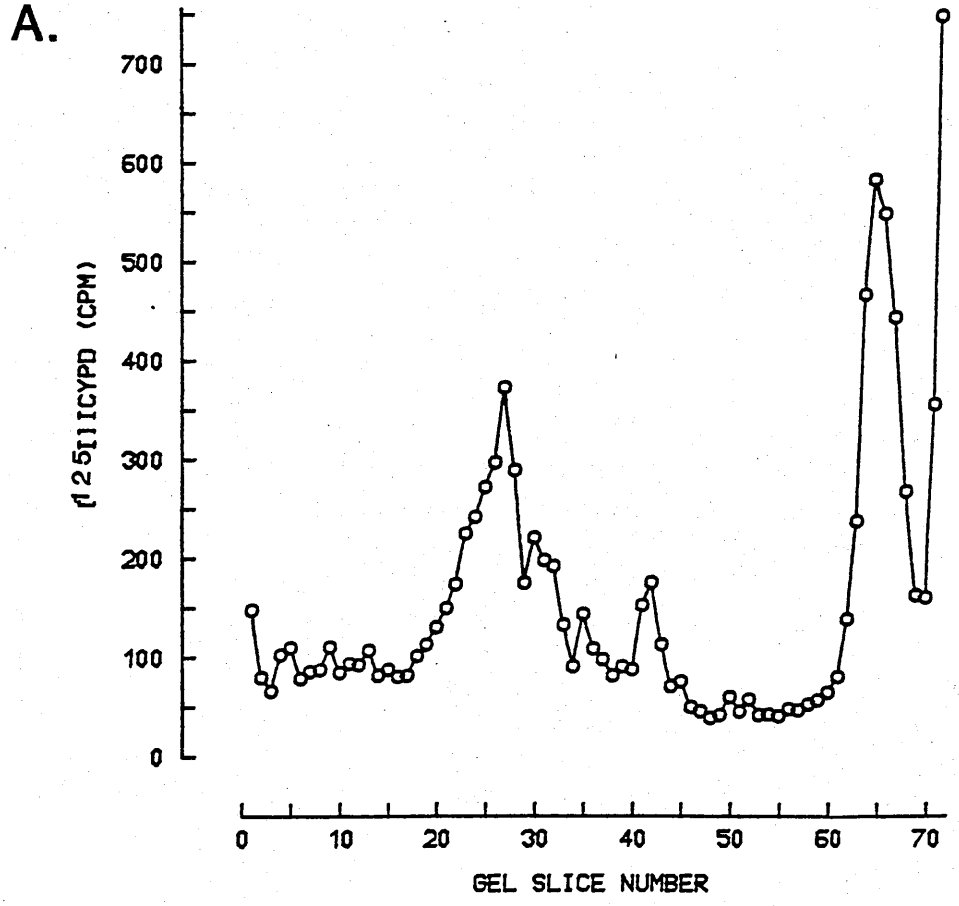
Fig. 3.16. Autoradiograph of two dimensional 16-BAC/SDS PAGE of β-adrenergic receptors of sheep adipose tissue photoaffinity-labelled in the presence of 100 μM (-)-isoproterenol

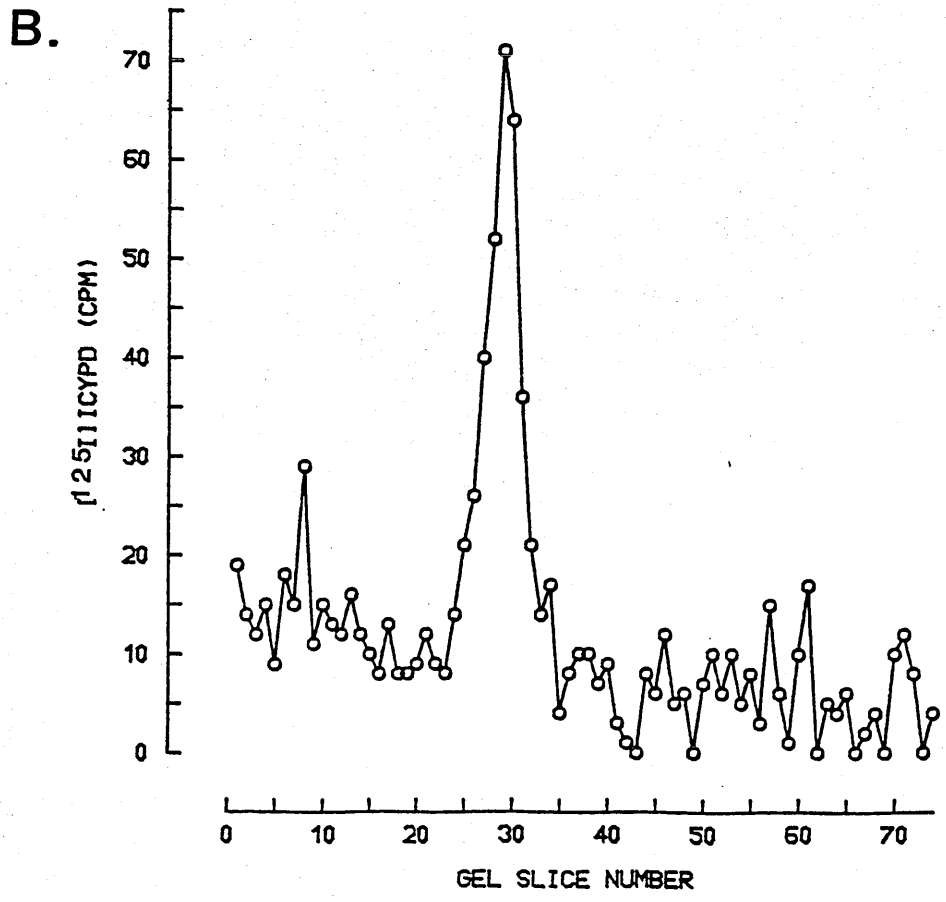
Legend as for Figure 3.15, except photoaffinity-labelling was performed in the presence of 100 μM (-)-isoproterenol, to determine non-specific labelling of proteins.

+
1stD
−
−
2ndD
+

Fig. 3.17. Two dimensional 16-BAC/SDS PAGE of photoaffinity-labelled sheep adipose tissue β-adrenergic receptors: slicing of gels

β-Adrenergic receptors from sheep (lactating ewe) omental adipose tissue were photoaffinity-labelled with [^{125}I]ICYPD, using an irradiation time of 1 h. Two identical samples (200 μg membrane protein each) were run on a 16-BAC PAGE gel. Following electrophoresis, one of the lanes was sliced (2 mm slices) and counted for [^{125}I]ICYPD (Graph A). A 6 mm slice, equivalent to gel slices 26-28 in the counted lane (Graph A), was cut from the second lane and run on an SDS PAGE gel. This gel was subsequently sliced (2 mm slices) and counted to determine the position of the [^{125}I]ICYPD-labelled protein (Graph B). Slice 1 represents the origin of the resolving gel.


A.
[125I]ICYPD (CPM)
0
100
200
300
400
500
600
700
0
10
20
30
40
50
60
70
GEL SLICE NUMBER


B.
[125I]ICYPD (CPM)
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
70
GEL SLICE NUMBER

CHAPTER FOUR

PROPERTIES OF THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

CHAPTER FOUR - PROPERTIES OF THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

4.1. Introduction

Determination of the physical properties of receptors allows the comparison of receptors from different tissues and species. The physiological effects mediated by the β-adrenergic receptors in sheep adipose tissue have been well studied by many laboratories (see Chapter 1; Fain & Garcia-Sainz, 1983). However, there is no information about the structure and physical properties of this receptor, and therefore studies were performed to characterise the β-adrenergic receptors in different adipose tissue depots, in sheep to compare them with those in the rat.

4.2. Experimental procedure

All sheep adipose tissue membranes used in this chapter were prepared from whole tissue (section 2.3.2), and were used within one week of preparation. The radioligands used were the antagonists [^{3}H]DHA and [^{125}I]ICYP, both of which are thought to bind specifically to β_1- and β_2-adrenergic receptors in a non-subtype selective manner (Stadel & Lefkowitz, 1983). β-Adrenergic ligands were dissolved in 1 mM ascorbate, and stored in aliquots at -20°C prior to use.

Competition curves for defining the subtypes of β-adrenergic receptors present in a sample were analysed using the ALLFIT computer program (De Lean et al., 1978). Other methods used are described in Chapter 2.

4.3. Results

4.3.1. Determination of dissociation constants (K_d) and maximum binding capacities (B_{MAX}) for the β-adrenergic receptor of sheep (wether) omental adipose tissue

Dissociation constants (K_d) and the maximal concentration of binding sites (B_{MAX}) were determined for [^{3}H]DHA and [^{125}I]ICYP binding to sheep (wether) omental adipose tissue membranes, using Scatchard analysis (Scatchard, 1949). [^{3}H]DHA had a high affinity for β-adrenergic receptors in sheep adipose tissue membranes, and bound in rapid, specific and saturable manner (Figure 4.1). Non-specific binding of the radioligand, determined in the presence of 100 μM (-)-isoproterenol, represented 15-25% of total binding at 10 nM [^{3}H]DHA. A Scatchard plot of [^{3}H]DHA binding data was linear, with a high correlation coefficient (r) of -0.962 (Figure 4.2). The K_d for [^{3}H]DHA was 3.23 ± 0.38 nM (n=4). B_{MAX} varied between 370 and 600 fmol/mg membrane protein.

[^{125}I]ICYP bound to sheep (wether) omental adipose tissue membranes with a higher affinity than [^{3}H]DHA. Binding was specific and saturable, with non-specific binding representing about 9% of total binding at 75 pM [^{125}I]ICYP (Figure 4.3). Analysis of the data in Figure 4.3 using the LIGAND computer program (Rodbard & Munson, 1980), gave a K_d of 53.5 pM and B_{MAX} of 1.048 pmol/mg membrane protein for [^{125}I]ICYP binding (data not shown). These values were from a single experiment, performed to determine the best concentration of [^{125}I]ICYP to use in other experiments.

4.4.2. Determination of the β-adrenergic receptor subtypes present in sheep adipose tissue

Characterisation of β-adrenergic receptor subtypes was achieved

using β-adrenergic agonists and antagonists. Omental adipose tissue from wethers was used exclusively for [^{3}H]DHA binding. The rank order of potencies of β-adrenergic agonist competition for [^{3}H]DHA binding was (-)-isoproterenol>(-)-adrenaline>(-)-noradrenaline (Figure 4.4 & Table 4.1). Competition curves were steep with no shoulders. IC_{50} and K_i values for each ligand are shown in Table 4.1. The antagonists selected were the β_2-selective ligand ICI 118,551, and the β_1-selective ligands CGP 20712A and atenolol. Competition curves for all antagonists were steep and sigmoidal (Figures 4.5 & 4.6). The rank order of potencies for competition of [^{3}H]DHA binding was ICI 118,551>>CGP 20712A>atenolol. IC_{50} and K_i values for each antagonist are presented in Table 4.1. The ratios between the IC_{50} value for ICI 118,551 and those of CGP 20712A and atenolol were 635 and 2111, respectively.

Based on the above findings, it was decided to determine the subtype(s) of β-adrenergic receptor in three adipose tissue depots in sheep (ewes). The depots studied were omental, subcutaneous (immediately anterior to the hind limb) and popliteal (inter-muscular depot in hind limb). The radioligand [^{125}I]ICYP was used because of its high affinity for β-adrenergic receptors and high specific activity, as compared to those of [^{3}H]DHA. Use of this ligand allowed the characterisation of receptors in less than 15 μg of membrane protein. Curves for the competition of [^{125}I]ICYP binding by ICI 118,551 and CGP 20712A were steep and monophasic in all three adipose depots (Figures 4.7-4.9). Data from eight sheep (4 non-lactating, 4 lactating) was analysed using the ALLFIT computer program. The rank order of IC_{50} values for CGP 20712A displacement of [^{125}I]ICYP binding was popliteal=subcutaneous>omental (Table 4.2). The IC_{50} values for

popliteal ($P<0.01$) and subcutaneous ($P<0.02$) adipose tissue were significantly different from omental adipose. There was no significant effect of lactation on the IC_{50} values for CGP 20712A (Table 4.2). The rank order of IC_{50} values for ICI 118,551 competition was omental>popliteal>subcutaneous (Table 4.3). The IC_{50} for subcutaneous adipose tissue was 45% lower than that in omental adipose ($P<0.02$). No effect of lactation was evident (Table 4.3). The ratio between IC_{50} values for ICI 118,551 and CGP 20712A displacement of [^{125}I]ICYP binding was determined for each depot. The higher the ratio (IC_{50} CGP 20712A/IC_{50} ICI 118,551), the more β_2-adrenergic receptor character is present. Analysis of $\log_{10}$-transformed results gave an order of β_2-character of subcutaneous=popliteal>omental ($P<0.01$). $\log_{10}$-transformation of some results was undertaken since it markedly reduced the coefficient of variation. Again there was no effect of lactation on this parameter.

In a separate experiment, adipocyte membranes and stroma-vascular membranes were prepared from sheep (ewe) omental adipose tissue (section 2.3.1). The β-adrenergic receptor subtype(s) present in each subcellular fraction was then investigated. Both fractions contained β_2-adrenergic receptors (Figures 4.10 & 4.11). The number of β-adrenergic receptors in adipocyte membranes and stroma-vascular membranes were 141.1 and 62.9 fmol/mg membrane protein, respectively.

4.4.3. Effect of lactation on β-adrenergic receptor ligand binding in three sheep adipose tissue depots

β-Adrenergic receptor ligand binding (at 75 pM [^{125}I]ICYP) present in sheep adipose tissue membranes prepared from omental, subcutaneous and popliteal depots, was quantified in samples from non-

lactating and lactating sheep. Analysis of $\log_{10}$-transformed results showed that lactation increased β-adrenergic receptor ligand binding in two out of three depots studied (Table 4.5 & Figure 4.12). The increases were significant in omental ($P<0.01$) and subcutaneous ($P<0.02$) depots. Percentage increases in β-adrenergic receptor ligand binding during lactation were 45% and 39% in omental and subcutaneous adipose tissue, respectively.

4.4.4. Determination of the β-adrenergic receptor subtype(s) present in rat adipocytes

Rat adipocyte membranes were prepared from combined abdominal adipose tissue depots i.e. parametrial, periovarian and lumbar. The rank order of potencies of β-adrenergic agonist competition for [^{3}H]DHA binding was (-)-isoproterenol>(-)-adrenaline>(-)-noradrenaline (Figure 4.13). Competition curves were similar to those obtained for sheep (wether) omental adipose tissue i.e. monophasic with steep gradients. IC_{50} values obtained from a single experiment were: (-)-isoproterenol, 3.5 μM; (-)-adrenaline, 27.2 μM; (-)-noradrenaline, 196.6 μM.

Competition of [^{3}H]DHA binding by antagonists gave the rank order of potency ICI 118,551>CGP 20712A>atenolol. Competition curves for all three ligands were rather shallow and monophasic (Figures 4.14 & 4.15). IC_{50} values were: ICI 118,551, 35.3 $\pm$ 9.2 nM (n=3); CGP 20712A, 2.5 $\pm$ 0.2 μM (n=3); atenolol, 38.2 μM (n=1).

To extend these studies further, adipocyte membranes were prepared separately from parametrial and lumbar adipose tissue depots. These experiments were performed because of apparent differences between our data for competition of [^{3}H]DHA binding to rat adipocyte membranes by adrenergic antagonists and the published findings of

Bahouth & Malbon (1988). [^{125}I]ICYP binding to β-adrenergic receptors in both depots, was competed out with increasing concentrations of ICI 118,551 and CGP 20712A. Competition curves for ICI 118,551 and CGP 20712A were biphasic, indicating the presence of high and low affinity binding sites (Figures 4.16 & 4.17). Simultaneous analysis of pooled data from three independent experiments with a computer operating non-linear iterative analysis (GraphPad InPlot, Ithica Street Software, Inc., U.S.A.) revealed that the lumbar depot possessed a higher percentage of high affinity CGP 20712A binding sites (62%; $P<0.02$) and a lower percentage of low affinity CGP 20712A sites (38%; $P<0.05$) compared to the parametrial depot (Table 4.6). In addition, the IC_{50} of the high affinity CGP 20712A site (0.533 nM) in the lumbar depot was greater ($P<0.001$) than the corresponding value for the parametrial depot (0.271 nM)(Table 4.6). In contrast, the parametrial depot possessed the highest percentage of high affinity ICI 118,551 binding sites (62%; $P<0.02$) and the lowest percentage of low affinity sites (38%; $P<0.05$). Also, the parametrial depot possessed a high affinity site with an IC_{50} lower than the corresponding site in lumbar adipose tissue. However, the low affinity binding sites for both CGP 20712A and ICI 118,551 in parametrial adipose possessed the same affinities (IC_{50}) as the corresponding values for the lumbar adipose tissue depot (Table 4.6).

4.4. Discussion

The dissociation constant (K_d) for [^{3}H]DHA binding to sheep (wether) omental adipose tissue membranes was 3.23 nM. Dissociation constants for β-adrenergic receptors in other species and tissues include: canine lung, 1.3 nM (Homcy et al., 1983); turkey erythrocyte, 8 nM (Vauquelin et al., 1979); frog erythrocyte, 2.2 nM (Caron & Lefkowitz,

1976); rat adipocyte, 20 nM (Cubero & Malbon, 1984), 19 nM (Malbon et al., 1978), 15 nM (Williams et al., 1976), 49 nM (Chiappe de Cingolani, 1986) and 2.2 nM (Bahouth & Malbon, 1988). The reasons for the variation in the K_d for rat adipocyte β-adrenergic receptor are unclear, and complicate direct comparison with the sheep adipose tissue β-adrenergic receptor. The K_d for [^{125}I]ICYP determined from a single experiment was 54 pM. Values obtained for other β-adrenergic receptors are: rat liver, 60 pM (Graziano et al., 1985); rat adipocyte, 30 pM (Bahouth & Malbon, 1988); human β_1-adrenergic receptor, 66 pM (Frielle et al., 1987); hamster lung, 50 pM (Engel et al., 1981); human β_3-adrenergic receptor, 490 pM (Emorine et al., 1989). The dissociation constants for sheep adipose tissue β-adrenergic receptor obtained for both radioligands, are therefore comparable to those of other β-adrenergic receptors. Scatchard plots of [^{3}H]DHA saturation binding data were linear, indicating the presence of a single class of high affinity binding sites.

B_{MAX} for β-adrenergic receptor density varies at least 100 fold between species and tissues. The B_{MAX} for [^{3}H]DHA binding to sheep (wether) omental adipose tissue membranes varied between 370 and 600 fmol/mg protein. This range encompasses the B_{MAX} for rat adipocytes, namely 500 fmol/mg membrane protein (Bahouth & Malbon, 1988). Rat liver and human placenta contain 17.7 fmol/mg membrane protein and 1.2-2.5 pmol/mg membrane protein of β-adrenergic receptors, respectively, and illustrate the range of values obtainable. Receptor densities of 200-600 fmol/mg plasma membrane protein are representative of the majority of tissues. Therefore sheep (wether) omental adipose tissue possesses a standard complement of β-adrenergic receptors. However, a B_{MAX} of

1.048 pmol/mg membrane protein was obtained for [^{125}I]ICYP binding to sheep (wether) omental adipose tissue membranes in one experiment. In contrast, using 75 pM [^{125}I]ICYP (about 70-80% of maximum binding), binding of 400 fmol/mg membrane protein was usual which is in agreement with estimates from [^{3}H]DHA binding. Therefore the B_{MAX} from the single experiment is probably not representative.

At present, three subtypes of β-adrenergic receptor have been cloned, called β_1-, β_2- and β_3-adrenergic receptors, respectively. β_1- and β_2-adrenergic receptors were originally characterised using agonists (Lands et al., 1967). Based on the classification of Lands et al., it is possible to define which subtype(s) of β-adrenergic receptor are present in a sample. The rank order of potencies for agonist competition of [^{3}H]DHA binding to sheep (wether) omental adipose tissue was (-)-isoproterenol>(-)-adrenaline>(-)-noradrenaline, indicative of β_2-character according to Lands et al. (1967). Competition curves were monophasic, indicating that a homogeneous population of β_2-adrenergic receptors is present in sheep (wether) omental adipose tissue. To characterise the receptor further, subtype-selective β-adrenergic antagonists were employed; CGP 20712A, atenolol and ICI 118,551. CGP 20712A is 10,000 fold more selective for β_1- as compared with β_2-adrenergic receptors in tissues containing mixed populations of β_1- and β_2-adrenergic receptors (Dooley et al., 1986; Bahouth & Malbon, 1987). Atenolol has a similar selectivity to CGP 20712A (Strader et al., 1989a). In contrast, ICI 118,551 is a potent, highly β_2-selective adrenergic antagonist (O'Donnell & Wanstall, 1979).

ICI 118,551 was the most potent ligand for the displacement of [^{3}H]DHA binding to sheep (wether) omental adipose tissue membranes,

being 635 and 2111 times more effective than CGP 20712A and atenolol, respectively. This result clearly defines this β-adrenergic receptor as of the β_2-subtype, which agrees with the results obtained for agonists. Again curves were smooth with no shoulders, indicating the presence of a single receptor population. Indeed the ratio between the IC_{50} values of CGP 20712A and ICI 118,551 of 635 is similar to that for the cloned human β_2-adrenergic receptor, which has a ratio between betaxolol (β_1-selective) and ICI 118,551 of 518 (Frielle et al., 1988). The human β_1-adrenergic receptor has a ratio (Betaxolol/ICI 118,551) of 0.1 (Frielle et al., 1988). The finding that sheep adipose tissue possesses β_2-adrenergic receptors correlates with the fact that many of the β-agonists found to alter carcass composition in livestock exhibit primarily β_2-adrenergic receptor selectivity (Cohen et al., 1982). It therefore seems likely that these agents reduce adiposity by stimulating the intracellular processes controlled by β_2-adrenergic receptors. Preparation of two fractions from sheep (ewe) omental adipose tissue, adipocyte membranes and stroma-vascular membranes showed that both fractions contained high affinity β_2-adrenergic receptors. This suggests that there is no difference between β-adrenergic receptor ligand binding in sheep adipocyte and adipose tissue membranes.

In many studies on β-adrenergic receptors, work has concentrated on a single tissue or depot in the case of adipose tissue. Results are then compared to those obtained for other tissues and species, often without any consideration of differences caused by the location of the tissue within the body of an animal. This is of particular importance when studying adipose tissue as it occurs in distinct depots at different sites in the body. β-Adrenergic receptors were studied in

three separate sheep adipose tissue depots, omental, subcutaneous and popliteal. All three depots contained a single population of β_2-adrenergic receptors. However there were differences in IC_{50} values for CGP 20712A and ICI 118,551 competition of [^{125}I]ICYP binding to membranes from these depots. CGP 20712A (β_1-selective) was most potent in omental adipose tissue, with an IC_{50} significantly lower than those of subcutaneous and popliteal depots, suggesting a higher degree of β_1-character in the omental depot. Conversely, ICI 118,551 (β_2-selective) was most effective in subcutaneous adipose, with omental and popliteal adipose tissue displaying equal affinities. Taken together, these results suggest that subcutaneous and popliteal adipose tissue contain β-adrenergic receptors displaying a higher degree of β_2-character than that of omental. Analysis of the ratio between IC_{50}'s for CGP 20712A and ICI 118,551, showed that popliteal and subcutaneous adipose tissue β-adrenergic receptors have the highest ratios, again indicative of β_2-adrenergic receptor character.

