BIOSYNTHESIS OF A PROLACTIN RECEPTOR

by

Harry Paul Elsholtz

A Thesis
Presented to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of
Master of Science

Department of Physiology
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ABSTRACT

The interaction of prolactin (PRL) and other lactogenic hormones with specific membrane receptors is the first step in the mechanism of hormonal action (1). Although the regulation of PRL-binding sites has been studied extensively in various target tissues, little is known of the mechanism of regulation. While a measurable increase in PRL-binding activity may indicate an increased rate of receptor biosynthesis alternative explanations such as the activation of pre-existing cryptic receptor sites may account for the elevation in binding. The purpose of the present investigation was to demonstrate directly de novo biosynthesis of PRL receptors in rat liver and in a PRL-responsive lymphoma cell line by the incorporation of labeled metabolic precursors into the receptors.

Two methods were used to study biosynthesis of PRL receptors in rat liver. Firstly, mRNA extracted from the tissue was translated in vitro in a lysate derived from rabbit reticulocytes and containing a radioactively labeled amino acid. A specific antiserum generated against partially purified PRL receptors was used in an attempt to precipitate newly synthesized receptors. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. No specific unique band(s) representing the receptor (or subunits thereof) were detectable.

Secondly, fine minces of fresh rat liver were incubated in ^{35}S-methionine-containing Krebs medium. Triton X-100-

solubilized extracts of the minces were examined by immunological methods for the presence of radioactively labeled PRL receptors. In addition to direct immunoprecipitation using the anti-PRL receptor antiserum, indirect precipitation, in which extracts were incubated first with human growth hormone (hGH) and then with anti-hGH antiserum, was used in an attempt to isolate newly synthesized receptors. Again, electrophoretic analysis of the immunoprecipitates failed to demonstrate de novo biosynthesis of PRL receptors.

Regulation and biosynthesis of PRL receptors was examined in tumor cells grown in culture. Recent studies have shown that PRL and other lactogenic hormones are mitogenic in the lymphoma cell line Nb2 (77). In this study receptors for ^{125}I-hGH were identified in both whole cell preparations and Triton X-100-solubilized extracts of the cells. Only lactogenic hormones were able to compete effectively for the binding sites.

Binding activity was low in cells cultured in the presence of fetal calf serum (FCS). Transfer of the cells to FCS-deficient medium resulted in a four-fold increase in binding after one day of culture. Cycloheximide at low concentrations (ng/ml) prevented the increase in binding. To determine whether the receptor molecules themselves were newly synthesized or whether the activation of cryptic sites was responsible for the increase in binding, density labeling of the receptors was attempted. Nb2 cells cultured in the presence of FCS were trans-

ferred to fresh medium (lacking FCS) containing either normal or $^{2}H,^{13}C$-amino acids. After 12 hours the cells were solubilized and the extracts were subjected to density equilibrium ultracentrifugation using CsCl gradients. Fractions of the gradient were assayed for ^{125}I-hGH binding activity. Incorporation of $^{2}H,^{13}C$-amino acids into PRL receptors was demonstrated by a density shift in the receptors in the gradient. Heavy isotope-labeled receptors banded at a position in the CsCl gradient distinctly below the normal 'light' PRL receptors.

The present study using density-labeling techniques provides direct evidence for the biosynthesis of functional PRL receptors. This approach to the study of PRL receptor synthesis circumvents the methodological difficulty inherent in studies employing radioactive isotopes which demand the purification and/or specific identification of labeled receptors. Finally, the present approach makes it feasible to investigate the regulation of synthesis, transport to the cell surface, and degradation of PRL receptors in cultured PRL-responsive cells.

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List of Abbreviations

Hormones

GH	growth hormone
PRL	prolactin
Rc	receptor
h	human (hormone prefix)
o	ovine (hormone prefix)

Sera

FCS	fetal calf serum
HS	horse serum
NGPS	normal guinea pig serum
NRS	normal rabbit serum

Units of measure

g	gram
mg	milligram
µg	microgram
ng	nanogram
pg	picogram
ml	millilitre
µl	microlitre
cm	centimetre
mm	millimetre
nm	nanometre
hr	hour
min	minute
sec	second
cpm	counts per minute
dpm	disintegrations per minute
µCi	microcuries
M	molar
mM	millimolar
N	normal
A_{260}	absorbance at 260 nanometres
ηD	refractive index
^{o}C	degrees Celsius
x g	times force of gravity

Miscellaneous

BSA	bovine serum albumin
CHX	cycloheximide
DMSO	dimethyl sulfoxide
DOC	deoxycholate
EBSS	Earl's balanced salt solution
EDTA	disodium ethylenediamine-tetraacetate
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
oligo(dT)	oligodeoxythymidylate
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
poly(A)	polyriboadenylate
PPO	2,5-diphenyloxazole
RSA	rat serum albumin
Sarkosyl	sodium lauryl sarcosinate
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Staph A	Staphylococcus aureus
TCA	trichloroacetic acid
TX-100	Triton X-100

INTRODUCTION

The binding of PRL to its plasma membrane receptor is the initial step in the action of the hormone. Similarly to receptors of other polypeptide hormones, the PRL receptor is believed to transmit the hormonal message into target cells triggering the sequence of intracellular events that generate the PRL effect. Unlike epinephrine, glucagon and a number of other hormones that bind to cell surface receptors, it appears that PRL-receptor interactions do not stimulate the well-known adenylate cyclase-protein kinase second messenger system to produce a lactogenic response. Indeed the mechanism of PRL action is poorly understood at present but may involve the participation of prostaglandins, polyamines such as spermine or spermidine and cyclic GMP. This problem has recently been addressed in a review by Shiu and Friesen (1).

Strong evidence supports the involvement of a plasma membrane receptor in the mediation of PRL action. A close correlation exists between the bindability of hormones to rabbit mammary gland membranes (2) and the lactogenic activity of those hormones in rabbit and mouse mammary gland organ cultures (3,4). Only those hormones with bioassayable lactogenic activity--PRL, hGH, and placental lactogens--are able to compete with radioiodinated PRL for binding sites in the radio receptor assay. Non-primate growth hormones, although structurally similar to hGH and placental lactogens, are ineffective

in both the bioassay and receptor assay. Furthermore, the extent to which a given preparation of ovine PRL can compete in the receptor assay is proportional to its biological potency.

A second line of evidence supporting the role of plasma membrane receptors in the mediation of PRL action has been reported by Shiu and Friesen (5). They have demonstrated that a guinea pig antiserum raised against purified PRL receptors from rabbit mammary gland not only specifically inhibits the binding of PRL to its receptor but also prevents prolactin-mediated incorporation of ^{3}H-leucine into casein and ^{14}C-aminoisobutyric acid transport in rabbit mammary gland explants. The same antiserum, however, does not affect insulin binding to its receptor or insulin-stimulated transport of amino acids; nor does it bind or alter the PRL molecule (6). An extension of these studies by Bohnet et al. (7) has provided further evidence that PRL receptors play an obligatory role in hormone action in vivo. They have found that the passive immunization of female rats with anti-PRL receptor antiserum results in an increased number of corpora lutea probably because the luteolytic action of PRL is inhibited. The lactogenic effect of PRL is also partially blocked in vivo. Pups of lactating rats treated with anti-PRL receptor antiserum weigh significantly less than control pups, suggesting that milk yield is reduced by the inhibition

of PRL binding. Also, the serum PRL levels of antisera-treated rats is nearly double control levels. This result may be caused by an antisera-activated feedback signal to the hypothalamus or pituitary gland following the blockage of hormone action on peripheral tissues (7). These findings provide direct evidence for an obligatory functional role of a membrane receptor in mediating the action of PRL.

To increase our understanding of how PRL interacts with its target tissues it is important to study the physical and chemical properties of the membrane receptors as well as their turnover and regulation. Similar properties of PRL receptors have been described in various species and in a number of organs including ovary, male genital tract, liver, mammary gland, and mammary tumors (8). PRL receptors in different tissues from various species also possess immunological similarities (6). The study of PRL receptors in a given tissue and species, therefore, may help to answer questions about these complex molecules in a variety of other target organs or animals.