The effect of lactation on β-adrenergic receptor properties was also studied because lactation has been shown to increase the number of β-adrenergic receptors in cattle (Jaster & Wegner, 1981), sheep (Watt et al., 1990a), and rat (Watt et al., 1990b) adipose tissue. In all of these studies only a single depot was tested. In this study, lactation increased β-adrenergic receptor ligand binding in two out of the three adipose tissue depots studied, namely omental and subcutaneous, thus it therefore seems possible that lactation modulates β-adrenergic receptors in a depot-specific manner. In contrast, no effect of lactation on β-adrenergic receptor affinities was observed in any of the depots, even though depots possess β-receptors with different ligand binding

properties. It is possible that these differences result from environmental effects e.g. changes in the composition of the lipid bilayer, or conformational changes induced in the receptor by post-translational modifications, in particular phosphorylation and glycosylation.

The β-adrenergic receptors of rat adipocytes were also studied. Initial experiments were performed on pooled abdominal adipose tissue i.e. parametrial, periovarian and lumbar. The rank order of potencies for agonist competition of [^{3}H]DHA binding was (-)-isoproterenol>(-)-adrenaline>(-)-noradrenaline, indicative of β_2-character (Lands et al., 1967). Characterisation with CGP 20712A and ICI 118,551 was also indicative of β_2-character, but not to the same degree as that seen in sheep adipose tissue. IC_{50} values for CGP 20712A and atenolol were lower in the rat than in the sheep, indicating the presence of more β_1-adrenergic receptor character. It therefore appeared that pooled abdominal rat adipose tissue possessed a β-adrenergic receptor of predominantly β_2-character with different binding characteristics from that present in sheep adipose tissue. In fact these results suggested that the β-adrenergic receptors in the rat adipocyte were atypical in nature i.e. not being definitively β_1- or β_2-adrenergic receptors.

The antagonist competition data discussed above was different from that obtained by Craig Malbon and co-workers (Bahouth & Malbon, 1988). Therefore it was decided to study the β-adrenergic receptors in two rat adipose tissue depots individually i.e. parametrial and lumbar, and refrain from pooling abdominal adipose tissue. Results for parametrial and lumbar adipose tissue suggested the presence of at least two β-adrenergic receptor populations, with differing affinities for the

subtype-selective antagonists CGP 20712A (β_1) and ICI 118,551 (β_2). The competition curves obtained for lumbar adipose tissue, resemble those shown for an identical characterisation of parametrial β-receptors (Bahouth & Malbon, 1988). The percentages of high and low affinity sites for CGP 20712A and ICI 118,551 in both adipose tissue depots suggest that there are two populations of receptors present in these depots (Table 4.6). One receptor population appears to possess high affinity for CGP 20712A and low affinity for ICI 118,551, normally associated with β_1-adrenergic receptors, and is most abundant in the lumbar depot. A second population exhibits high affinity for ICI 118,551 and low affinity for CGP 20712A, characteristics of β_2-adrenergic receptors, and is most prevalent in parametrial adipose tissue. Such a close correlation was not evident in the paper, Bahouth & Malbon (1988), and no discussion of the presence of the high affinity ICI 118,551 binding site which they also found, was given. However, the ratio between the IC_{50} values for these two hypothetical receptor populations are quite different from those of typical β_1- and β_2-adrenergic receptors (Frielle et al., 1988), and therefore the correlation between the percentages of each binding site may be coincidental. The ligand binding characteristics of β-adrenergic receptors in sheep and rat adipose tissue again serve to distinguish the β-receptors in these species. On the one hand, sheep possess a homogeneous population of β_2-adrenergic receptors, whilst rat adipose tissue contains a heterogeneous population of β_1- and atypical β-adrenergic receptors.

The subtypes of β-adrenergic receptor seen in rat adipose tissue in this study represent a controversial area for discussion. Lands et al. (1967) originally proposed that the β-adrenergic receptor mediating

catecholamine stimulation of lipolysis in the rat was of the β_1-subtype, based upon the relative lipolytic potencies of several β-adrenergic agonists. This finding has apparently been substantiated by other workers (Williams et al., 1976; Malbon et al., 1978; Cubero & Malbon, 1984; Bahouth & Malbon, 1988). In direct contrast, Harms et al. (1974), and De Vente et al. (1980) suggested that a hybrid or atypical type of β-adrenergic receptor mediates lipolysis in rat adipocytes, with low affinities for β_1- and β_2-selective and non-selective ligands. Further studies by other groups have shown that isoproterenol-stimulated lipolysis in rat adipocytes is mediated by atypical β-adrenergic receptors (Wilson et al., 1984; Bojanic et al., 1985; Hollenga & Zaagsma, 1989; Hollenga et al., 1990). The adipocyte receptor also has different stereo-selectivity ratios for antagonist enantiomers compared to typical β_1- and β_2-adrenergic receptors (Harms et al., 1977). In addition, it was established that the atypical characteristics of antagonists was not due to a heterogeneous population of both β_1- and β_2-adrenergic receptors (De Vente et al., 1980; Bojanic et al., 1985).

The existence of atypical β-adrenergic receptors in rat adipocytes was dismissed in a controversial paper by Bahouth & Malbon (1988). In this report extensive radioligand binding assays were performed using four different radioligands. Their conclusion was that rat adipocytes contain solely β_1-adrenergic receptors. This paper has recently been challenged by a proposal that the rat adipocyte β-adrenergic receptor is a form of the recently cloned atypical human β_3-adrenergic receptor (Zaagsma & Nahorski, 1990). They dismiss Bahouth and Malbon's conclusions because of a lack of correlation between inhibition constants for radioligand binding data, with those for

functional studies on inhibition of isoproterenol-stimulated lipolysis by ICI 118,551 and CGP 20712A. Also it has been demonstrated that isoproterenol-stimulated lipolysis in rat white adipose tissue is mediated predominantly by atypical β-adrenergic receptors, whereas typical β_1-adrenergic receptors play a small subordinate role (Hollenga & Zaagsma, 1989). In a previous study from the same laboratory, it was suggested that the atypical β-adrenergic receptor was coupled to adenylate cyclase (Bojanic et al., 1985). The existence of atypical β-adrenergic receptors in sheep adipose tissue cannot be ruled out as yet. The results from radioligand binding assays detailed in this thesis, must be shown to correlate with functional studies on lipolysis in adipocytes, before the presence of atypical β-adrenergic receptors can be dismissed.

A review of some key papers on the rat adipocyte reveals that the adipose tissue depot used varies between laboratories and includes parametrial (Cubero & Malbon, 1984; Bahouth & Malbon, 1988) and epididymal (Lands et al., 1967; Wilson et al., 1984; Bojanic et al., 1985; Hollenga & Zaagsma, 1989; Hollenga et al., 1990) adipose tissue. In light of the findings of this study, it would appear that some of the conflicting reports could be the result of the use of different adipose tissue depots, and animals of different sex. Indeed, how real are apparent published discrepancies in lipolytic activity induced by β-adrenergic agonists in human and rat adipocytes, when the human adipose tissue was subcutaneous and rat adipose tissue was epididymal (Hollenga et al., 1990)? In the same study, the human subjects were predominately female (14 female : 5 male), whilst rats were all male. Obviously, detailed investigation into sex differences in β-adrenergic receptor subtype characteristics in adipose tissue is required.

Table 4.1. Displacement of [^{3}H]DHA binding to sheep omental adipose tissue membranes by adrenergic agonists and antagonists

Competition curves for displacement of 4 nM [^{3}H]DHA binding to sheep (wether) omental adipose tissue membranes were analysed using the ALLFIT computer program. Inhibition contants (K_i) for the ligands were calculated from the resulting IC_{50} values using the equation $K_i=IC_{50}/(1+L/K_d)$, where L is the concentration of [^{3}H]DHA used (Cheng & Prussoff, 1973). A K_d of 3.23 nM was used in the calculations. Results are expressed as the mean ± S.E.M. n = number of experiments.

Ligand	IC_{50} (nM)	K_i (nM)	n
Agonists			
(-)-Isoproterenol	869 ± 122	299 ± 90	3
(-)-Adrenaline	10764 ± 709	3583 ± 743	3
(-)-Noradrenaline	24913 ± 2913	8534 ± 1680	3
Antagonists			
ICI 118,551	24.4 ± 4.7	12.2 ± 2.1	5
CGP 20712A	15499 ± 5976	7844 ± 2119	3
Atenolol	51517 ± 12583	24043 ± 6287	3

Table 4.2. Displacement of [^{125}I]ICYP binding to sheep adipose tissue membranes from three depots by CGP 20712A

IC_{50} values for competition of [^{125}I]ICYP binding to sheep adipose tissue membranes by CGP 20712A were obtained by analysis of competition curves, using the ALLFIT computer program. [^{125}I]ICYP (75 pM) binding was measured at eight different concentrations of CGP 20712A (10^{-10}-10^{-3} M). Results are the means of data from eight animals (4 non-lactating & 4 lactating ewes), analysed by analysis of variance.

i) Depot differences

Depot	IC_{50} (μM)
Omental	1.60*
Subcutaneous	2.99
Popliteal	3.55
s.e.d.	0.472

* value significantly different from those for subcutaneous ($P<0.02$) and popliteal ($P<0.01$) depots.

ii) Effect of lactation

	IC_{50} (μM)	
Depot	Non-lactating	Lactating
Omental	1.47	1.72
Subcutaneous	3.09	2.90
Popliteal	2.99	4.11

S.e.d. between depots with the same physiological state = 0.667; s.e.d. for the effect of lactation for each depot = 1.004. No significant effect of lactation.

Table 4.3. Displacement of [^{125}I]ICYP binding to sheep adipose tissue membranes from three depots by ICI 118,551

IC_{50} values for competition of [^{125}I]ICYP binding to sheep adipose tissue membranes by ICI 118,551 were obtained by analysis of competition curves using the ALLFIT computer program. [^{125}I]ICYP (75 pM) binding was measured at eight different concentrations of ICI 118,551 (10^{-10}-10^{-3} M). Results are means of data from eight animals (4 non-lactating & 4 lactating ewes), analysed by analysis of variance.

i) Depot differences

Depot	IC_{50} (nM)
Omental	9.37*
Subcutaneous	5.14
Popliteal	6.82
s.e.d.	1.516

* value significantly different from that for subcutaneous depot ($P<0.02$).

ii) Effect of lactation

	IC_{50} (nM)	
Depot	Non-lactating	Lactating
Omental	9.37	9.25
Subcutaneous	3.76	6.51
Popliteal	7.77	5.88

S.e.d. between depots with the same physiological state = 2.144; s.e.d. for the effect of lactation for each depot = 2.655. No significant effect of lactation.

Table 4.4. Displacement of [^{125}I]ICYP binding to sheep adipose tissue membranes from three depots by CGP 20712A and ICI 118,551 - ratio of IC_{50} values

IC_{50} ratios (CGP 20712A/ICI 118,551) for displacement of [^{125}I]ICYP binding to sheep adipose tissue membranes, by CGP 20712A and ICI 118,551, were obtained from analysis of competition curves, using the ALLFIT computer program. [^{125}I]ICYP (75 pM) binding was measured at eight different concentrations of antagonist (10^{-10}-10^{-3} M). Results are means of data from eight animals (4 non-lactating & 4 lactating ewes), analysed by analysis of variance using log_{10} transformed data to reduce the coefficient of variation.

i) Depot differences

	IC_{50} (CGP/ICI)	
Depot	mean	log_{10} of mean
Omental	266.3	2.226*
Subcutaneous	706.8	2.692
Popliteal	641.8	2.743
Log_{10} s.e.d.		0.1245

* value significantly different from those for subcutaneous ($P<0.01$) and popliteal ($P<0.01$) depots.

ii) Effect of lactation

	IC_{50} (CGP/ICI)			
	Non-lactating		Lactating	
Depot	mean	log_{10} of mean	mean	log_{10} of mean
Omental	254.9	2.126	277.6	2.327
Subcutaneous	778.6	2.792	635.0	2.591
Popliteal	473.0	2.617	810.4	2.868

Log_{10} s.e.d. between depots with the same physiological state = 0.1761; log_{10} s.e.d. for the effect of lactation for each depot = 0.2838. No significant effect of lactation.

Table 4.5. Effect of lactation on β-adrenergic receptor ligand binding in three sheep adipose tissue depots

Sheep adipose tissue membranes (15 μg protein) from omental, subcutaneous and popliteal depots were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the absence or presence of 100 μM (-)-isoproterenol. [^{125}I]ICYP binding displaced by isoproterenol was assumed to represent specific binding to β-adrenergic receptors. Results are means of data from eight animals (4 non-lactating & 4 lactating ewes), analysed by analysis of variance.

i) Effect of lactation

	[^{125}I]ICYP bound (fmol/mg protein)	
	mean	$\log_{10}$ of mean
Non-lactating	175.3	2.225
Lactating	280.8	2.434*
$\log_{10}$ s.e.d.		0.0689

* value significantly different from that for non-lactating sheep ($P<0.02$).

ii) Depot differences

	[^{125}I]ICYP bound (fmol/mg protein)			
	Non-lactating		Lactating	
Depot	mean	$\log_{10}$ of mean	mean	$\log_{10}$ of mean
Omental	164.2	2.192	300.1	2.466**
Subcutaneous	190.8	2.264	311.9	2.484*
Popliteal	170.9	2.219	230.5	2.352

*, **, value significantly different, $P<0.02$ and $P<0.01$, respectively, compared to the corresponding value for non-lactating sheep. $\log_{10}$ s.e.d. between depots with the same physiological state = 0.0743; $\log_{10}$ s.e.d for the effect of lactation for each depot = 0.0918

Table 4.6. Displacement of [^{125}I]ICYP binding to rat adipocyte membranes by CGP 20712A and ICI 118,551

Results for the displacement of [^{125}I]ICYP (92.9 pM) binding to rat parametrial and lumbar adipocyte membranes by the β-adrenergic receptor antagonists CGP 20712A and ICI 118,551, were analysed using non-linear iterative analysis (GraphPAD InPlot, GraphPAD Software, 10855 Sorrento Valley Road, San Diego, CA 92121, U.S.A). The quality of curve fitting for 1 and 2 site models was assessed using an F-test. Each value is the mean ± S.E.M, calculated from three separate experiments performed in duplicate.

	ADIPOSE TISSUE DEPOT			
	PARAMETRIAL		LUMBAR	
	R_H	R_L	R_H	R_L
i) CGP 20712A				
IC_{50}	0.271 ± 0.011 nM	4.797 ± 0.101 µM	0.553 ± 0.007 nM***	4.662 ± 0.131 µM
Percentage of sites	37.98 ± 3.84	62.02 ± 6.24	61.89 ± 4.02**	38.11 ± 2.49*
ii) ICI 118,551				
IC_{50}	3.518 ± 0.120 nM	0.862 ± 0.020 µM	14.292 ± 0.486 nM***	0.871 ± 0.019 µM
Percentage of sites	61.71 ± 5.17	38.29 ± 3.22	35.77 ± 3.79**	64.23 ± 6.83*

R_H and R_L represent high and low affinity ligand binding sites, respectively. * P<0.05, ** P<0.02, *** P<0.001, value significantly different from the corresponding value for parametrial adipose tissue.

Fig. 4.1. [^{3}H]DHA binding to sheep omental adipose tissue membranes

Sheep (wether) omental adipose tissue membranes (100 μg protein) were incubated for 10 min at 37°C, with the indicated concentrations of [^{3}H]DHA, in the absence (closed circles) or presence (open circles) of 100 μM (-)-isoproterenol. Specific binding (half-filled circles) was defined as binding sensitive to competition by isoproterenol. Each point is the mean of triplicate determinations from a single representative experiment.

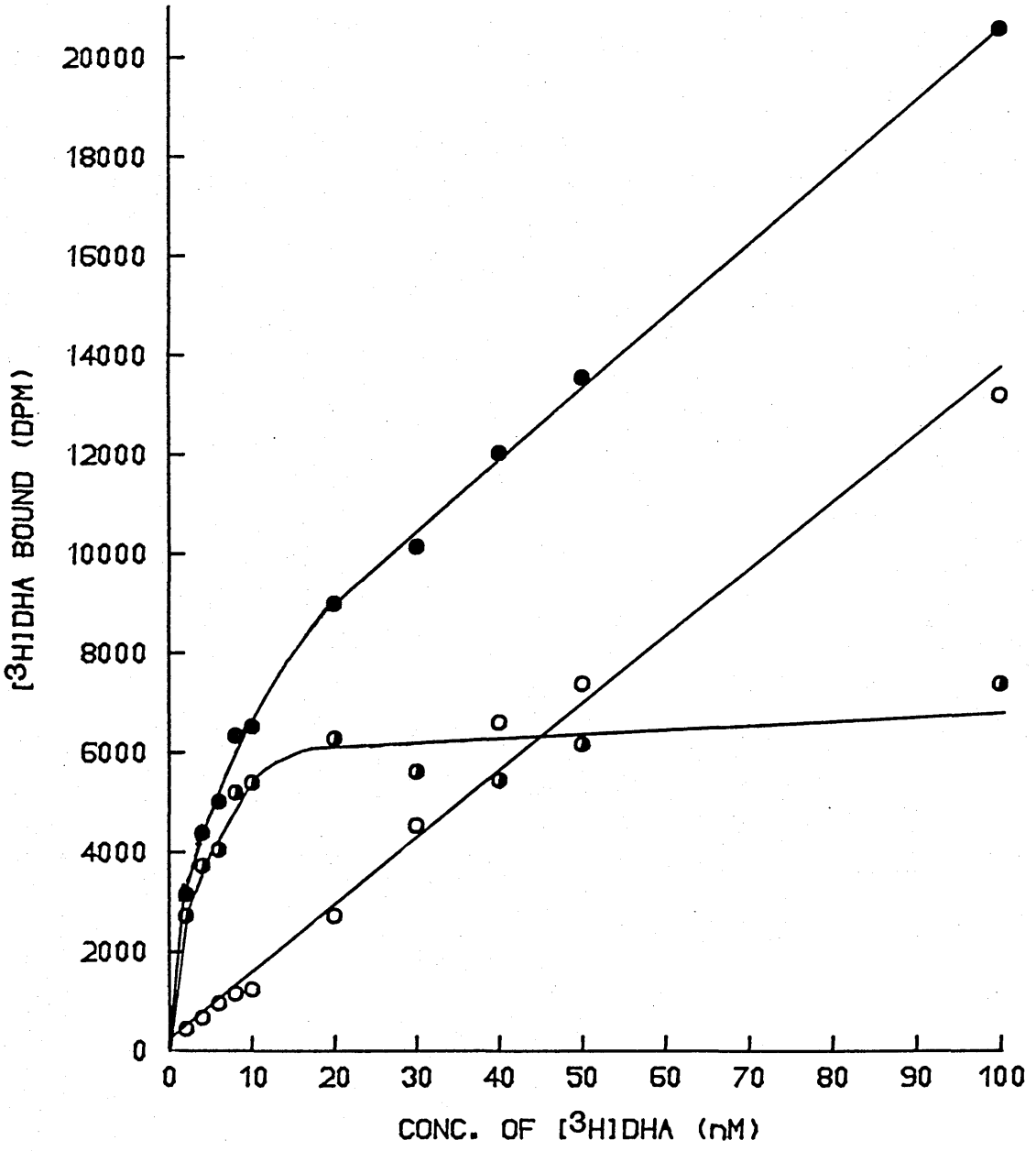

[3H]DHA BOUND (DPM)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
10
20
30
40
50
60
70
80
90
100
CONC. OF [3H]DHA (nM)

Fig. 4.2. Scatchard analysis of [^{3}H]DHA binding to sheep omental adipose tissue membranes

For assay conditions see Figure 4.1. The data is a Scatchard plot of the data presented in Figure 4.1. The correlation coefficient of regression (r) for the transformed data was -0.962. In this experiment the dissociation constant for [^{3}H]DHA was 3.603 nM while the maximum binding was 607.5 fmol/mg membrane protein.

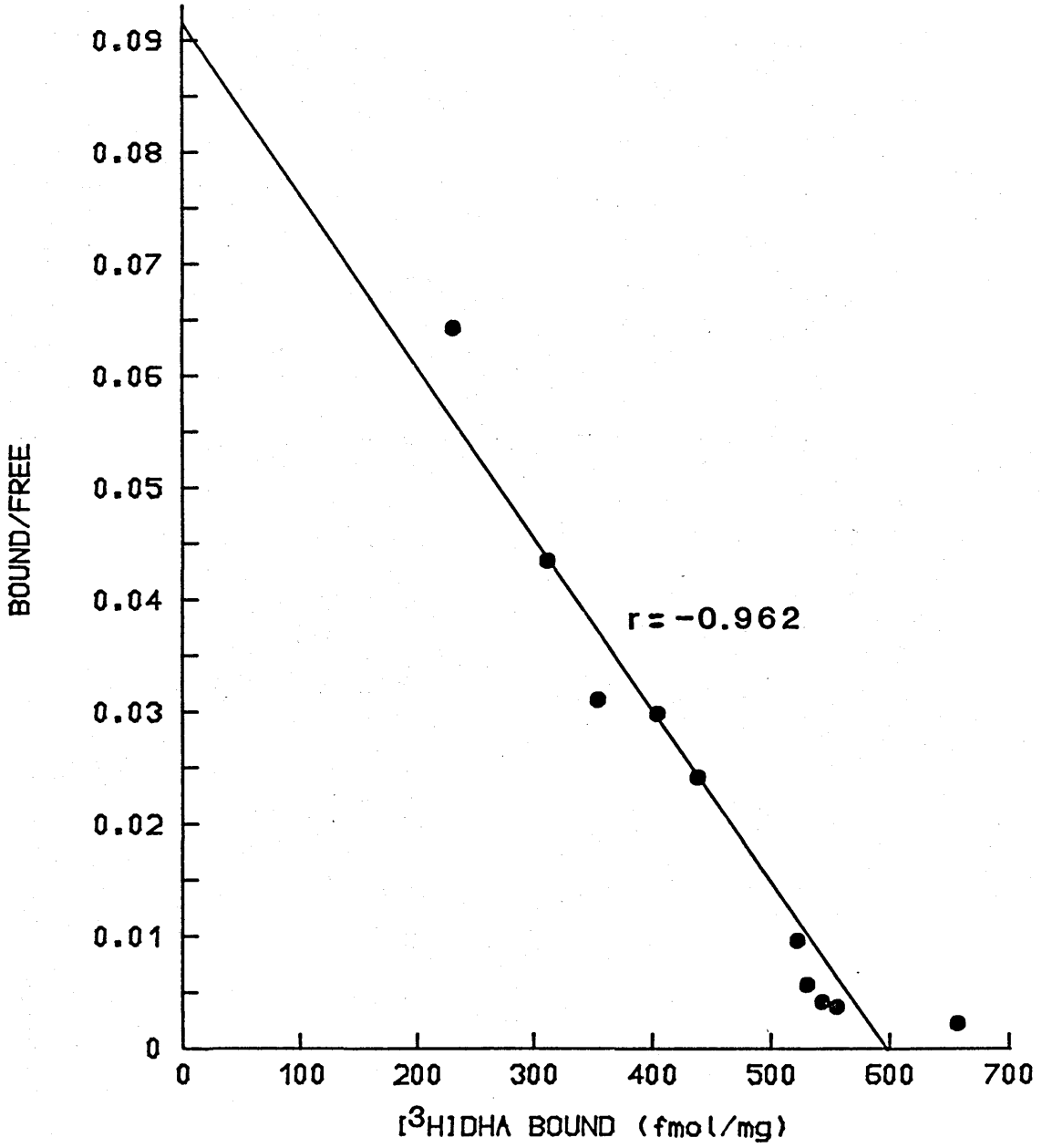

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
BOUND/FREE
r = -0.962
0
100
200
300
400
500
600
700
[3H]DHA BOUND (fmol/mg)

Fig. 4.3. [^{125}I]ICYP binding to sheep omental adipose tissue membranes

Sheep (wether) omental adipose tissue membranes (15 μg protein) were incubated for 1 h at 22°C, with the indicated concentrations of [^{125}I]ICYP, in the absence (closed circles) or presence (open circles) of 100 μM (-)-isoproterenol. Specific binding (half-filled circles) was defined as binding sensitive to competition by isoproterenol. Each point is the mean of triplicate determinations from a single experiment.