Shiu and Friesen (9) have demonstrated that PRL receptors are enriched in membrane fractions containing the highest specific activity of 5'-nucleotidase, a plasma membrane marker enzyme (10), confirming that receptors for PRL are located on the plasmalemma. They have also shown that PRL binding to its receptor in rabbit mammary gland exhibits many of the features used to define hormone-receptor inter-

actions (11,12). These include specificity, high affinity, saturability and reversibility. As for other hormones, the rate and extent of PRL binding increases if the concentration of either labeled hormone or receptors is increased (9). Binding of ^{125}I-PRL to its receptor is strongly influenced by pH and ionic conditions whereas many low-molecular weight compounds including steroids, nucleotides and various drugs do not affect binding (9). The stimulatory effect of divalent cations on lactogen-receptor interactions may involve alterations in ligand structure such that receptor association is enhanced (13). Protease or phospholipase C digestion of membranes destroys receptor activity (8,14) suggesting that PRL receptors are lipoprotein complexes. Carbohydrate moieties appear to be part of the receptor since the binding of ^{125}I-ovine PRL to female rat liver membranes is inhibited by concanavalin A (a plant lectin which binds to specific carbohydrate determinants) and detergent solubilized ^{125}I-PRL-receptor complexes are bound by concanavalin A-Sepharose (15). However, that carbohydrate moieties are required for PRL-receptor interactions has not been established. Neuraminidase, unlike proteolytic enzymes or phospholipases, has no effect on receptor activity in rabbit mammary gland membranes (9). A recent study (14) has shown that neuraminidase may in fact stimulate PRL receptor activity in female rat liver.

If the PRL receptor is a mediator of hormone action,

then investigating receptor regulation becomes as important as studying the control of PRL secretion. An increase in either the concentration of the circulating hormone or the receptor would increase the number of hormone-receptor complexes in the target tissue, and should therefore enhance the hormone effect. Despite the discovery of spare receptprs in various hormonal systems (11) whose occupancy does not further promote hormone action, the number of receptors per target cell are nevertheless an index of hormone sensitivity or responsiveness. This means, for example, that a very low serum level of hormone could more readily interact with a target tissue sensitized by an increase in receptor activity.

The regulation of PRL receptor activity in a number of tissues and species under various conditions has been reviewed recently (8). Steroid (16-20), thyroid (21,22) and polypeptide (23-25) hormones including PRL (26,27) play important roles in regulating the number of PRL binding sites. At the present time, however, many of the regulatory aspects are poorly understood. Binding studies have revealed that the factors controlling receptor levels interact in a complex manner, being dependent on the tissue, sex and species tested. At the onset of lactation in rats, for example, hepatic binding levels decrease while those of the mammary gland increase (28); yet both tissues are exposed to the same hormonal milieu. Castration causes a decrease in the binding of ^{125}I-PRL in the prostate whereas in the liver this procedure results in a major

increase in binding activity (18). Estrogen treatment of male rats or ovariectomized females stimulates hepatic PRL binding activity and although a similar effect is seen in male mice, female mice exhibit a binding increase after ovariectomy while estrogen replacement reduces PRL binding to control levels or lower (29). Another intriguing aspect of PRL receptor regulation is the involvement of the hormone in modulating its own receptor levels. Receptor down-regulation, characteristic of many hormone systems (11), has been identified in PRL-responsive tissues, as well, following i.v. injection of large doses of PRL (30), but unlike most hormones, sustained high levels of PRL cause an induction or an up-regulation of the PRL receptor (26,27). Many, if not most, of the details of PRL receptor regulation are still missing and further investigation in this area is necessary.

One of the shortcomings of most studies on PRL receptor regulation is the failure to demonstrate what actually causes the changes in binding activity. While it may be implied or assumed that a substance which increases the number of measurable binding sites does so by turning on the PRL receptor gene or promoting de novo receptor biosynthesis post-transcriptionally, this need not be the case. Alternatively, an elevation in the number of assayable sites may be due to:

1. dissociation of endogenously bound lactogenic hormones from receptors as a result of lowered plasma levels of those hormones;

2. activation of cryptic receptor sites which are present in the cell but are incapable of binding PRL prior to an appropriate trigger mechanism;
3. a decreased rate of receptor degradation.