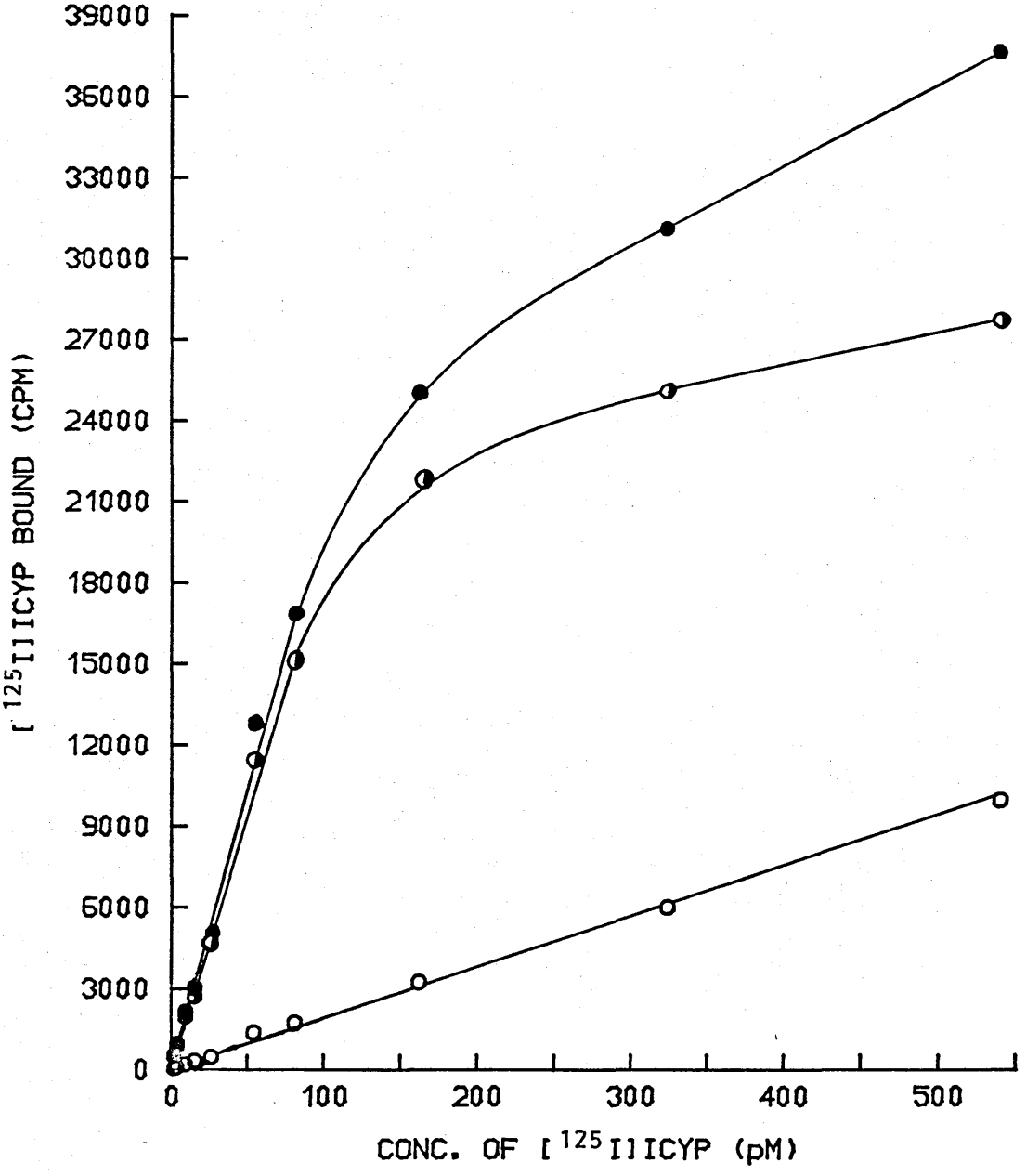

39000
36000
33000
30000
27000
24000
21000
18000
15000
12000
9000
6000
3000
0
[125I]ICYP BOUND (CPM)
0
100
200
300
400
500
CONC. OF [125I]ICYP (pM)

Fig. 4.4. Competition of [^{3}H]DHA binding to sheep omental adipose tissue membranes by isoproterenol, adrenaline and noradrenaline

Sheep (wether) omental adipose tissue membranes (100 μg protein) were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the agonists (-)-isoproterenol (open squares), (-)-adrenaline (closed squares) and (-)-noradrenaline (open circles). Total binding represents [^{3}H]DHA bound in the absence of competing agonist. Curves were analysed using the ALLFIT computer program. This is a representative experiment, which was performed three times with similar results. Each point represents the mean of triplicate observations.

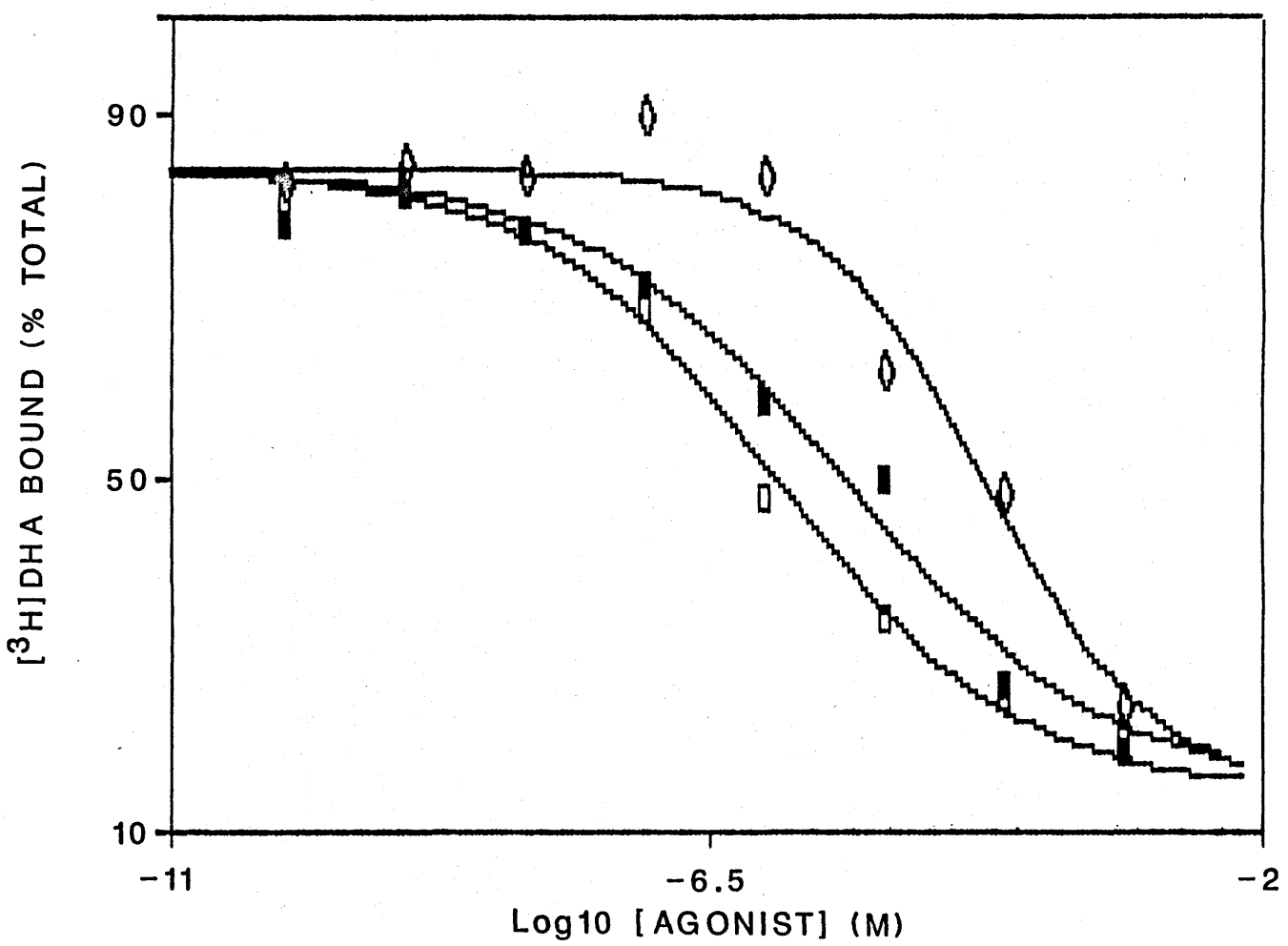

90
50
10
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [AGONIST] (M)

Fig. 4.5. Competition of [^{3}H]DHA binding to sheep omental adipose tissue membranes by ICI 118,551 and CGP 20712A

Sheep (wether) omental adipose tissue membranes (100 µg protein) were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the antagonists ICI 118,551 (closed squares) and CGP 20712A (open squares). Total binding represents [^{3}H]DHA bound in the absence of antagonist. Curves were analysed using the ALLFIT computer program. This is a representative experiment, which was performed three times with similar results. Each point represents the mean of triplicate observations.

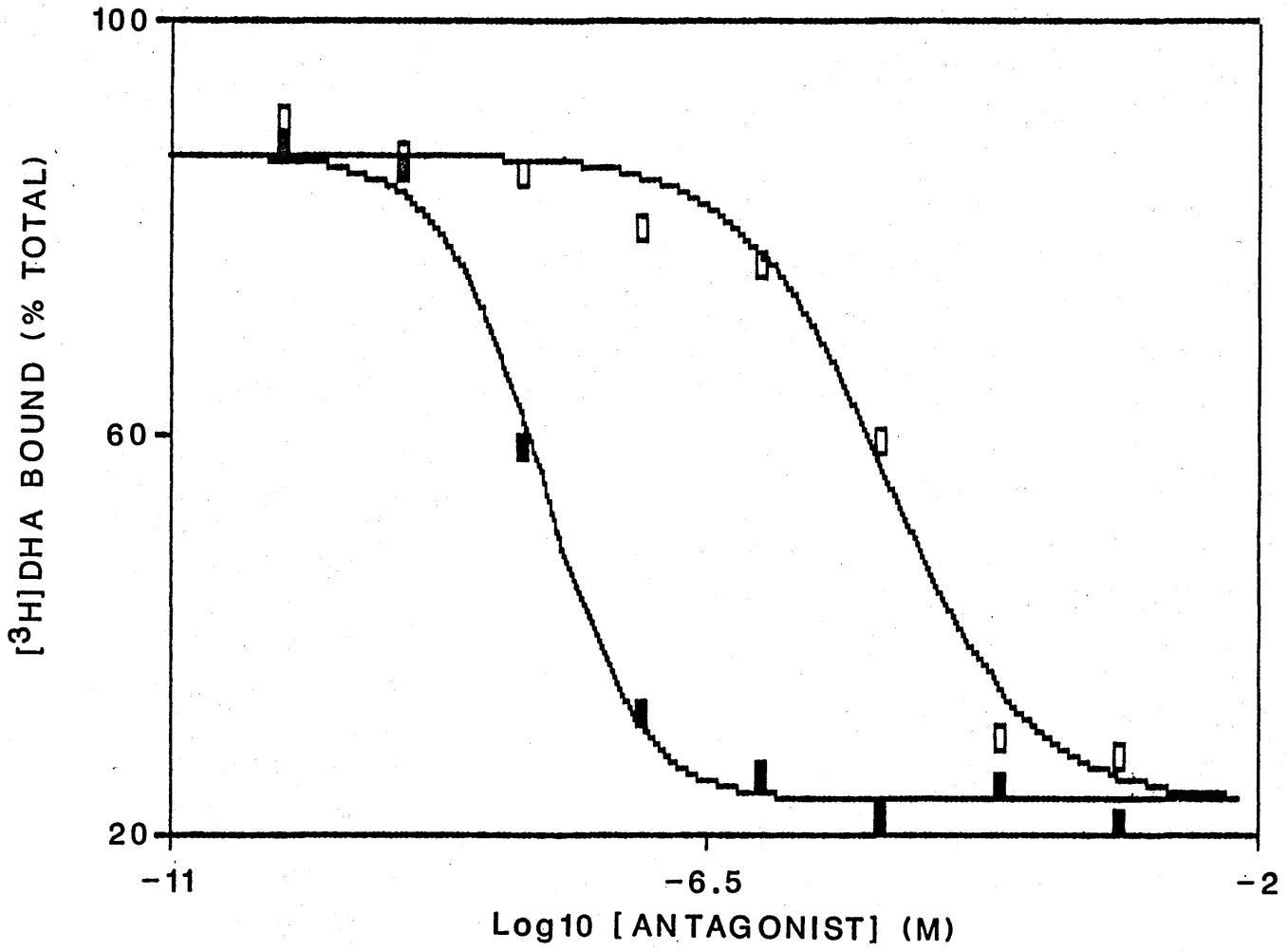

100
60
20
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.6. Competition of [^{3}H]DHA binding to sheep omental adipose tissue membranes by ICI 118,551 and atenolol

Sheep (wether) omental adipose tissue membranes (100 μg protein) were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the antagonists ICI 118,551 (open squares) and atenolol (closed squares). Total binding represents [^{3}H]DHA bound in the absence of antagonist. Curves were analysed using the ALLFIT computer program. This is a representative experiment, which was performed three times with similar results. Each point represents the mean of triplicate observations.

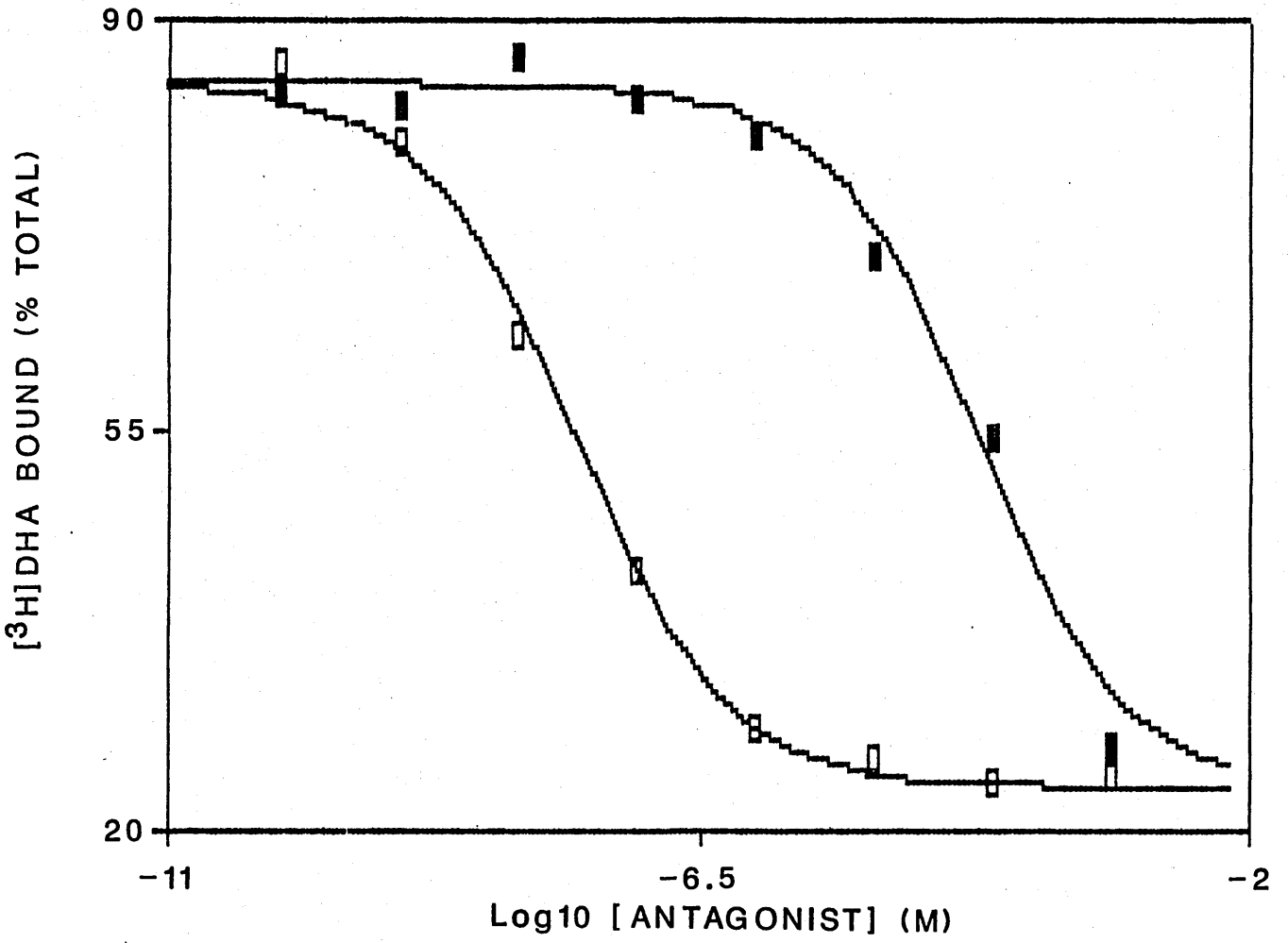

90
55
20
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.7. Competition of [^{125}I]ICYP binding to sheep omental adipose tissue membranes by CGP 20712A and ICI 118,551

Omental adipose tissue membranes (15 µg protein) from 3-4-year old ewes were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (open squares) and ICI 118,551 (closed squares). Total binding represents [^{125}I]ICYP bound in the absence of any antagonist. Curves were analysed using the ALLFIT computer program. IC_{50} values for CGP 20712A and ICI 118;551 were 1.6 µM (s.e.d.=0.472) and 9.37 nM (s.e.d.=1.516), respectively (n=8). This is a representative experiment (lactating ewe), which was performed eight times with preparations from different sheep (4 non-lactating & 4 lactating) with similar results. Each point represents the mean of duplicate observations.

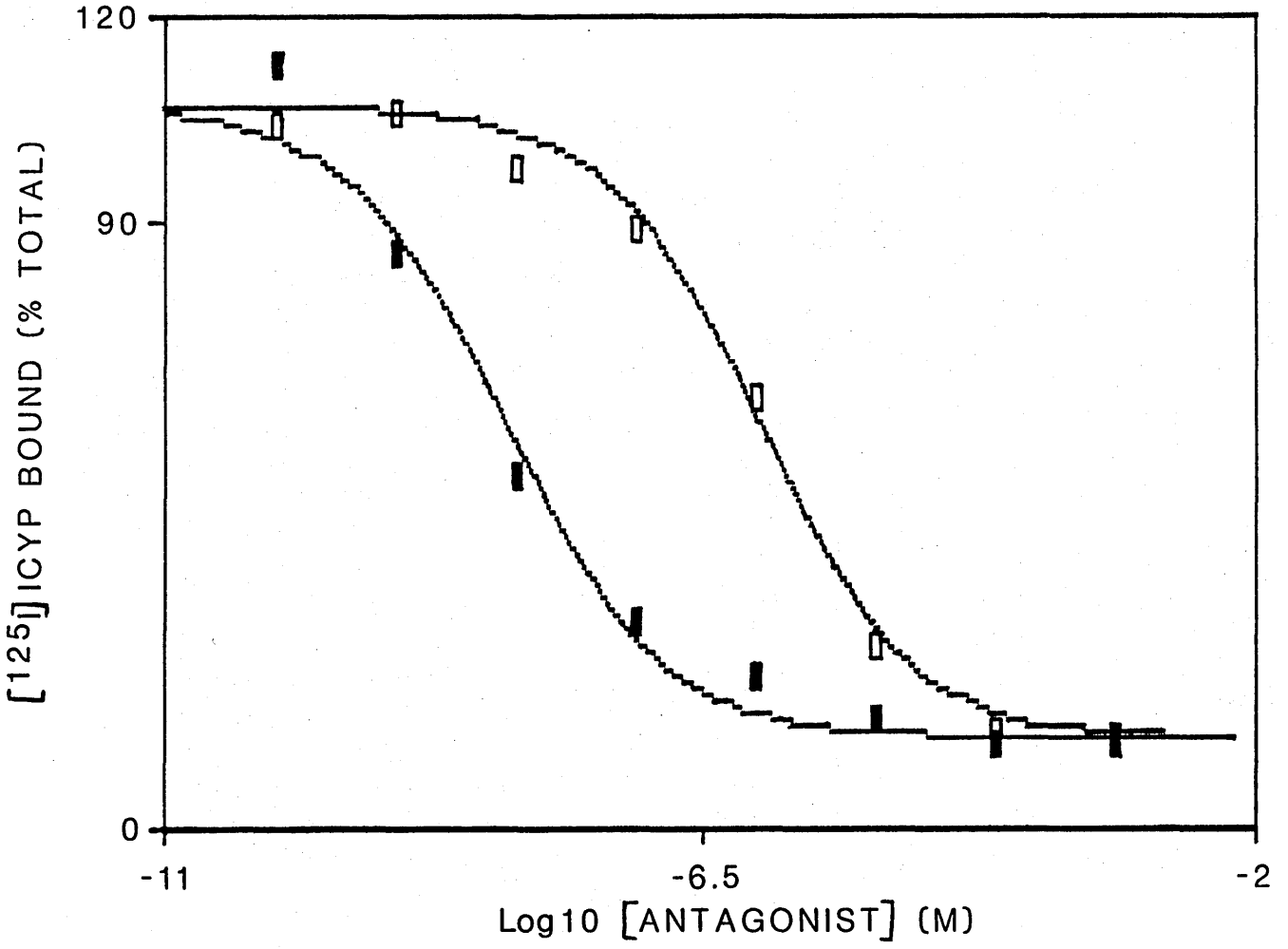

120
90
0
[125I]ICYP BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.8. Competition of [^{125}I]ICYP binding to sheep subcutaneous adipose tissue membranes by CGP 20712A and ICI 118,551

Subcutaneous adipose tissue membranes (15 µg protein) from 3-4-year old ewes were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (open squares) and ICI 118,551 (closed squares). Total binding represents [^{125}I]ICYP bound in the absence of any antagonist. Curves were analysed using the ALLFIT computer program. IC_{50} values for CGP 20712A and ICI 118,551 were 2.99 µM (s.e.d.=0.472) and 5.14 nM (s.e.d.=1.516), respectively (n=8). This is a representative experiment (lactating ewe), which was performed eight times with preparations from different sheep (4 non-lactating & 4 lactating) with similar results. Each point represents the mean of duplicate observations.

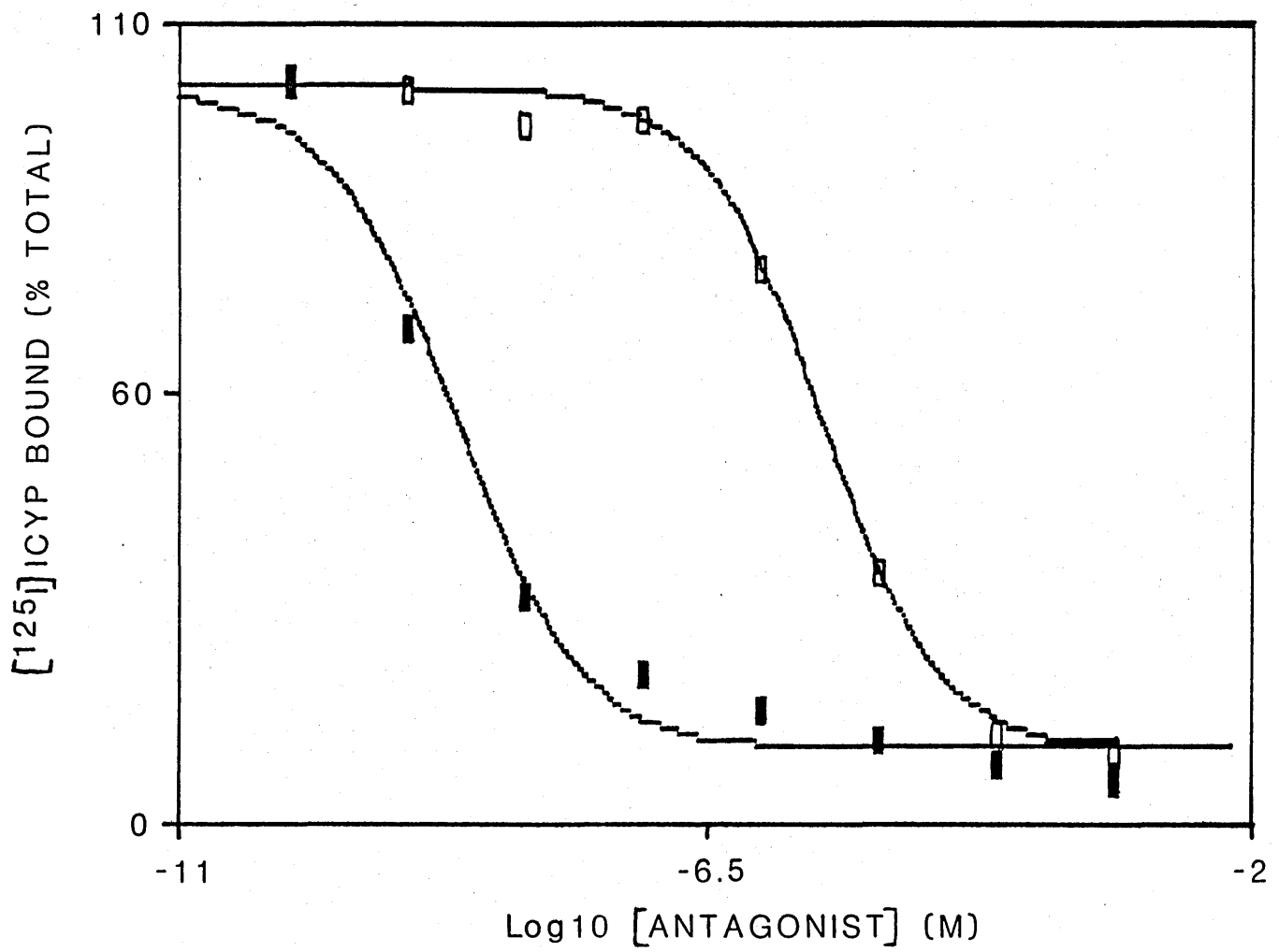

110
60
0
[125I]ICYP BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.9. Competition of [^{125}I]ICYP binding to sheep popliteal adipose tissue membranes by CGP 20712A and ICI 118,551

Popliteal adipose tissue membranes (15 µg protein) from 3-4-year old ewes were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (open squares) and ICI 118,551 (closed squares). Total binding represents [^{125}I]ICYP bound in the absence of any antagonist. Curves were analysed using the ALLFIT computer program. IC_{50} values for CGP 20712A and ICI 118,551 were 3.55 µM (s.e.d.=0.472) and 6.82 nM (s.e.d.=1.516), respectively (n=8). This is a representative experiment (lactating ewe), which was performed eight times with preparations from different sheep (4 non-lactating & 4 lacating) with similar results. Each point represents the mean of duplicate observations.

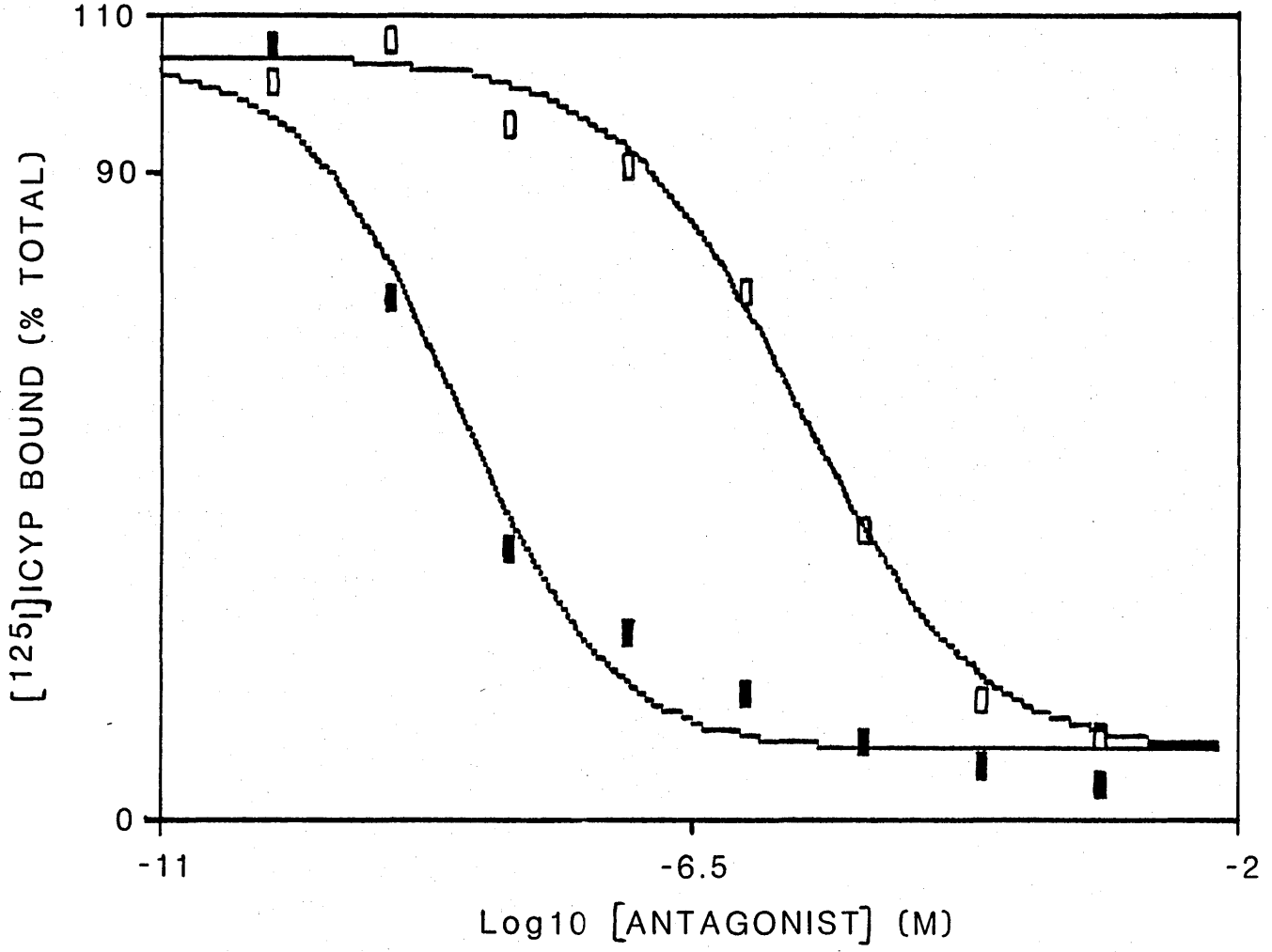

110
90
0
[125I]ICYP BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.10. Competition of [^{125}I]ICYP binding to sheep omental adipocyte membranes

Adipocyte membranes and stroma-vascular membranes were prepared from sheep (non-lactating ewe) omental adipose tissue, by collagenase digestion and differential centrifugation (section 2.3.1). Adipocyte plasma membranes (15 μg protein) were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (open squares) and ICI 118,551 (closed squares). Total binding represents [^{125}I]ICYP bound in the absence of antagonist. β-Adrenergic receptor ligand binding was 141.1 fmol/mg membrane protein. This experiment was performed once, and each point represents the mean of duplicate observations.

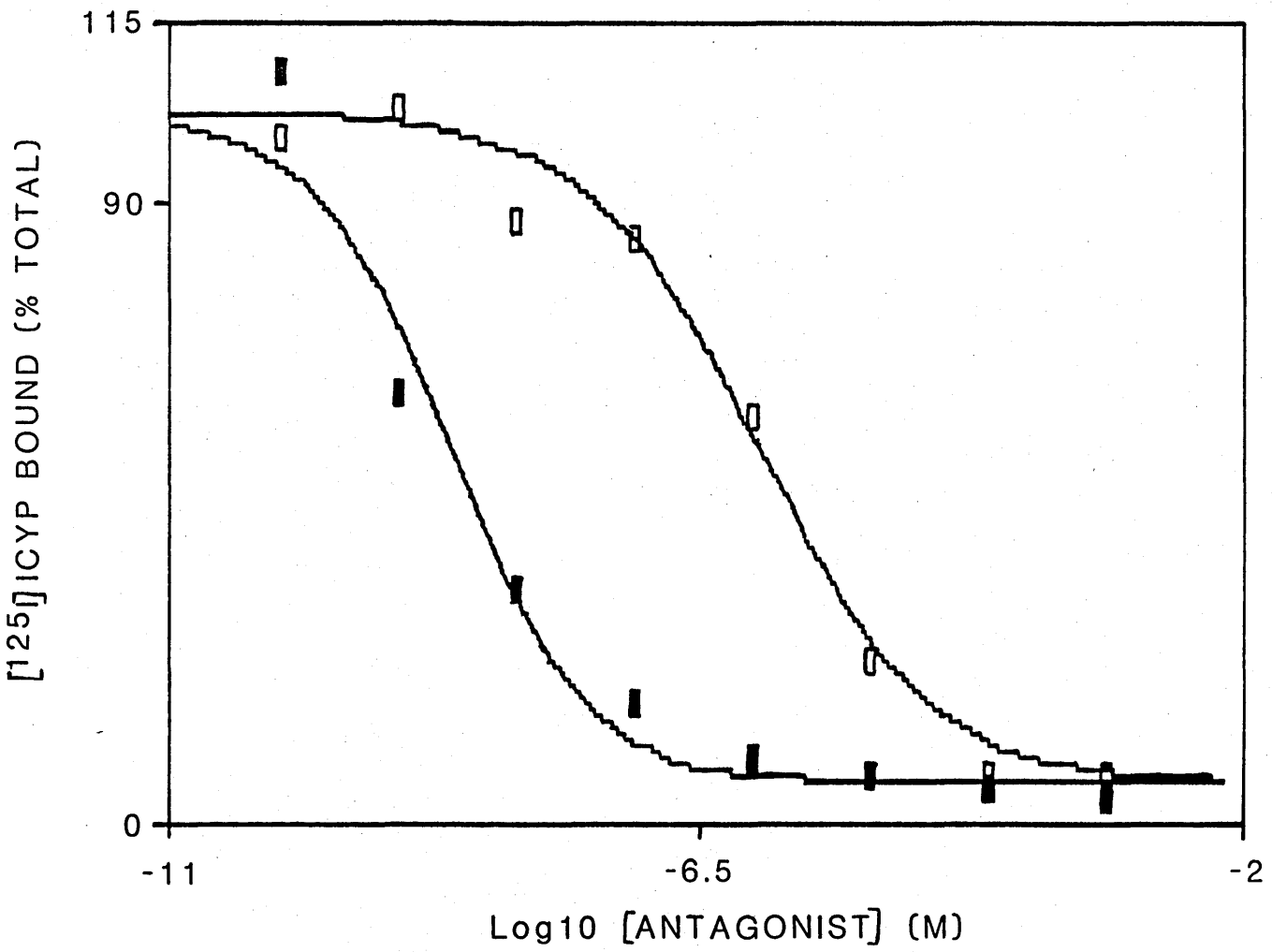

115
90
0
[125I]ICYP BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.11. Competition of [^{125}I]ICYP binding to stroma-vascular membranes from sheep omental adipose tissue

Adipocyte membranes and stroma-vascular membranes were prepared from sheep (non-lactating ewe) omental adipose tissue, by collagenase digestion and differential centrifugation (section 2.3.1). Stroma-vascular membranes (15 μg protein) were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (open squares) and ICI 118,551 (closed squares). Total binding represents [^{125}I]ICYP bound in the absence of antagonist. β-Adrenergic receptor ligand binding was 62.9 fmol/mg membrane protein. This experiment was performed once, and each point represents the mean of duplicate observations.

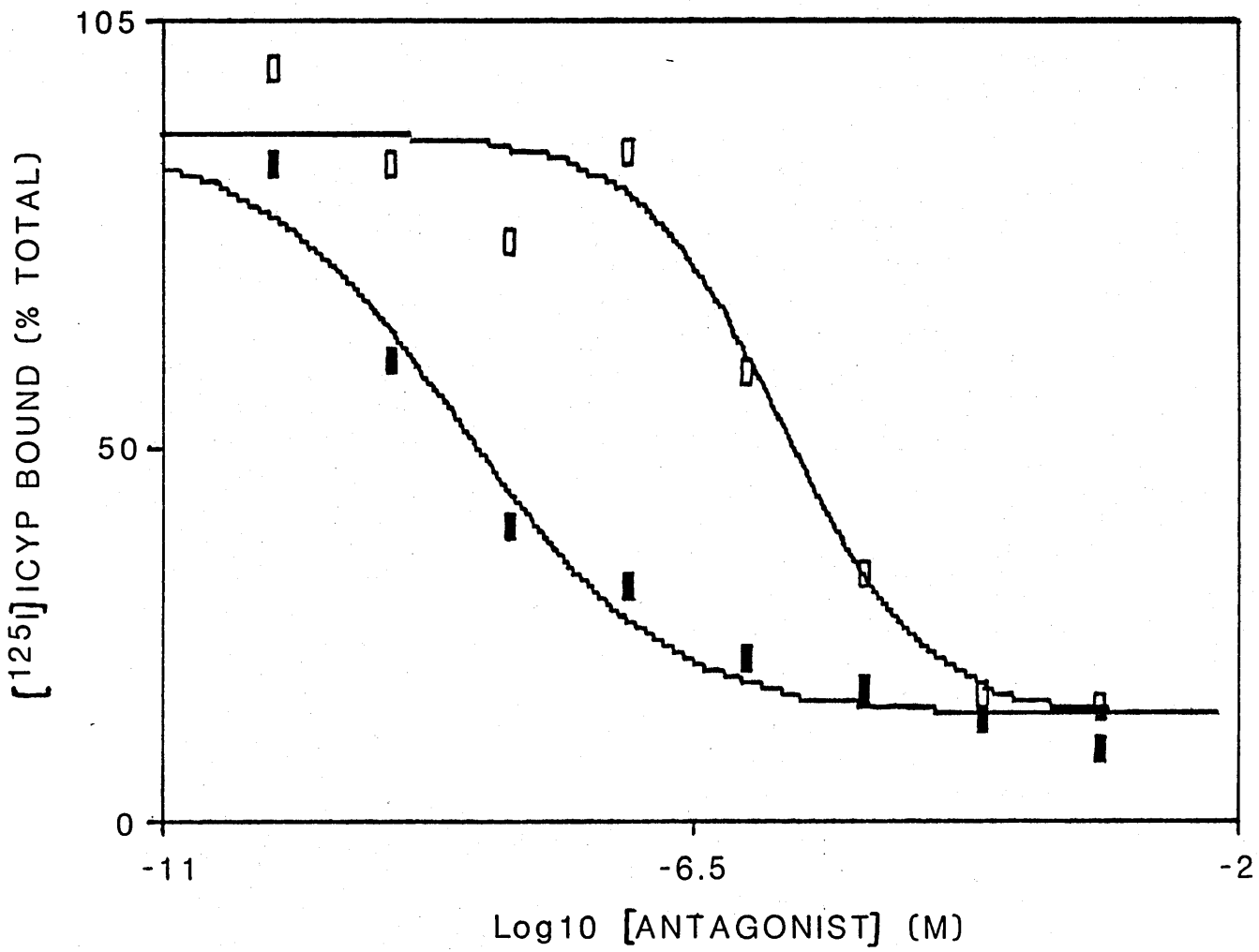

105
50
0
[125I]ICYP BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.12. Effect of lactation on β-adrenergic receptor ligand binding in three sheep adipose tissue depots

Sheep (ewes) adipose tissue membranes were prepared from omental, subcutaneous and popliteal adipose tissues (section 2.3.2). Membranes (15 μg protein) were incubated with 75 pM [^{125}I]ICYP for 60 min at 22°C. Non-specific binding was determined in the presence of 100 μM (-)-isoproterenol. Results are means of four independent observations in each case, analysed by analysis of variance. The results presented are tabulated in Table 4.5. *, **, value significantly different, $P<0.02$ & $P<0.01$, respectively, from corresponding value for non-lactating sheep for $\log_{10}$ transformed results. $\log_{10}$ s.e.d. between depots within the same physiological state = 0.0743; $\log_{10}$ s.e.d. for the effect of lactation for each depot = 0.0918

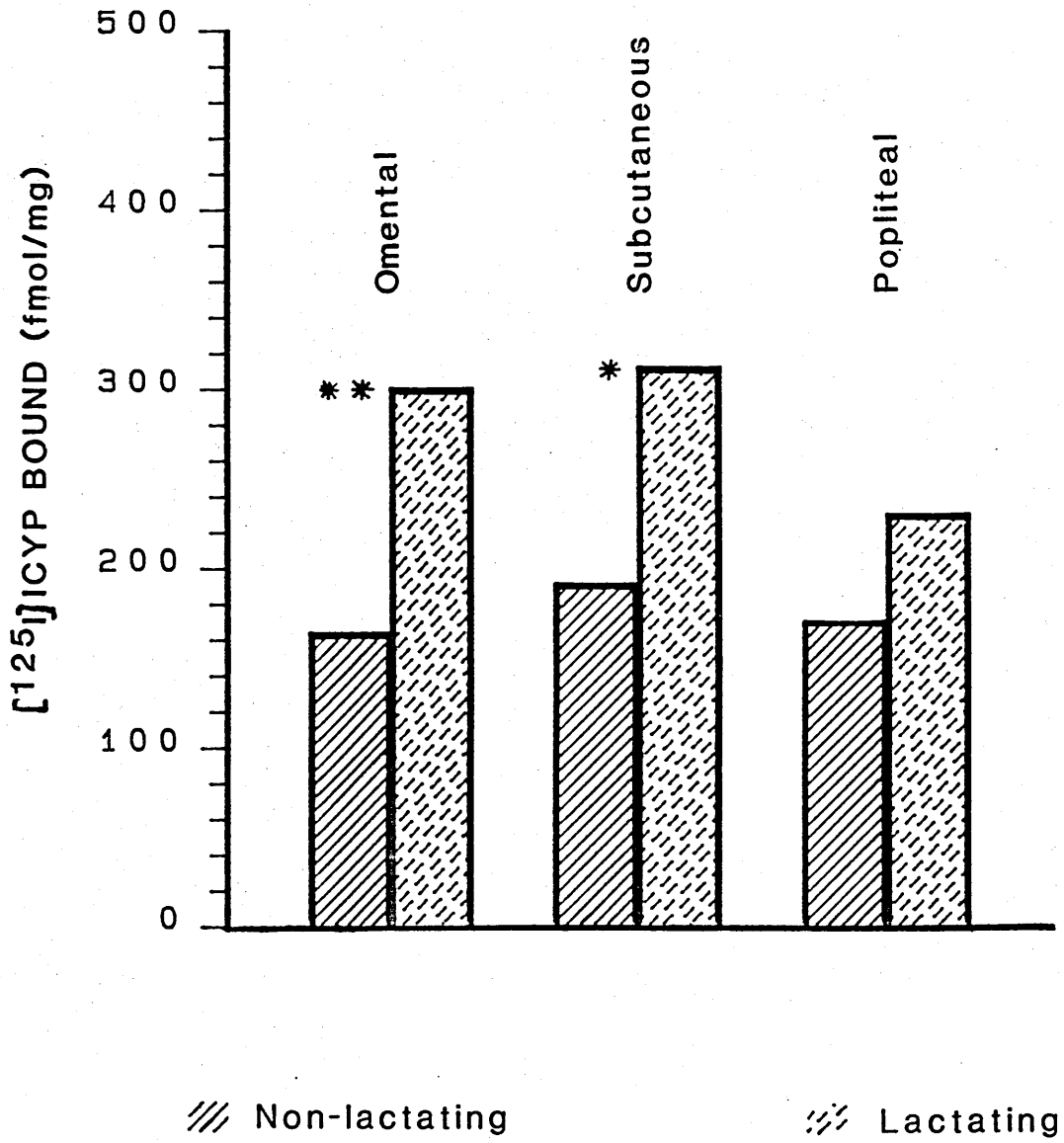

500
400
300
200
100
0
[125I]ICYP BOUND (fmol/mg)
Omental
Subcutaneous
Popliteal
**
*
Non-lactating
Lactating

Fig. 4.13. Competition of [^{3}H]DHA binding to rat adipocyte membranes by isoproterenol, adrenaline and noradrenaline

Rat adipocyte membranes (100 µg protein) prepared from abdominal adipose tissue were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the agonists (-)-ispoproterenol (open squares), (-)-adrenaline (closed squares) and (-)-noradrenaline (open circles). Total binding represents [^{3}H]DHA bound in the absence of agonist. Curves were analysed using the ALLFIT computer program. This data is from a single experiment. Each point represents the mean of triplicate observations.

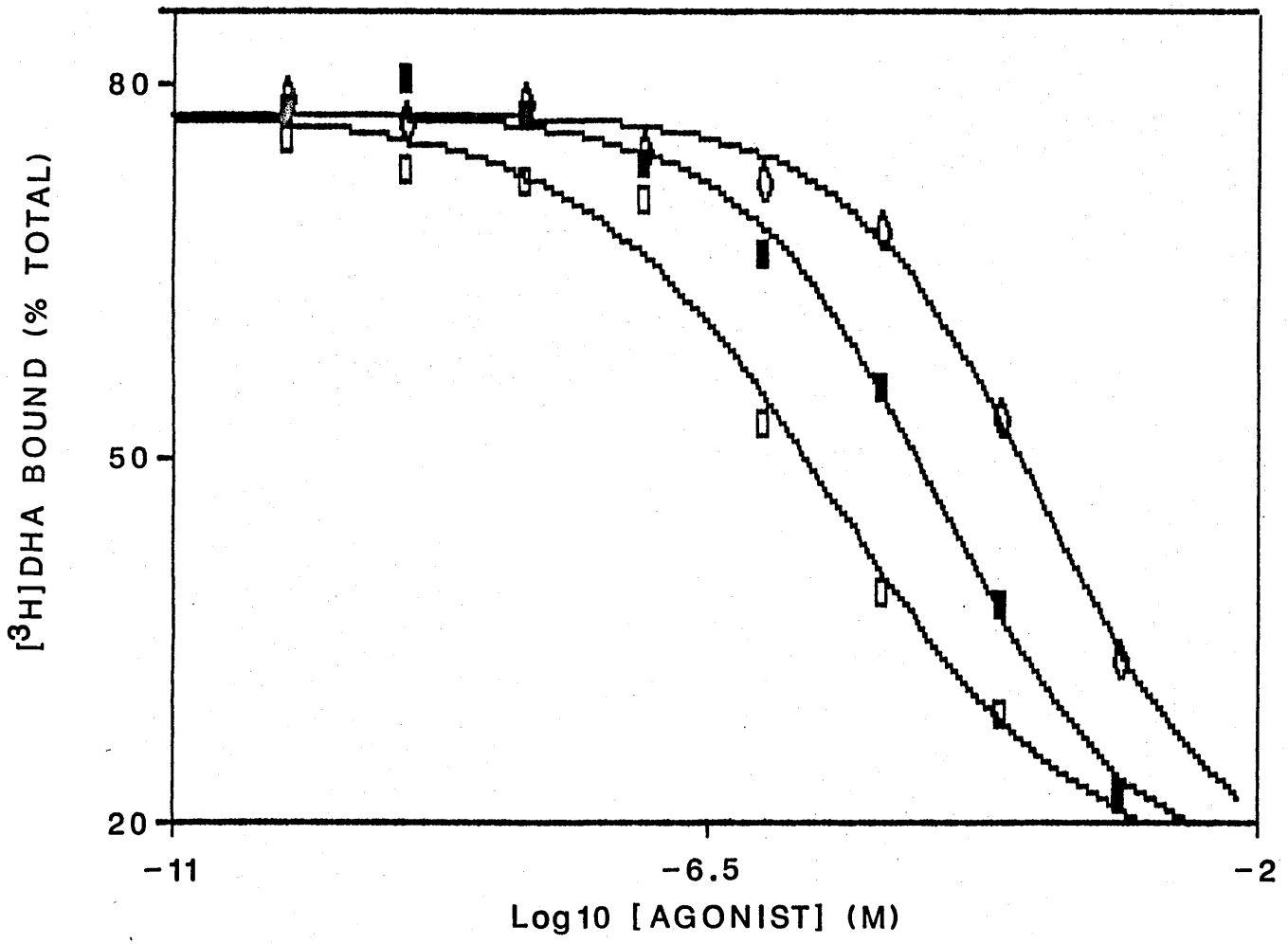

80
50
20
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [AGONIST] (M)

Fig. 4.14. Competition of [^{3}H]DHA binding to rat adipocyte membranes by ICI 118,551 and CGP 20712A

Rat adipocyte membranes (100 μg protein) prepared from abdominal adipose tissue were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the antagonists ICI 118, 551 (closed squares) and CGP 20712A (open squares). Total binding represents [^{3}H]DHA bound in the absence of antagonist. Curves were analysed using the ALLFIT computer program. This is a representative experiment, repeated three times with similar results. Each point represents the mean of triplicate observations.

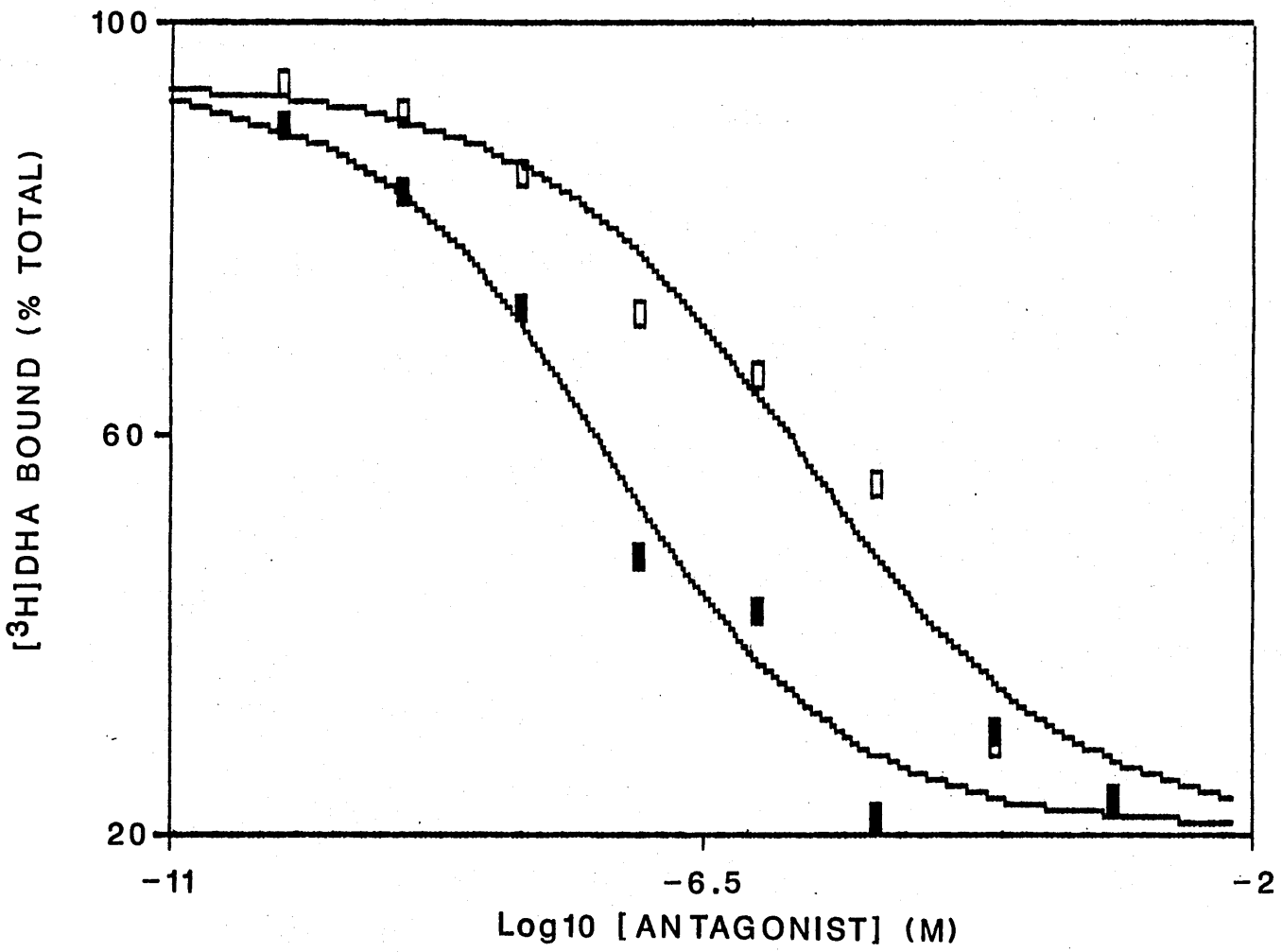

100
60
20
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.15. Competition of [^{3}H]DHA binding to rat adipocyte membranes by ICI 118,551 and atenolol

Rat adipocyte membranes (100 µg protein) prepared from abdominal adipose tissue were incubated with 4 nM [^{3}H]DHA for 10 min at 37°C, in the presence of the indicated concentrations of the antagonists ICI 118, 551 (closed squares) and atenolol (open squares). Total binding represents [^{3}H]DHA bound in the absence of antagonist. Curves were analysed using the ALLFIT computer program. This data is from a single experiment. Each point represents the mean of triplicate observations.

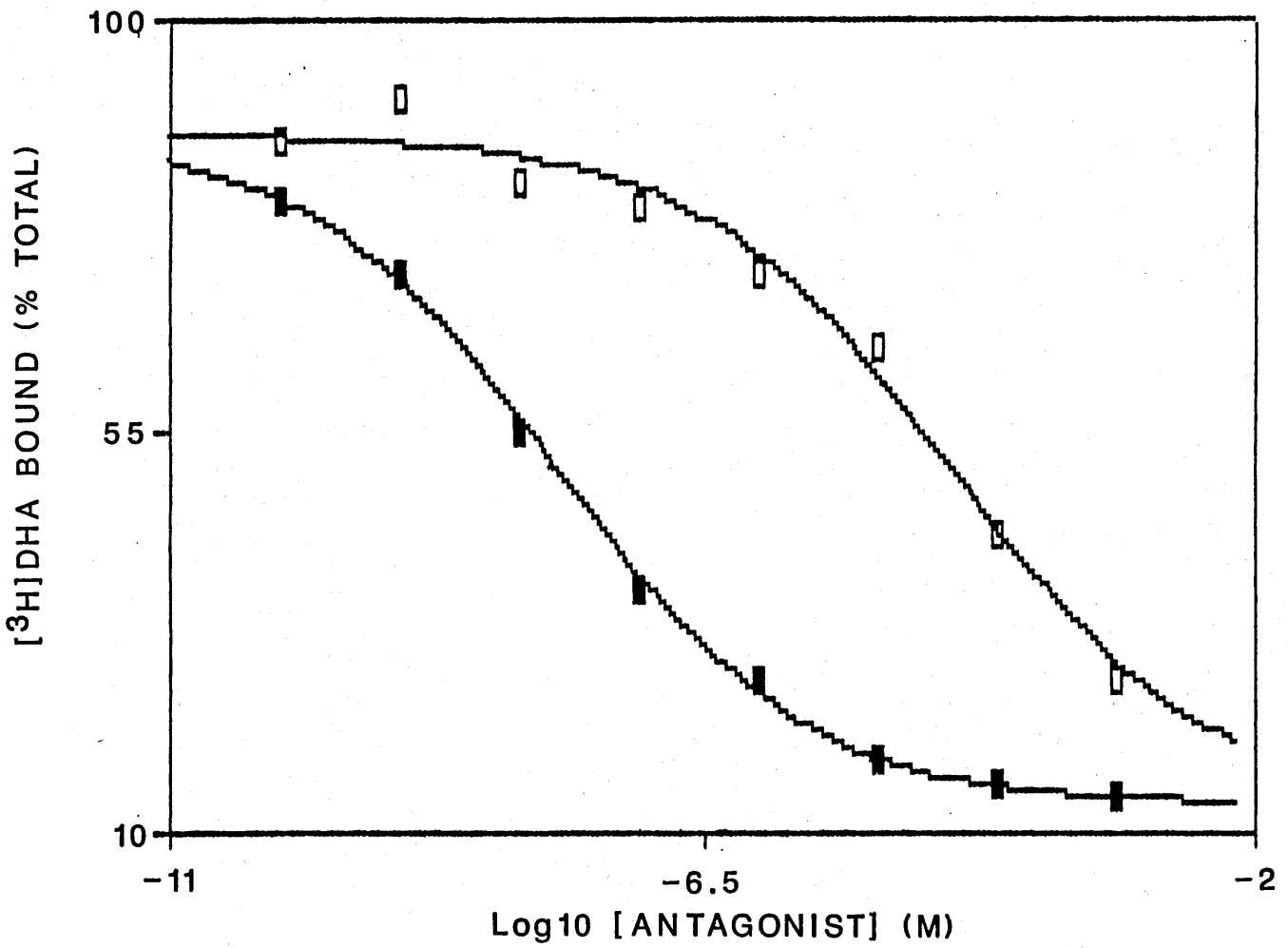

100
55
10
[3H]DHA BOUND (% TOTAL)
-11
-6.5
-2
Log10 [ANTAGONIST] (M)

Fig. 4.16. Competition of [^{125}I]ICYP binding to rat parametrial adipocyte membranes by CGP 20712A and ICI 118,551

Rat parametrial adipocyte membranes (20 µg protein) were incubated with 85 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (closed circles) and ICI 118,551 (open circles). Total binding represents [^{125}I]ICYP bound in the absence of antagonist. This is a representative experiment, which was performed three times with similar results. Each point represents the mean of duplicate observations.

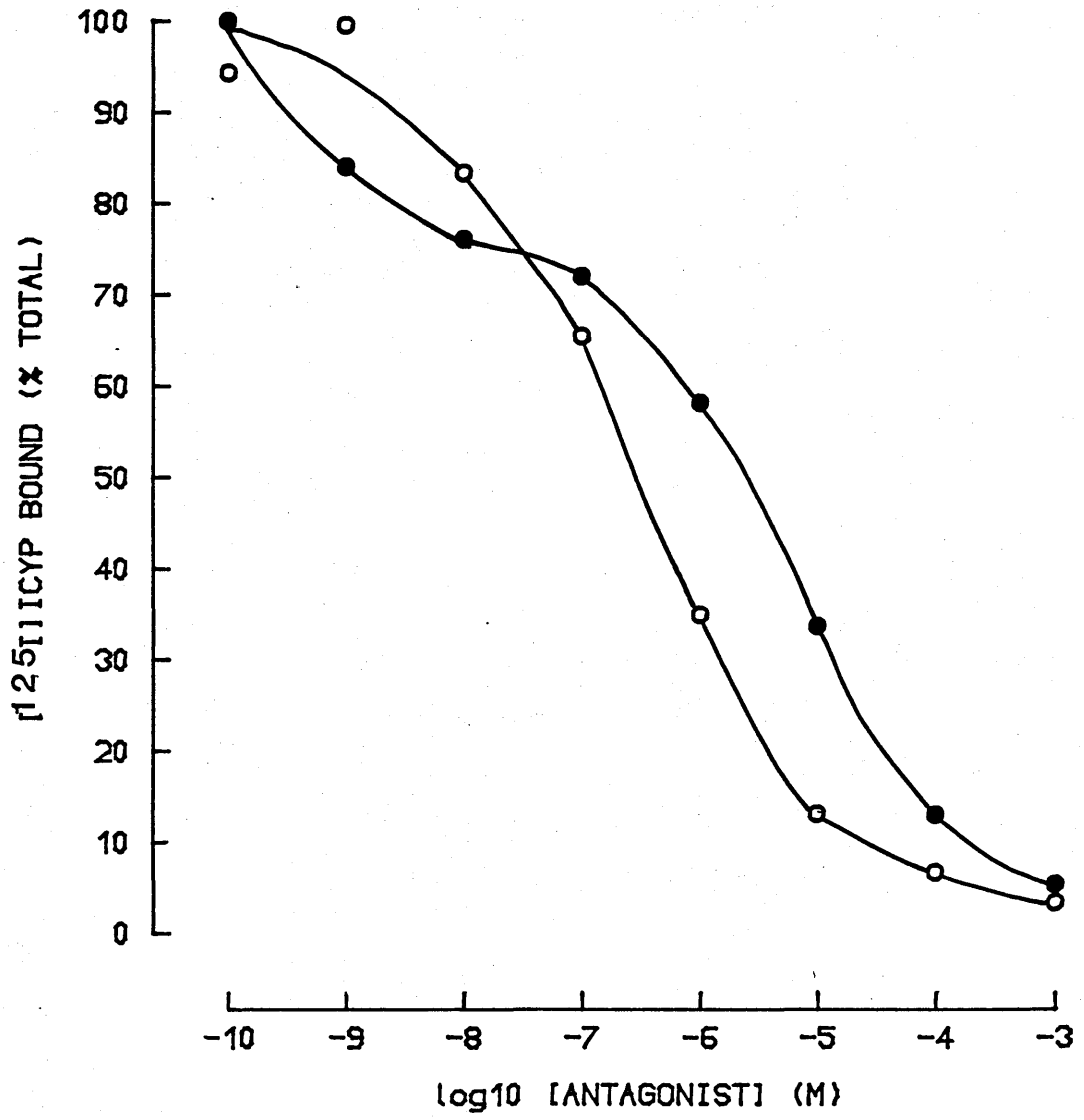
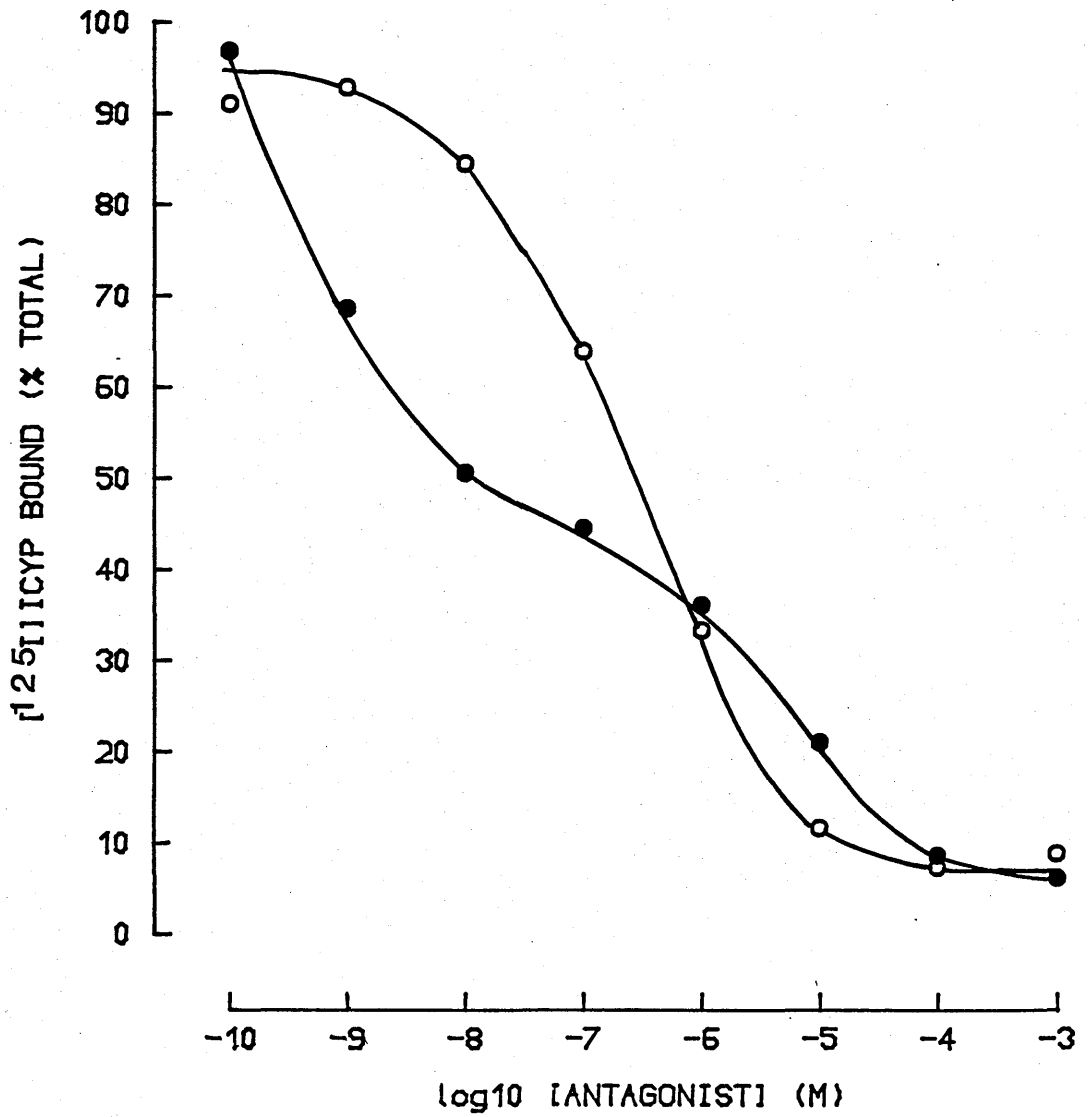

100
90
80
70
60
50
40
30
20
10
0
[125I]ICYP BOUND (% TOTAL)
-10
-9
-8
-7
-6
-5
-4
-3
log10 [ANTAGONIST] (M)

Fig. 4.17. Competition of [^{125}I]ICYP binding to rat lumbar adipocyte membranes by CGP 20712A and ICI 118,551

Rat lumbar adipocyte membranes (20 µg protein) were incubated with 85 pM [^{125}I]ICYP for 60 min at 22°C, in the presence of the indicated concentrations of the antagonists CGP 20712A (closed circles) and ICI 118,551 (open circles). Total binding represents [^{125}I]ICYP bound in the absence of antagonist. This is a representative experiment, which was performed three times with similar results. Each point represents the mean of duplicate observations.


100
90
80
70
60
50
40
30
20
10
0
[125I]ICYP BOUND (% TOTAL)
-10
-9
-8
-7
-6
-5
-4
-3
log10 [ANTAGONIST] (M)

CHAPTER FIVE

IMMUNOLOGICAL STUDIES ON THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

CHAPTER FIVE - IMMUNOLOGICAL STUDIES ON THE β-ADRENERGIC RECEPTOR OF SHEEP ADIPOSE TISSUE

5.1. Introduction

The use of immunologically-based techniques in biochemistry has increased markedly in the last decade. The specificity of antibody-antigen interaction allows the identification of both solid-phase (EIA) and soluble (RIA, EIA) antigens/antibodies. In the field of receptor characterisation, antibodies raised against receptors can be used to determine structural and even sequence homology between receptors from different species and tissues, or between pharmacologically distinct receptor subtypes. These studies can often provide additional information to further substantiate results from receptor photaffinity-labelling and purification. Antibodies can be used for quantification or localisation of receptor protein, either by immunoblotting or immunocytochemistry. This has the advantage over radioligand binding assays of being able to identify receptors which are incapable of binding ligands e.g. proreceptors or partially-degraded receptors.

The aim of this work was the production of antibodies directed against the β-adrenergic receptor of sheep adipose tissue. Collaboration with other researchers was initiated to obtain samples of antisera specific for β-adrenergic receptors from other species and tissues.

5.2. Experimental procedure

All methods used in this study are fully described in Chapter 2. Membranes were prepared from sheep omental adipose tissue. Antiserum CM 13 (Wang et al., 1989c) was a generous gift from Dr Craig Malbon, Department of Pharmacological Sciences, State University of New York, New York, U.S.A. CM 13 is a rabbit polyclonal antiserum raised against a

synthetic decapeptide whose sequence is contained in hamster lung β_2-adrenergic receptor (Wang et al., 1989c). Monoclonal antibody BRK2 was provided by Professor A. D. Strosberg, Institut Pasteur, Paris, France. BRK2 was raised against the β_2-adrenergic receptor of human A431 cells, and recognises β_1- and β_2-adrenergic receptors in many species (Kaveri et al., 1987).

5.3. Results

5.3.1. Screening of mouse antisera for antibodies against the β-adrenergic receptor

5.3.1.1. EIA

Mice were immunised as detailed in section 2.12.1. Serum samples obtained were initially screened by EIA (section 2.12.4) for reactivity against sheep omental adipose tissue membranes and sheep erythrocyte ghosts. Using this assay it was hoped to select mice exhibiting strong anti-sheep adipose tissue responses and weak activity towards sheep erythrocyte ghosts. It was hypothesised that such reactivity may reflect the presence of antibodies against the β-adrenergic receptor, since sheep erythrocyte ghosts lack such receptors.

Sera from all mice exhibited reactivity towards sheep (lactating ewe) omental adipose tissue membranes (Figure 5.1). Mouse 4 (immunised with NaOH-treated sheep adipose tissue membranes) was the best responder against both membrane preparations, indicating a lack of selectivity towards sheep adipose tissue (Figures 5.1 & 5.2). Three mice immunised with partially-purified β-adrenergic receptor exhibited specific binding towards sheep adipose tissue membranes, with mouse 2 having the same response as normal mouse serum against sheep erythrocyte ghosts (Figures 5.1 & 5.2). With regards to mice immunised

with electroeluted 58 kDa proteins from SDS PAGE of sheep adipose tissue membranes, animals 5 and 6 possessed the best antibody responses against sheep adipose tissue membranes (Figure 5.1). Mouse 7 did not respond well, and had almost identical antibody titre against both membranes. High backgrounds in the EIA were due to non-specific binding of the alkaline-phosphatase second antibody to the plates. In later assays, this was significantly reduced by blocking the plate with 1% (w/v) BSA instead of Tween 20.

5.3.1.2. Immunoblotting

Sera from immunised mice were screened against sheep (ewe) omental adipose tissue membrane proteins by immunoblotting (section 2.16). The monoclonal antibody BRK2 was also tested in the same manner. All antisera except BRK2 recognised antigens in sheep adipose tissue membranes (Figure 5.3). The batch of BRK2 antibody supplied also failed to recognise β-adrenergic receptors in sheep adipose tissue, sheep lung, rat liver and human placenta in an EIA. These results are quite different from those published (Kaveri et al., 1987). The sample of BRK2 must therefore have contained an abnormally low concentration of antibodies (Professor A. D. Strosberg, personal correspondence). No binding of NMS was evident at a dilution of 1:50. Sera from mice immunised with partially-purified β-adrenergic receptors (AS1-3) recognised two major proteins, with approximate molecular weights of 55 kDa and 100 kDa. AS4, from a mouse immunised with NaOH-treated sheep adipose tissue membranes, recognised a whole spectrum of proteins, indicative of a poorly selective antiserum. Mice immunised with 58 kDa electroeluted proteins bound proteins ranging in molecular weight from 55-75 kDa. No molecular weight markers were run on the immunoblot in

Figure 5.3 and therefore the molecular weights quoted are estimates based on Rf values obtained from later immunoblotting experiments.

5.3.1.3. Inhibition of [^{125}I]ICYP binding to sheep adipose tissue membranes

A further screen for antibodies against the β-adrenergic receptor involved the competition of antisera for [^{125}I]ICYP binding sites on sheep omental adipose tissue membranes. Sera from mice immunised with partially-purified β-adrenergic receptor all inhibited binding of [^{125}I]ICYP, with AS2 inhibiting 22-27% of the binding obtained in the presence of NMS (Figure 5.4). Interestingly, AS5 also competed out [^{125}I]ICYP as efficiently as AS2, and therefore this ability was produced by immunisation with two of the three immunogens used. All other antisera exhibited little or no competition of binding. The experiment was only performed twice due to the limited availability (amounts) of antisera (20 µl/tube). No antibodies against [^{125}I]ICYP were detectable in the antisera using direct precipitation of the first antibody with 30% (w/v) PEG (data not shown).

After analysing all the results, mouse 2 was chosen for monoclonal antibody production since its antiserum; i) bound specifically to sheep adipose tissue membrane antigens, ii) recognised few antigens on immunoblots, some with molecular weights close to that of the β-adrenergic receptor, iii) inhibited [^{125}I]ICYP binding to sheep adipose tissue membranes, iv) contained no antibodies against [^{125}I]ICYP, v) was raised against an antigen which had been used successfully before, and was of the greatest purity. Antiserum (AS2) obtained from this mouse prior to fusion was screened by EIA for antibodies against sheep adipose tissue membranes. AS2 reacted very strongly against sheep adipose

tissue membranes, even at a dilution of 1:10,000 (Figure 5.5a). AS2 had a slightly higher titre against sheep erythrocyte ghosts compared to normal mouse serum, which is probably a function of an increased serum antibody concentration in this immuno-stimulated animal. Binding of second antibody again caused an increase in background absorbance of ~0.8 units.

5.3.2. Production of monoclonal antibodies

Splenocytes from a mouse (mouse 2) immunised four times with 1 pmol of partially-purified β-adrenergic receptors were fused with non-secreting myeloma cells. The efficiency of fusion was relatively poor, with less than a quarter of the wells containing sustainable growth of hybridomas in the presence of HAT medium. The number of wells containing hybridomas in plates 1-3 were 23%, 18% and 23%, respectively. Supernatants from cultures of fused cells were screened twice by EIA for the presence of antibodies against sheep adipose tissue membranes (section 2.12.4). Absorbance changes for wells deemed positive against sheep adipose tissue membranes were small, typically 0.15-0.2. However, there was a good correlation between wells deemed positive by EIA and wells containing growing hybridomas.

Twenty-four wells which were positive in EIA and contained good growth (61 wells possessed growth) were expanded into a 24-well plate for further screening. Analysis by EIA after expansion revealed that 11 out of 24 wells contained specific antibodies against sheep adipose tissue membranes. These wells were studied using more elaborate assays to detect specific antibodies against the β-adrenergic receptor, rather than activity towards other sheep adipose tissue membrane antigens. Hybridoma culture supernatants were initially tested for their ability to

immunoprecipitate solubilised β-adrenergic receptors (section 2.14). Direct measurement of β-adrenergic receptors in supernatants following immunoprecipitation indicated that no precipitation of receptors was evident (Table 5.1). Suprisingly, more β-adrenergic receptors were measurable in samples containing hybridoma supernatants, relative to those incubated with conditioned HT medium alone. Culture supernatants also failed to inhibit binding of [^{125}I]ICYP to sheep adipose tissue membranes (Table 5.2). In fact an enhancement of binding was obtained, which also occurred in the presence of the irrelevant monoclonals 2H11 and 2C12 raised against rGH (provided by Dr L. Q. Stevenson).

Immunoblotting with supernatants against sheep omental adipose tissue membranes showed that some wells contained hybridomas secreting antibodies against the 100 kDa protein recognised by antiserum 2 (AS2). However this result was seen at the detection limit of immunoblotting. In order to overcome this problem and also that of interference of culture supernatants with [^{125}I]ICYP binding, photoaffinity-labelled β-adrenergic receptors were incubated with hybridoma supernatants to see if they could be immunoprecipitated (section 2.15). Analysis of autoradiographs following immunoprecipitation and SDS PAGE showed that no specific precipitation of photoaffinity-labelled β-adrenergic receptors had occurred. However, Coomassie-blue staining of the SDS PAGE gel revealed that proteins with molecular weights of 55 kDa and 95 kDa were immunoprecipitated by some supernatants (Table 5.3).

5.3.3. Immunoblotting against sheep adipose tissue β-adrenergic receptors with antiserum CM 13

CM 13 is a rabbit polyclonal antiserum directed against the peptide Gln-Asp-Asn-Leu-Ile-Pro-Lys-Glu-Val-Tyr-Cys. The first ten

amino acids in this peptide correspond to residues 299-308 in the cloned hamster lung β_2-adrenergic receptor (Figure 5.6). The antiserum was received as a lyophilised powder, and was resuspensed in 500 µl of PBS, 1% (w/v) Marvel with no idea of the final protein concentration.

Immunoblots of sheep adipose tissue membranes with CM 13 showed prominent immunoreactivity against four proteins (Figure 5.7). Staining comprised of a doublet and two higher molecular weight bands. A repeat immunoblot, which possessed molecular weight markers, was performed using a peroxidase anti-peroxidase amplification system. On this blot the doublet of proteins was very clear, with little immunoreactivity towards other proteins (Figure 5.8, lane 3). The doublet comprised of polypeptides with molecular weights of 59 kDa and 61 kDa. No staining was observed with a 1:50 dilution of normal rabbit serum (Figure 5.8, lane 1). The immunostaining of AS2 against sheep adipose tissue membranes was greater in Figure 5.7 than in Figure 5.8, relative to CM 13 activity. The reasons behind this shift in relative immunoreactivity are not clear, but it could have resulted from the use of different second antibodies (i.e. AS2 - mouse; CM 13 - rabbit) in Figure 5.7, and differential activity of peroxidase anti-peroxidase and bridging antibodies in Figure 5.8.

5.4. Discussion

The aim of this work was to develop antibodies specific for the β-adrenergic receptor. From the purification work described in Chapter 3, it became clear that it was not possible to purify the β-adrenergic receptor from sheep adipose tissue membranes to homogeneity using the available techniques, without excessive loss of receptors. To purify the β-adrenergic receptor to homogeneity from membranes requires a

purification of about 40,000 fold (Shorr et al., 1981; Homcy et al., 1983; Benovic et al., 1984; Cubero & Malbon, 1984). SDS PAGE of partially-purified β-adrenergic receptor preparations indicated the presence of a whole spectrum of protein contaminants. It was therefore decided to use monoclonal antibody technology to produce the desired antibody, since a polyclonal antiserum raised against this immunogen would have been non-specific.

Three distinct immunogens were used for this purpose. One group of mice received 1 pmol of active β-adrenergic receptors, partially-purified by affinity chromatography. This amount of receptor has been successfully used for the production of monoclonal antibodies to the turkey erythrocyte β_1-adrenergic receptor (Couraud et al., 1983; Chapot et al., 1989), calf lung β_2-adrenergic receptor (Fraser & Venter, 1980) and human A431 β_2-adrenergic receptor (Kaveri et al., 1987). A second group of animals were immunised with proteins electroeluted from SDS PAGE gels of sheep adipose tissue membranes (with approximate molecular weights of 58 kDa). It was predicted that the β-adrenergic receptor would be contained in this preparation, based on the apparent molecular weight of photoaffinity-labelled receptor. About a 10-fold purification factor was also inherent in this method. A single mouse received NaOH-treated sheep omental adipose tissue membranes, which it was hoped contained all the integral membrane proteins, including the β-adrenergic receptor. NaOH-treatment strips off peripheral proteins which are non-covalently bound to the membrane (Mueller & Morrison, 1981). This immunogen was included because of the success of Deckman and Shinitzky (1989), who raised monoclonal antibodies against the S_2-serotonin receptor from rat brain using a similar method. Only 2.4% of protein

remained associated with sheep adipose tissue membranes after NaOH treatment, and represented a possible purification of the β-adrenergic receptor.

Antisera from all mice bound to sheep omental adipose tissue membranes in EIA, but animals immunised with partially-purified β-adrenergic receptors exhibited the greatest specific response. Two of these mice failed to recognise antigens in sheep erythrocyte ghosts, which is probably due to the relatively high purity of the immunogen used. Indeed, in immunoblotting experiments antisera from this group of animals stained only a few proteins. Mice immunised with 58 kDa proteins produced less specific antisera, but nonetheless immunoreactivity was limited to a few major proteins whose molecular weights centred around 58 kDa. The range of proteins recognised was most likely due to the size of the slice cut from the SDS PAGE gel (1 cm), and also perhaps from binding to precursors of 58 kDa proteins. This result also indicated that these antigens retained their immunoreactivity following electroelution and immunisation in the presence of SDS detergent. Antiserum from the single mouse which received integral membrane proteins bound equally well to sheep omental adipose tissue membranes and sheep erythrocyte ghosts in EIA, and also recognised a host of proteins on immunoblots. These results indicated that this antiserum was non-specific towards sheep adipose tissue membranes, but served to illustrate the selectivity obtainable by EIA for sheep adipose tissue membrane antigens prepared in a more specific fashion.

To select a mouse for the production of monoclonal antibodies, two further assay systems were employed. Antisera from all mice failed

to immunoprecipitate digitonin-solubilised β-adrenergic receptors. The reasons for this are unclear since no positive control was available to validate the assay protocol. However some antisera inhibited binding of [^{125}I]ICYP to sheep omental adipose tissue membranes. Although this suggests that antibodies were present that were binding to the β-adrenergic receptor, it is possible that [^{125}I]ICYP binding could have been reduced by steric hindrance from antibodies binding to non-receptor antigens in close proximity to the receptor. However, the degree of inhibition of two antisera, AS2 and AS5, was encouraging, especially since AS4 from the mouse immunised with NaOH-treated membranes did not inhibit binding, even though it recognised many antigens and had the highest titre in EIA.

Screening by EIA of hybridoma supernatants during the production of monoclonals revealed that some antibodies were present that bound sheep adipose tissue membrane antigens. This assay system has been successfully used by several groups, and appeared to be an ideal choice of first screen (Couraud et al., 1983; Kaveri et al., 1987; Chapot et al., 1989). In reality immunoreactivity in EIA was poor and absorbance changes were small. This was probably due to the limiting amounts of proteins on the wells and the low concentrations of antibodies in the culture supernatants (~1-5 μg/ml). Having been committed to this assay as a first screen, twenty-four wells with statistically higher titres against sheep adipose tissue membranes compared to sheep erythrocyte ghosts were expanded. Due to the low efficiency of fusion (~20% growth in each plate) the choice of wells was reduced from a possible 288 wells to only 61.

Further characterisation of the resulting hybridomas failed to

show the presence of antibodies against the β-adrenergic receptor. The hybridomas failed to; i) immunoprecipitate digitonin-solubilised β-adrenergic receptors, ii) inhibit [^{125}I]ICYP binding to sheep adipose tissue membranes, iii) immunoprecipitate solubilised photoaffinity-labelled β-adrenergic receptor, even though microgram quantities of other antigens were precipitated, iv) recognise a 58 kDa protein (or doublet) on immunoblots against sheep omental adipose tissue membranes. However, most of the assays for hybridoma characterisation used detergent-solubilised β-adrenergic receptor, and therefore it is possible that antibodies capable of binding native receptor were present but their binding was affected by the detergent.

The failure to produce a monoclonal antibody specific for β-adrenergic receptor was possibly due to a number of factors. Firstly, the amount of partially-purified β-adrenergic receptor was limiting. Only three animals could be immunised, and there was insufficient preparation for use as an antigen in the first EIA screen. Secondly, the EIA employed as a first screen was not sufficiently specific for antibodies against β-adrenergic receptors. Because the receptor represented less than 1% of protein in the immunogen, and even less in the membranes coated on the EIA plate, it is likely that any specific binding to β-adrenergic receptors would have been insignificant relative to immunoreactivity towards other major antigens. Thirdly, the growth of hybridomas post-fusion was poor. Since β-adrenergic receptor protein represented <1% of the immunogen, less than 1 in 100 hybridomas was likely to secrete antibodies against this receptor, or even less since the β-adrenergic receptor is probably poorly immunogenic due to its conserved sequence. However, some wells could have contained many

clones, and so even with this poor growth there was a chance of screening out a positive clone. Finally, the accurate measurement of β-adrenergic receptors using radioligand binding assays was not possible in the presence of hybridoma supernatants, because of an enhancement of binding due to an unknown factor.

A number of improvements could be made for subsequent studies: i) Increase the yield and purity of β-adrenergic receptor preparations used for immunisation, possibly by using larger amounts of membranes and a larger affinity column. ii) Following fusion of splenocytes and myeloma cells use only 24-well plates (2 ml wells) for growing hybridomas, and thus allow the use of more elaborate assays for the initial screens. iii) Use the immunoprecipitation of photoaffinity-labelled β-adrenergic receptor as a primary screen, since it involves direct interaction of antibodies with β-adrenergic receptor, and the analysis is not dependent on radioligand binding assays. iv) Perform more studies with CM 13 or BRK2 antibodies, with a view to using them as positive controls in assays.

Two antibodies were provided as gifts from other researchers, namely CM 13 (Dr Craig Malbon) and BRK2 (Professor A. D. Strosberg). Unfortunately BRK2 failed to recognise β-adrenergic receptors in sheep adipose tissue, or membrane-bound β-adrenergic receptors from various species and tissues in EIA which it had previously recognised with high affinity (Kaveri et al., 1987). A new sample of BRK2 has been promised at the time of writing. It is probable that BRK2 will recognise the sheep adipose tissue β-adrenergic receptor because of its lack of specificity with regards to species and tissue, and β-adrenergic receptor subtype (Kaveri et al., 1987; Raposo et al., 1989). Rabbit polyclonal

antiserum CM 13 is a site-directed anti-peptide antiserum raised against the hamster lung β_2-adrenergic receptor (Wang et al., 1989c). CM 13 recognised a doublet of proteins on immunoblots of sheep adipose tissue membranes, whose molecular weights were 59 kDa and 61 kDa. These bands most likely represent two isoforms of β-adrenergic receptor since CM 13 has already been shown to bind β-adrenergic receptors in mouse S49 lymphoma cells, hamster lung, and both human β_1- and β_2-adrenergic receptor subtypes (Wang et al., 1989c). Interestingly, CM 13 displayed no significant immunoreactivity towards the rat adipocyte β-adrenergic receptor (Wang et al., 1989c). CM 13 recognises a decapeptide sequence in the predicted exo-facial domain linking transmembrane helices 6 and 7 (see Figure 5.6). It therefore appears that the β-adrenergic receptor in sheep adipose tissue shares homology in this area with β-adrenergic receptors in S49 lymphoma cells, hamster lung, and human β_1- and β_2-adrenergic receptors, but not with the rat adipocyte β-receptor. This may reflect a divergence of the genes encoding these receptors during their evolution.

Human placental β_1- (Frielle et al., 1987) and β_2-adrenergic receptors (Kobilka et al., 1987a) share 2 out of 10 and 9 out of 10 amino acids in the decapeptide sequence to which CM 13 was raised, respectively. Since CM 13 binds to both these β-adrenergic receptors on immunoblots, it appears that this antiserum recognises the secondary structure of this extracellular loop rather than the primary amino acid sequence. Studies with CM 13 were limited by the amount of antiserum supplied. It would therefore be useful to synthesise the decapeptide antigen 'in house' and produce our own antiserum. The antiserum could then be used for immunocytochemistry, immunoblotting and for screening

an expression library of sheep adipose tissue proteins, with a view to cloning the β-adrenergic receptor. Some additional characterisation of the antiserum would have to carried out intially to confirm that it binds to the β-adrenergic receptor of sheep adipose tissue. Experiments could also be performed to see if the antiserum inhibits binding of adrenergic ligands to the β-adrenergic receptor, and immunoprecipitates digitonin-solubilised β-receptors.

In summary, the work detailed in this chapter has laid a good foundation for further studies in this area, having highlighted particular methodological problems. It appears that the major problem with the production of antibodies against the β-adrenergic receptor is the low abundance of this protein in cell plasma membranes. This hampers the production of a good immune response due to the use of small amounts of impure immunogens, and restricts the type of assays which can be used to detect antibodies against a single antigen. By overcoming this fundamental problem, it should be possible to raise antibodies against the β-adrenergic receptor of sheep adipose tissue using monoclonal antibody technology.

Table 5.1. Immunoprecipitation of digitonin-solublised β-adrenergic receptors by hybridoma culture supernatants

Hybridoma culture supernatants were tested for their ability to immunoprecipitate digitonin-solubilised β-adrenergic receptors from sheep (lactating ewe) omental adipose tissue, as detailed in section 2.14. Culture supernatants are from wells containing hybridomas secreting antibodies against sheep omental adipose tissue membrane antigens. Results are expressed as means of triplicate observations. The experiment was performed twice with similar results.

Sample	β-Adrenergic receptor ligand binding in supernatants after immunoprecipitation [^{125}I]ICYP bound specifically (CPM)
HT medium, 20% (v/v) FCS	3164
Normal mouse serum	3010
Polyclonal antiserum	
Antiserum 2 (AS2)	3078
Hybridoma supernatants	
A1	4045
A2	3884
A5	3736
A6	3720
B2	3828
B3	3844
B4	4333
C1	4314
C2	4752
C4	4330
C5	4539

Table 5.2. Inhibition of [^{125}I]ICYP binding to sheep adipose tissue membranes by hybridoma culture supernatants

Hybridoma culture supernatants were tested for their ability to inhibit binding of 75 pM [^{125}I]ICYP to sheep (lactating ewe) omental adipose tissue membranes, as detailed in section 2.13. 2H11 and 2C12 - anti-rGH hybridoma culture supernatants (controls provided by Dr L. Q. Stevenson). All other wells are culture supernatants from wells containing hybridomas secreting antibodies against sheep omental adipose tissue membrane antigens. Results are expressed as mean +/- S.E.M. of triplicate observations. This is a representative experiment which was performed twice with the same results. Binding was independent of the order in which samples were assayed.

Sample	[^{125}I]ICYP bound (CPM)
HT medium, 20% (v/v) FCS	4184 ± 280
Hybridoma culture supernatants	
A1	4612 ± 178
A2	4383 ± 154
A5	5360 ± 395
A6	5155 ± 225
B2	5318 ± 162
B3	5163 ± 274
B4	5266 ± 567
C1	5212 ± 291
C2	5512 ± 369
C4	4970 ± 65
C5	5439 ± 368
Anti-rGH hybridomas	
2H11	4899 ± 83
2C12	5289 ± 267

Table 5.3. Immunoprecipitation of sheep adipose tissue membrane proteins with hybridoma culture supernatants

Sheep (lactating ewe) omental adipose tissue membrane proteins were immunoprecipitated with hybridoma supernatants during precipitation of digitonin-solubilised photoaffinity-labelled β-adrenergic receptors (section 2.15). Coomassie blue staining of the resulting SDS PAGE gel revealed the immunoprecipitation of two separate protein species.

Hybridoma supernatant	Protein immunoprecipitated	
	55 kDa	95 kDa
A4	+	-
A6	+	-
B2	+	+
B6	+	+
C2	-	+
C4	+	+
D4	+	+

Fig. 5.1. Screening of mouse antisera against sheep adipose tissue membranes by EIA

Sheep (non-lactating ewe) omental adipose tissue membranes were coated onto 96-well microtitre plates (Immulon 2, Dynatech) overnight. Serial dilutions of mouse sera were incubated in the plates, and antibody binding quantified by EIA (section 2.12.4). Further details of immunogens are given in section 2.12.1. NMS - Normal mouse serum.

		Immunogen
●----●	NMS	
o----o	Mouse 1	Partially-purified β-adrenergic receptor
□----□	" 2	" " " "
△----△	" 3	" " " "
⊡----⊡	" 4	NaOH-treated sheep adipose tissue membranes
●----●	" 5	Electroeluted 58 kDa proteins
■----■	" 6	" " " "
▲----▲	" 7	" " " "

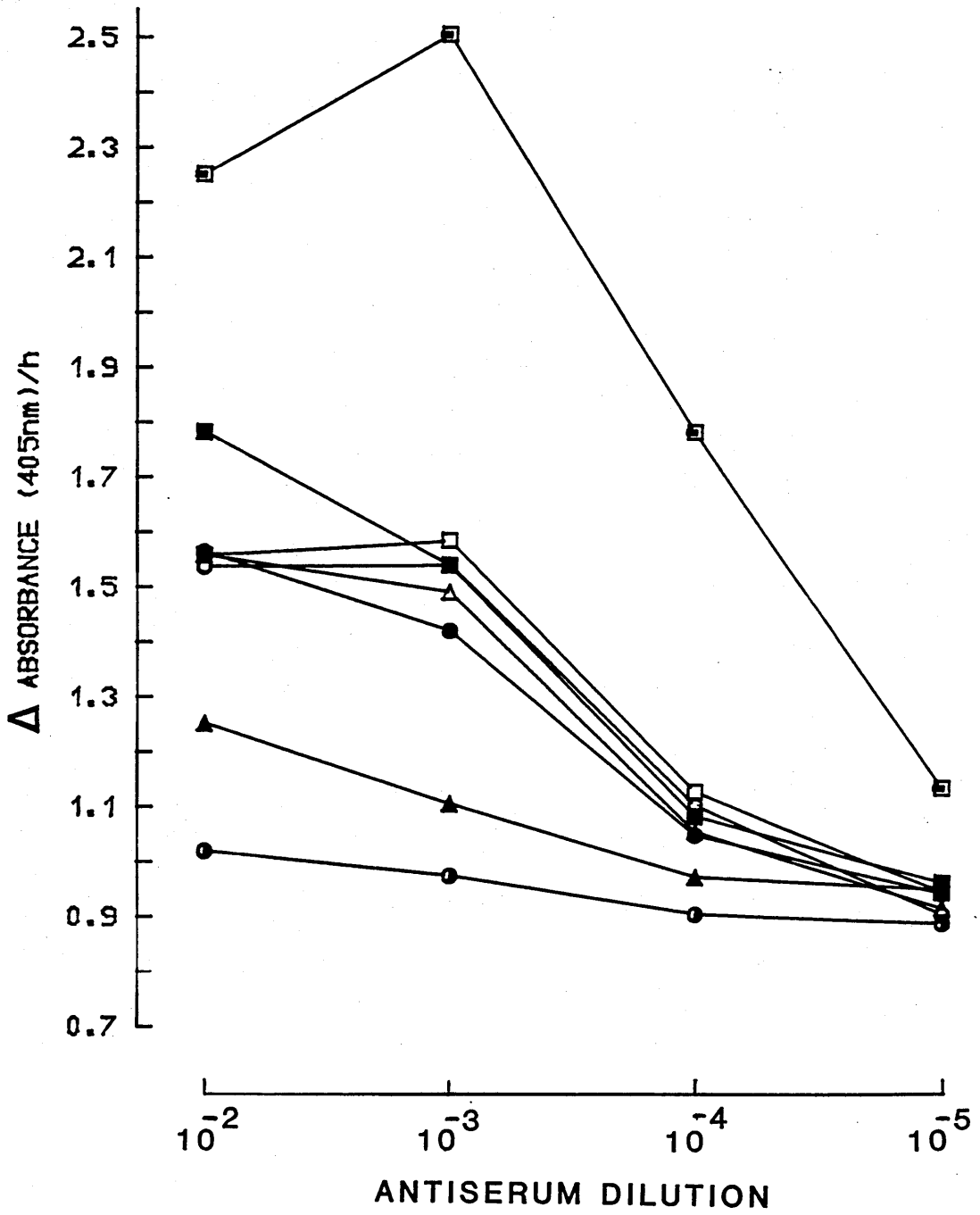

2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
Δ ABSORBANCE (405nm)/h
10-2
10-3
10-4
10-5
ANTISERUM DILUTION

Fig. 5.2. Screening of mouse antisera against sheep erythrocyte ghosts by EIA

Sheep erythrocyte ghosts were coated onto 96-well microtitre plates (Immulon 2, Dynatech) overnight. Serial dilutions of mouse sera were incubated in the plates, and antibody binding quantified by EIA (section 2.12.4). Further details of immunogens are given in section 2.12.1.

		Immunogen
●----●	NMS	
○----○	Mouse 1	Partially-purified β-adrenergic receptor
□----□	" 2	" " " "
△----△	" 3	" " " "
⊡----⊡	" 4	NaOH-treated sheep adipose tissue membranes
●----●	" 5	Electroeluted 58 kDa proteins
■----■	" 6	" " " "
▲----▲	" 7	" " " "

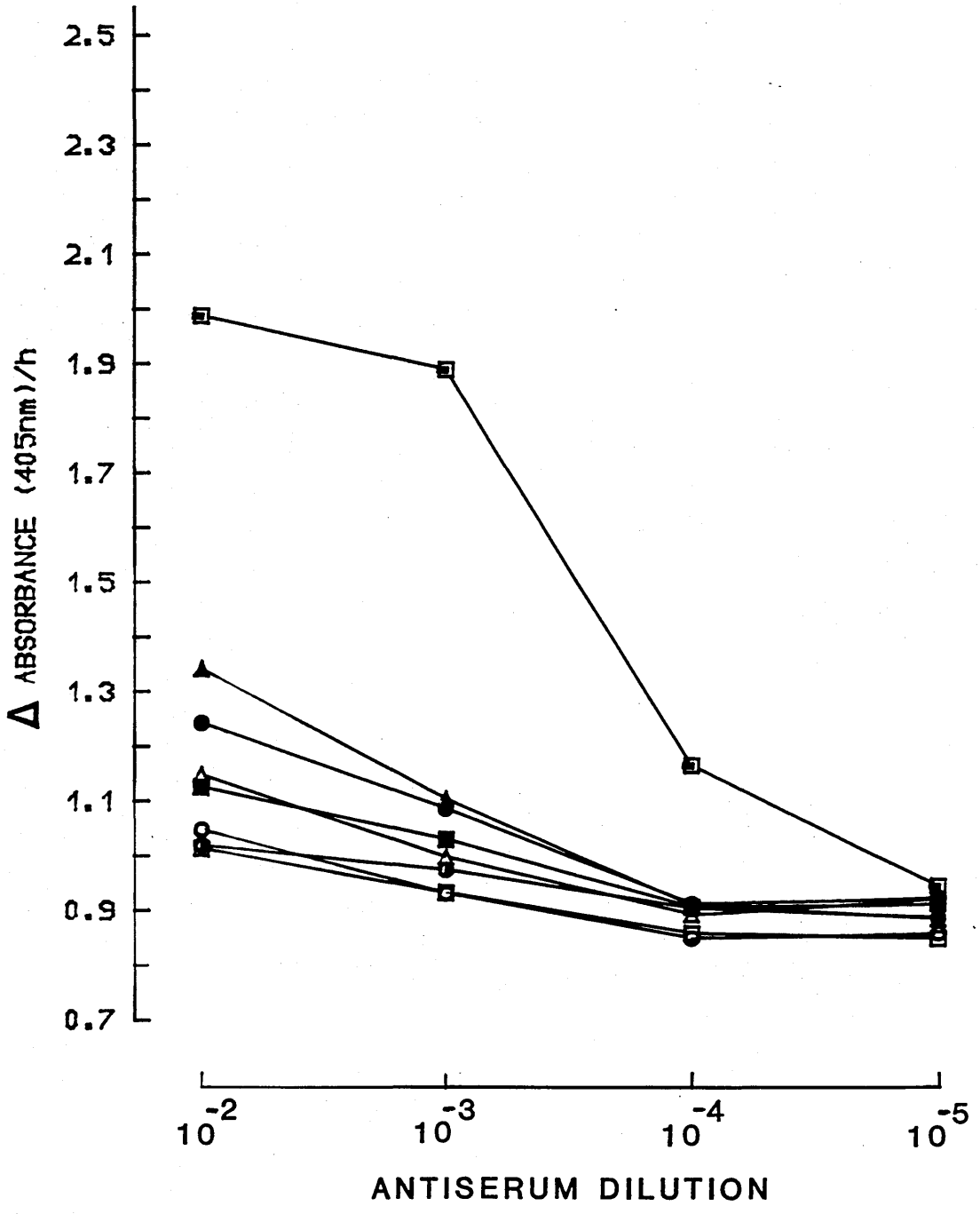

2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
Δ ABSORBANCE (405nm)/h
10-2
10-3
10-4
10-5
ANTISERUM DILUTION

Fig. 5.3. Immunoblotting against sheep adipose tissue membranes with mouse polyclonal antisera and BRK2 monoclonal antibody

Sheep (non-lacating ewe) omental adipose tissue membrane proteins (5 μg protein/well) were subjected to SDS PAGE, transferred to nitrocellulose, and probed with antisera (section 2.16).

Lane	Immunogen	Dilution
1	Normal mouse serum	1:50
2	Partially-purified β-adrenergic receptor	"
3	" " " "	"
4	" " " "	"
5	NaOH-treated sheep adipose tissue membranes	"
6	Electroeluted 58 kDa proteins	1:300
7	" " " "	"
8	" " " "	1:100
9	Normal mouse serum	1:50
10	BRK2 monoclonal antibody	1:50

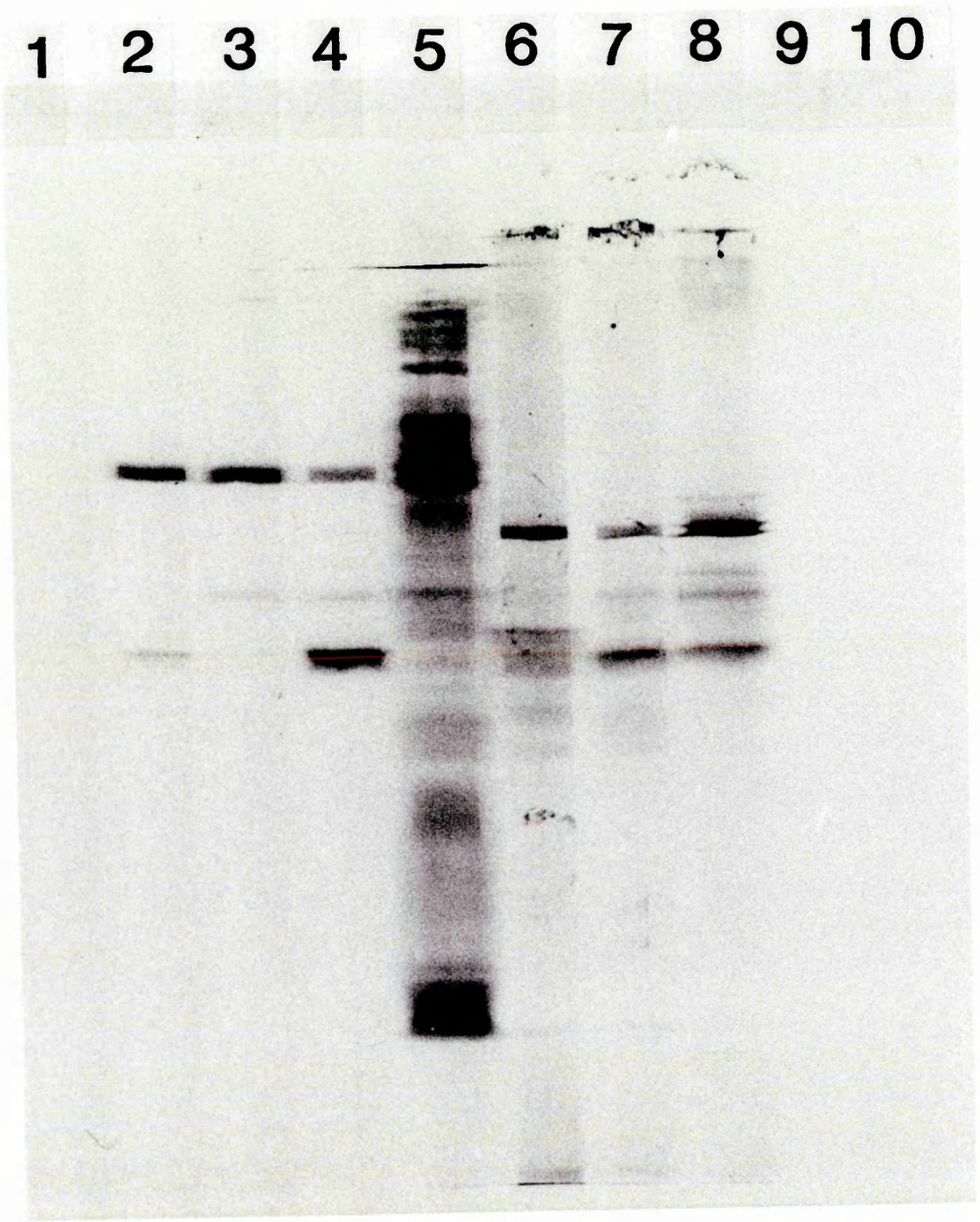

1 2 3 4 5 6 7 8 9 10

Fig. 5.4. Inhibition of [^{125}I]ICYP binding to solubilised sheep adipose tissue membranes by mouse antsera

Digitonin-solubilised sheep (non-lactating ewe) omental adipose tissue membranes (25 μg protein) were incubated with mouse antisera (1:10 final dilution) for 2 h at 22°C. [^{125}I]ICYP was added to a final concentration of 75 pM, and samples incubated for a further 2 h at 22°C. [^{125}I]ICYP binding was then quantified using the PEG precipitation assay for soluble β-adrenergic receptors. The data represents results from two independent experiments, and are expressed as percentage of [^{125}I]ICYP binding in the presence of normal mouse serum (NMS). Samples were analysed in duplicate. See section 2.14.1 for further details of immunogens.

	Immunogen
NMS	
Mouse 1	Partially-purified β-adrenergic receptor
" 2	" " " "
" 3	" " " "
" 4	NaOH-treated sheep adipose tissue membranes
" 5	Electroeluted 58 kDa proteins
" 6	" " " "
" 7	" " " "

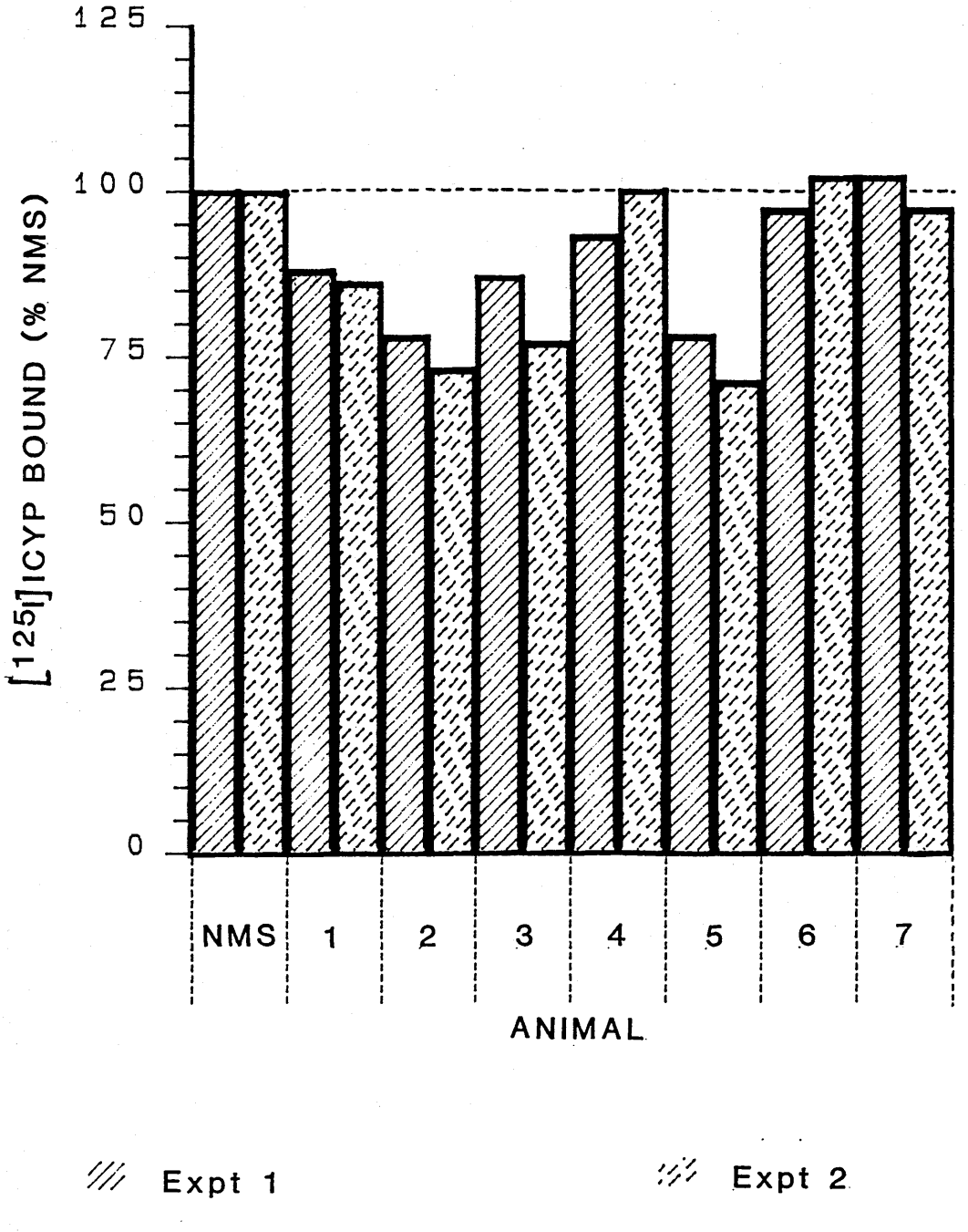

125
100
75
50
25
0
[125I]ICYP BOUND (% NMS)
NMS
1
2
3
4
5
6
7
ANIMAL
Expt 1
Expt 2

Fig. 5.5. Screening of mouse antiserum AS2 against sheep adipose tissue membranes and sheep erythtocyte ghosts

Sheep (lactating ewe) omental adipose tissue membranes and sheep erythrocyte ghosts were coated onto 96-well plates (Immulon 2, Dynatech). Serum from a pre-fusion bleed of mouse No. 2 (filled circles) and NMS (open circles) were screened for anti-membrane activity by EIA (section 2.12.4). In A, sheep omental adipose tissue membranes; B, sheep erythrocyte ghosts.

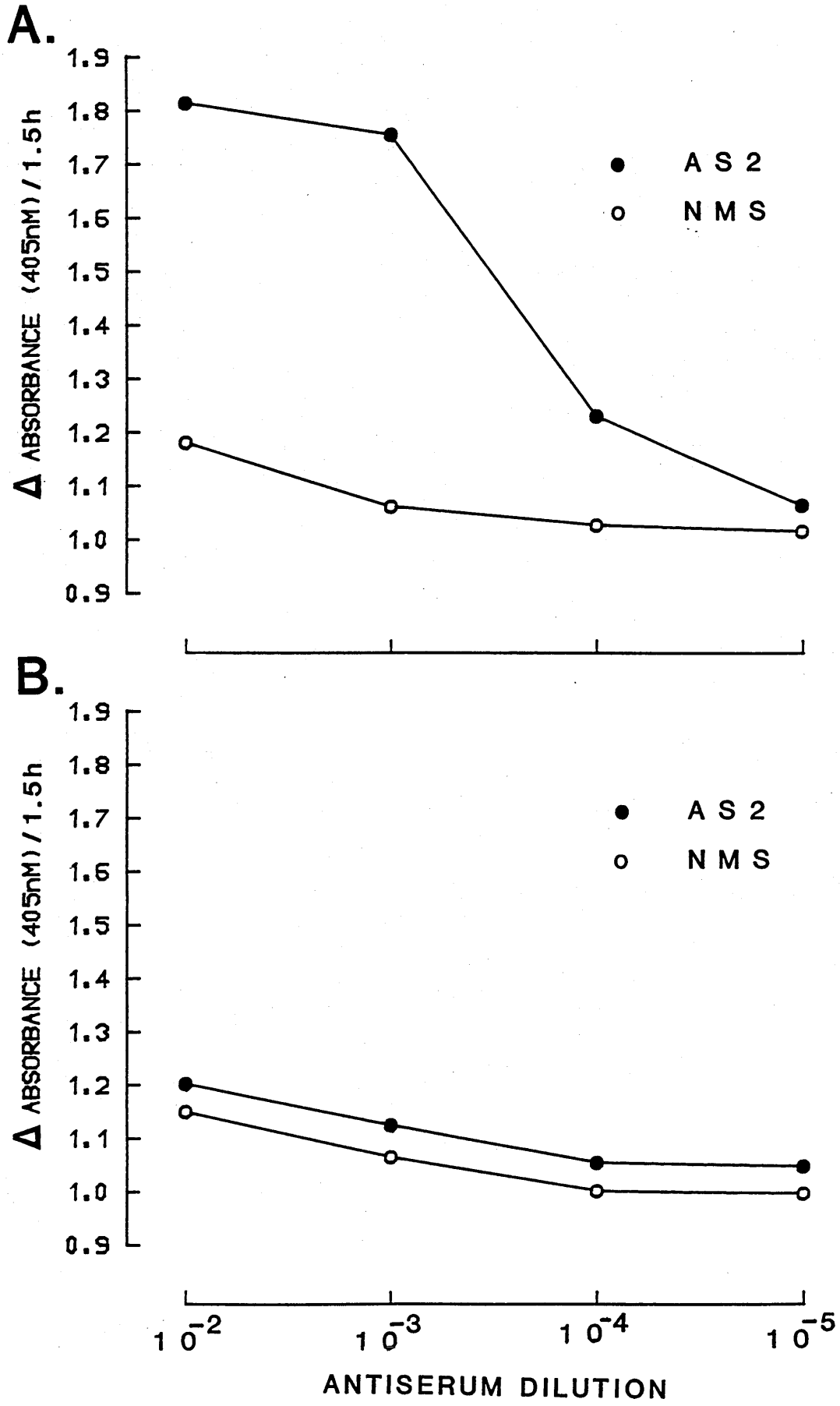

A.
B.
Δ ABSORBANCE (405nM) / 1.5h
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
● AS 2
o NMS
10^-2
10^-3
10^-4
10^-5
ANTISERUM DILUTION

Fig. 5.6. Topography of the hamster lung β_2-adrenergic receptor

The primary amino acid sequence for the hamster lung β_2-adrenergic receptor, deduced from the cloned cDNA, is shown. The β-adrenergic receptor is predicted to possess seven hydrophobic membrane-spanning domains. CM 1-13 define the amino acid sequences used for preparing anti-peptide antibodies.

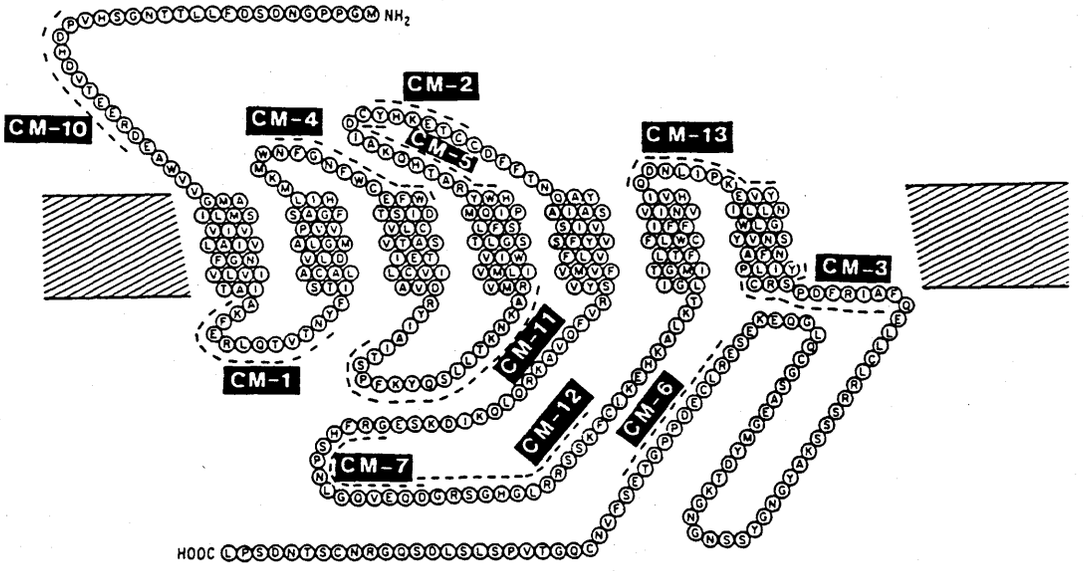


Fig. 5.7. Immunoblotting against sheep omental adipose tissue membranes with CM 13 and AS2

Immunoblotting as described for Figure 5.3, except that the adipose tissue came from a lactating ewe. Key: lane 1 - normal mouse serum (1:50); lane 2 - CM 13 (1:50); lane 3 - CM 13 (1:1); lane 4 - antiserum from mouse 2 (AS2) (1:50). Dilutions quoted for CM 13 represent dilution of the lyophilised preparation after its resuspension in 500 µl of PBS, 1% (w/v) Marvel.

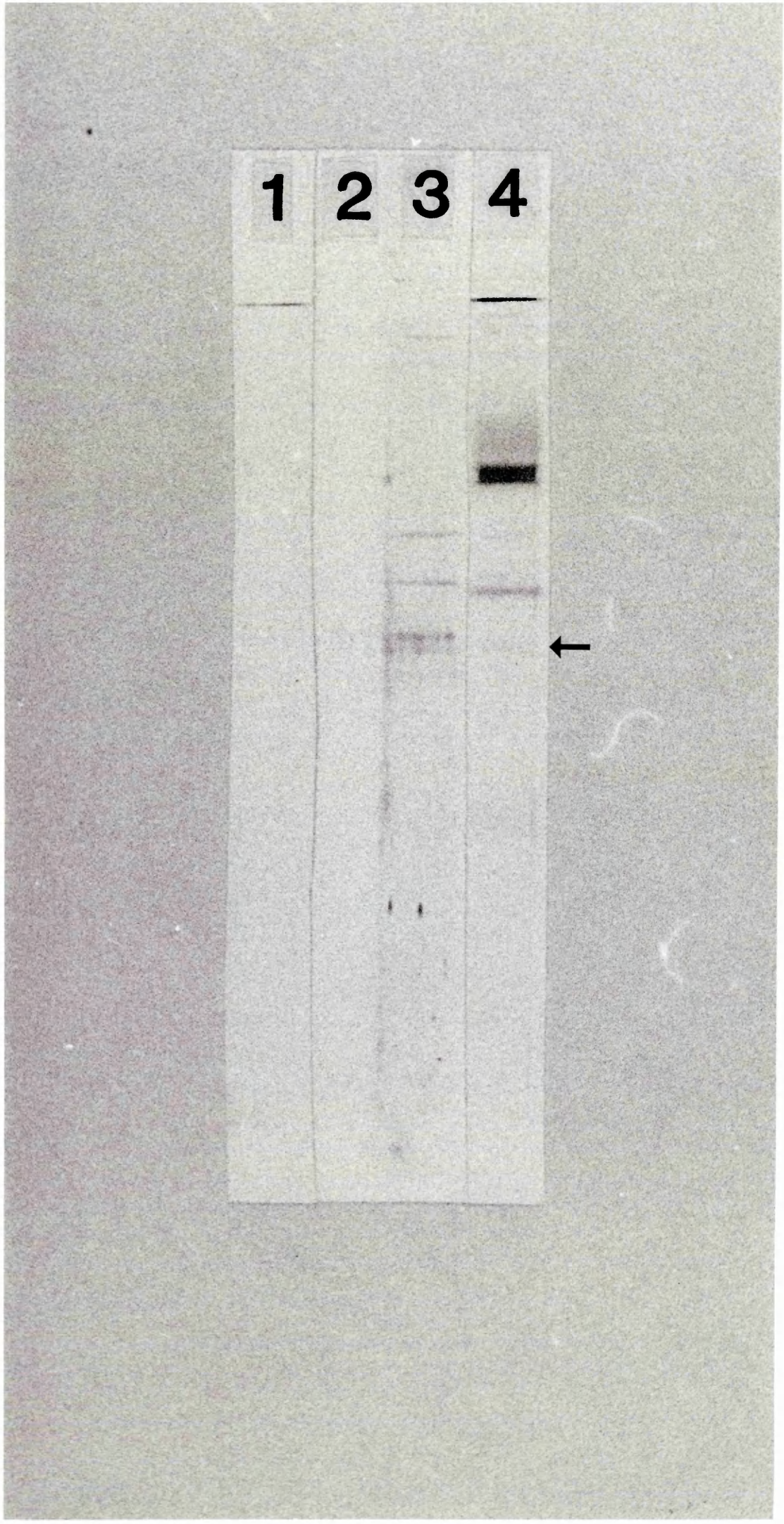
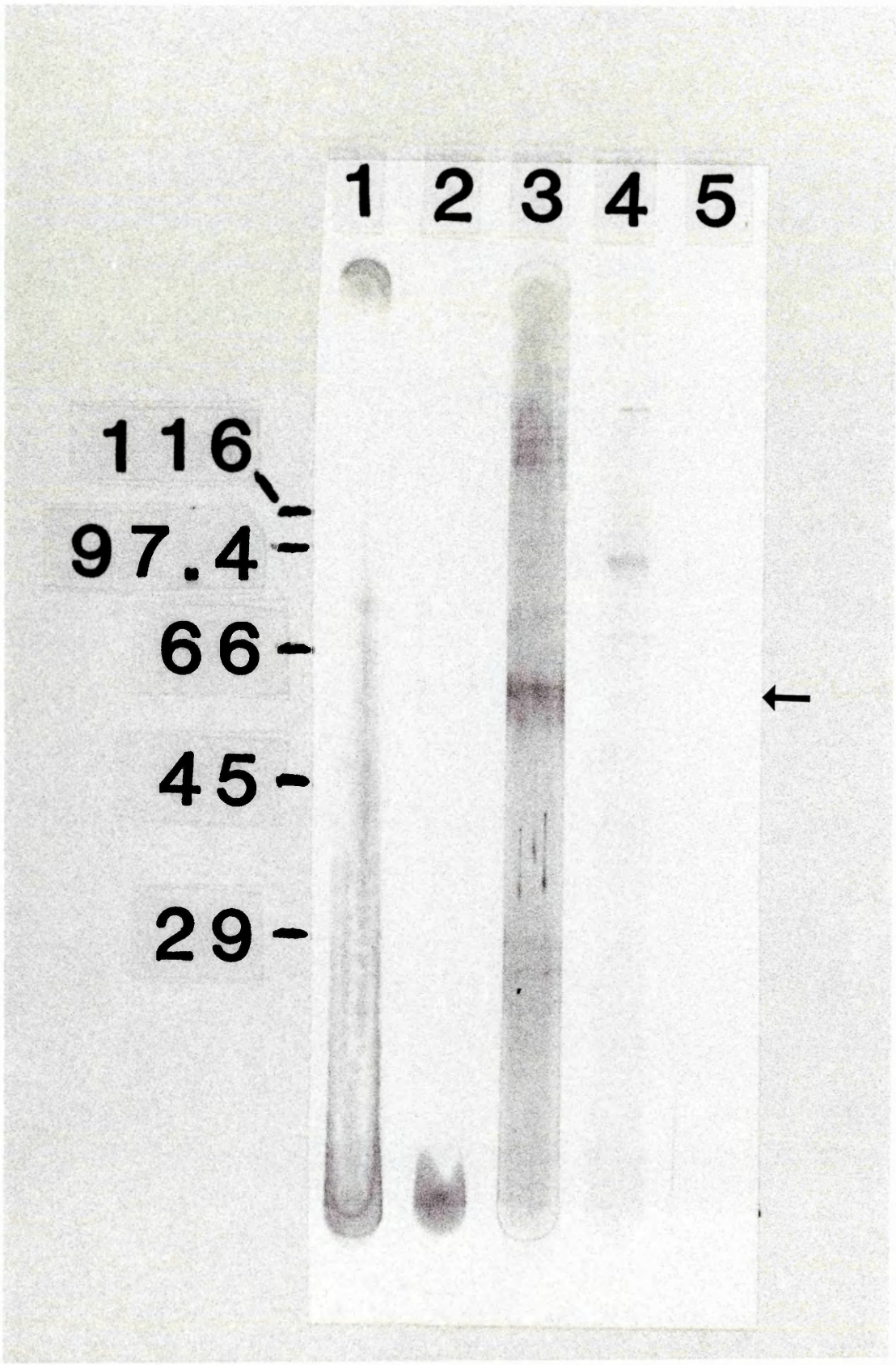

1
2
3
4

Fig. 5.8. Immunoblotting against sheep omental adipose tissue membranes with CM13 and AS2 - Molecular weight determination

Immunoblotting as described for Figure 5.3, except that a peroxidase anti-peroxidase complex was used to amplify the immunostaining and the adipose tissue came from a lactating ewe. Key: lane 1 - normal rabbit serum (1:50); lanes 2 and 3 - CM13 (1:50 and 1:1 respectively); lane 4 - antiserum from mouse 2 (AS2) (1:50); lane 5 - normal mouse serum (1:50). Molecular weights for markers (SDS-6H, Sigma) are in kDa.


1 2 3 4 5
116
97.4
66
45
29

CHAPTER SIX

GENERAL DISCUSSION

CHAPTER SIX - GENERAL DISCUSSION

When this study began, nothing was known about the structure of the β-adrenergic receptor of sheep adipose tissue, and little about its ligand binding properties. This apparent 'gap' in the literature made it difficult to correlate direct effects of physiological state and β-adrenergic ligands on adrenergic mechanisms in sheep adipose tissue. Indeed there was a strong possibility that sheep adipose tissue contained an atypical β-adrenergic receptor, in light of the controversy surrounding the rat adipocyte β-adrenergic receptor (Bahouth & Malbon, 1988; Zaagsma & Nahorski, 1990). Also, the current interest in the use of β-agonists in livestock production (including sheep), which act directly on adipose tissue to reduce adiposity in carcasses, meant that characterisation of the sheep adipose tissue β-adrenergic receptor might lead to a better understanding of β-agonist action, and possibly allow the development of better and more selective drugs and/or therapies.

Initial radioligand binding studies confirmed the presence of β-adrenergic receptors in sheep adipose tissue, in sufficiently high numbers to attempt their purification. The level of β-adrenergic receptor expression reflects the importance of adrenergic regulation in adipose tissue metabolism. The β-adrenergic receptor of sheep omental adipose tissue was purified greater than 300 fold (compared to levels found in membranes) by affinity chromatography, which allowed limited studies on partially-purified receptors. The amount of β-adrenergic receptor in these preparations meant that direct staining of receptor protein on SDS PAGE gels was not feasible. Therefore, photoaffinity-labelling of the β-adrenergic receptor was performed to determine the apparent molecular weight of the receptor. As a result, photoaffinity-

labelling of β-adrenergic receptors in membranes and partially-purified preparations identified a 58 kDa protein, which appeared to be heavily glycosylated. Glycosylation is a common feature of membrane proteins, the sugar moieties being added in the Golgi apparatus prior to the protein's transport and insertion into the plasma membrane.

Classification of β-adrenergic receptor subtypes is a subject which has received much attention over the past two decades. The multiplicity of β-adrenergic receptor 'selective' radioligands (both agonists and antagonists), and the study of many tissues, has culminated in the positive identification of three β-adrenergic receptor subtypes, termed β_1, β_2, and β_3. β-Adrenergic receptors were first described by Ahlquist (1948), with β_1- and β_2-adrenergic receptors being differentiated by Lands et al. (1967). This characterisation remained intact until investigations into the properties of the rat adipocyte β-adrenergic receptor revealed atypical characteristics (Grana et al., 1972; Stanton, 1972). This contrasted with Lands et al.'s classification of the rat adipocyte β-adrenergic receptor as a β_1-subtype. Detailed study of both the ligand binding and functional properties of this β-adrenergic receptor defined it as being a 'hybrid' receptor i.e. possessing both β_1 and β_2 characteristics (De Vente et al., 1980), and culminated in the development of adrenergic agonists with selective action on adipose tissue (Arch et al., 1984; Wilson et al., 1984). However, the classification of a β_3-adrenergic receptor was only confirmed unequivocally with the isolation of a cDNA encoding a third human β-adrenergic receptor (Emorine et al., 1989).

Characterisation of the properties of the β-adrenergic receptor of sheep adipose tissue identified it as primarily β_2-subtype, with no

β_1-characteristics and with high affinities for non-subtype selective β-adrenergic radioligands. Its properties are therefore different from the rat adipocyte β-adrenergic receptor. As a result of Lands et al.'s classification, β_1-adrenergic receptors became associated with lipolysis and cardiac stimulation, whilst β_2-receptors were proposed to mediate bronchodilation and vasodilation. For many years researchers regarded these arbitary divisions as being absolute, and were reluctant to accept change. The cloning of the human β_3-adrenergic receptor discredited the β_1/β_2 subtype classification, and adds weight to proposals of atypical β-adrenergic receptor properties in other species and tissues. Indeed, Kaumann (1989) has proposed that a third β-adrenergic receptor exists in the heart. The finding that sheep adipose tissues possess mostly the β_2-subtype provides further evidence against the early proposal of Lands et al. (1967), supported by Bahouth & Malbon (1988), that adipocytes possess β_1-adrenergic receptors.

Adipose tissue is found in distinct locations throughout the body e.g. omental, parametrial, subcutaneous, intramuscular. This distribution shows little variation between mammalian species, and appears to have occurred early in mammalian evolution (Pond, 1984). There is an increasing body of evidence suggesting that both anatomic and metabolic characteristics of adipocytes vary between adipose tissue depots. Firstly, adipocyte size and volume varies between different depots within a species (Pond, 1984; Sinnett-Smith & Woolliams, 1987). Also, the rate of lipolysis increases with adipocyte size (Hood, 1982), and larger cells in sheep have greater noradrenaline-stimulated lipolytic rates (Vernon & Finley, 1985). The development and growth of adipose depots occurs non-uniformly in sheep (Hammond et al., 1971; Leat et al., 1976; Wood &

Butler-Hogg, 1986), guinea pigs (Pond et al., 1986), rats (Cryer & Jones, 1978), pigs and cattle (Wood & Butler-Hogg, 1986). Castration of cattle results in greater growth of subcutaneous relative to perirenal adipose tissue (Prescott & Lamming, 1964).

On a more biochemical theme, several metabolic activities vary between adipose depots. Lipoprotein lipase activity varies amongst fat depots in growing sheep (Sidhu et al., 1973), wether lambs (Haugebak et al., 1974), and lean rats (Savard & Greenwood, 1988). Differences in acetyl CoA carboxylase activity are apparent between subcutaneous adipose depots in sheep (Sinnett-Smith & Woolliams, 1987). This correlates with earlier findings suggesting depot-differences in the rate of fatty acid synthesis in growing lambs (Ingle et al., 1972a,b). Undernutrition of wether lambs results in the preferential loss of lipids from subcutaneous adipose tissue depots (Vernon, 1980). In the rat, regional differences occur in glucocorticoid action (Hauner & Pfeiffer, 1989), glucose metabolism (Fried et al., 1982), and the sensitivity of noradrenaline-stimulated lipolysis (Hartman & Christ, 1978). In humans, depot differences in the control of lipolysis (Ostman et al., 1979; Rebuffe-Scrive et al., 1989) and lipoprotein lipase activity (Rebuffe-Scrive et al., 1989) have been shown. Thus it is clear that all adipose tissues are not identical, and so differences in the properties of β-adrenergic receptors might have been predicted.

Study of β-adrenergic receptors in different sheep adipose tissue depots revealed that differences in their properties were present. Subcutaneous and popliteal depots both possessed β-adrenergic receptors with high selectivity towards the β_2-selective antagonist ICI 118,551. In contrast, omental adipose tissue possessed a β-adrenergic receptor

with less β_2-character. The reasons for these findings are not clear. They may represent a complex modulation of β-adrenergic receptor properties, which correlates the location of adipose tissue within the body with β-adrenergic receptor function. Periods of under-nutrition do not lead to uniform fat loss from all depots (Vernon, 1980), and such differences may well be due to differences in β-adrenergic receptor properties of individual depots.

In the rat, parametrial and lumbar adipose tissue depots possessed two β-adrenergic receptor subtypes. One subtype exhibited β_1-character, whilst a second displayed atypical β-adrenergic receptor characteristics. The number of each receptor species in the two depots studied was different. This was suprising since anatomically the parametrial and lumbar depots are virtually juxtaposed in the abdomen of the rat. Competition of [^{125}I]ICYP binding to rat lumbar adipocyte membranes by ICI 118,551 and CGP 20712A gave graphs similar to those obtained by Bahouth & Malbon (1988) in an identical experiment performed on parametrial adipose tissue. Why such profound differences should occur between laboratories is not immediately apparent, but they may result from methodological differences during the preparation of adipocyte membranes. No discussion of the atypical binding present was included in Bahouth & Malbon's paper, and only the parametrial depot was studied. The authors also dismissed the concept of atypical β-adrenergic receptors in rat adipose tissue by other groups without any consideration of depot and sex differences. Indeed, Zaagsma's group, firm proponents of atypical β-adrenergic receptors, used male rats and the epididymal adipose tissue, a depot not present in female animals (Hollenga & Zaagsma, 1990). In light of this controversy, the influence

of sex on β-adrenergic receptor characteristics deserves further study.

This study has revealed marked differences between the β-adrenergic receptors present in sheep and rat adipose tissue. The receptors differ in their structural characteristics, notably molecular weight, susceptibility to reduction by disulphide reducing agents, and possibly primary amino sequence, with respect to the binding of the polyclonal antisera CM 13. Sheep possess β_2-adrenergic receptors whilst rats have atypical and β_1-adrenergic receptors on their adipocytes. Therefore when studying β-adrenergic mechanisms in adipose tissue, it is not always possible to directly extrapolate findings between species.

Since adipose is not a homogeneous tissue with many differences between fat depots, it is feasible that drugs acting on adipose tissue may exhibit depot-selective effects. It may therefore be possible to design β-adrenergic agonists/antagonists which selectively bind and activate β-adrenergic receptors in a particular depot. This therapy could be used in humans where adiposity needs to be reduced in a particular depot for medical/cosmetic reasons, assuming that the β-adrenergic receptors in human adipose tissue also differ between depots.

If the study had been more successful, and monoclonal antibodies were raised against the β-adrenergic receptor of sheep adipose tissue, it might have been possible to develop immunological alternatives to β-agonists. Monoclonal antibodies have already been produced which possess agonist activity, but these were anti-idiotypic antibodies raised against monoclonal antibodies against the β-adrenergic ligand alprenolol (Guillet et al., 1985). By using purified β-adrenergic receptors as an immunogen, it may be possible to raise monoclonal antibodies with selective agonist activity against a single adipose tissue depot. These

antibodies would be a welcome alternative to the β-agonists used in livestock production, as they would not leave any potentially harmful residues in the animal after treatment.

With regards to future research, it is important to correlate the ligand binding properties of the β-adrenergic receptor of sheep adipose tissue with its physiological function i.e. control of lipid metabolism. Initial work could investigate receptor stimulation of lipolysis, and confirm that β_2-adrenergic receptors mediate this effect in sheep adipose. Also during such functional studies, the possible presence of an atypical β-adrenergic receptor could be investigated using the novel β-agonist BRL 37344, and the approach of Hollenga & Zaagsma (1989). By studying the effect of subtype selective antagonists (e.g. CGP 20712A and ICI 118,551) on (-)-isoproterenol- and BRL 37344-stimulated lipolysis, a clear indication of which subtype(s) of β-adrenergic receptor mediate catecholamine regulation of lipolysis in sheep adipose tissue should be attainable. These experiments could also examine adipose tissue depots not studied in this work, to determine the degree of heterogeneity in β-adrenergic receptor properties throughout the entire body.

In addition, the production of polyclonal antisera against the β-adrenergic receptor of sheep adipose tissue should continue to be a key aim. Such antisera are useful for immunoblotting and immunoprecipitation, and can also be used to screen cDNA expression libraries from sheep adipose tissue for β-adrenergic receptor cDNAs. Cloning of a β-adrenergic receptor from adipose tissue would allow the generation of anti-peptide antisera once the primary amino acid sequence was known. An alternative approach would be to obtain a suitable cDNA from another

laboratory e.g. that encoding the hamster lung β_2-adrenergic receptor (Dixon et al., 1986), and use it to screen a cDNA library. Who knows what such studies will reveal? Perhaps sheep adipose tissue receptors will be shown to be yet another β-adrenergic receptor subtype!

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