The desaturation of receptors occupied by endogenous lactogens is noteworthy because it represents, in essence, an artifact rather than true regulation of the PRL receptor molecule. In rats, due to the elevated plasma levels of PRL which occur at noon of proestrus and at estrus (31), during a spontaneous afternoon surge (32), and as a result of pregnancy, a variable portion of the PRL receptors in target tissues may be saturated by endogenous lactogenic hormones. Tissue homogenization and isolation of microsomal membranes does not appreciably strip endogenously bound lactogen from the PRL receptor, presumably due to a slow rate of dissociation (9,33). As a result an apparent increase in PRL binding sites can occur if circulating lactogen levels are lowered in the test animal, generating a greater proportion of unoccupied receptors. However, the actual number of receptors has not changed. Recently Kelly et al. (34) have demonstrated that chaotropic agents like 4 M $MgCl_2$, which effectively remove endogenously bound hormone from membranes in vitro, can facilitate the measurement of 'total' (rather than unoccupied) binding sites. Inhibitors of PRL secretion have been reported to desaturate lactogenic binding sites in vivo (35).

A measured increase in PRL binding may in certain situ-

ations arise from an activation of cryptic receptors. Support for the concept of preexisting, activatable binding sites comes from insulin, β-adrenergic, and chemotactic peptide receptor studies in which agents that perturb membrane integrity enhance binding activity. Exposure of cells or membranes to phospholipases (36,37), trypsin (38), neuraminidase (38), alcohols (39), ketones (40) and S-adenosyl-L-methionine (41) increases binding. Recent evidence indicates that membrane-modifying procedures can also reveal 'hidden' PRL receptors. As mentioned above, neuraminidase digestion of rat liver membranes enhances PRL binding by 30% (14), a result which is similar to the neuraminidase-stimulated increase in insulin binding reported for chick embryo fibroblasts (38). S-adenosyl-L-methionine, an amino acid derivative that acts as a specific substrate for phospholipid methylation, increases the number of PRL binding sites in mouse mammary gland membranes (42). Consistent with these in vitro observations is the finding by Knazek and Liu (43) that certain dietary fatty acids are required for the expression of PRL receptors in mouse liver. The authors suggest that the decreased PRL binding of fatty acid-deprived mice may be caused by alterations in the receptor's lipid environment such that PRL-receptor interactions cannot occur.

Further support for cryptic PRL receptors comes from studies on PRL receptor induction. In the Snell dwarf mouse, injections of bovine GH cause a rapid induction of hepatic PRL

receptors (23). Simultaneous injections of cycloheximide are unable to prevent the appearance of new binding sites suggesting that de novo protein biosynthesis is not a prerequisite. Thyroid hormones, too, can stimulate lactogenic binding in mouse mammary gland by a mechanism not blocked by puromycin even though the drug reduces total incorporation of ^{3}H-leucine by 20-fold (22). One explanation given by the authors is that the lipolytic activity of thyroid hormones may produce changes in the lipid composition of mammary membranes. A second explanation may be that thyroid hormones can act upon cell membranes directly by way of a plasma membrane receptor (44).

In a recent study Costlow and Hample (45) have examined the effects of uncouplers of oxidative phosphorylation and inhibitors of electron transport on PRL binding in monolayer cultures and fresh slices of rat mammary tumors. They have found that agents which block energy production cause an 8 - 20 fold increase in binding. Similarly, lowering the incubation temperature from $37^{o}C$ to $4^{o}C$ enhances binding by more than 20-fold. One interpretation offered by the authors is that metabolic energy may be necessary to maintain PRL receptors in an inactive (cryptic) form and that energy depletion allows the activation or expression of these pre-existing binding sites.

The study of membrane receptor biosynthesis and turnover is a new and relatively undeveloped field. By far the most rapid progress has occurred in acetylcholine receptor

has generally been the approach taken to demonstrate that the receptor is indeed newly synthesized. Purification steps are usually necessary to verify that the metabolic precursors have been incorporated specifically into receptor protein. Thus Hall and Reiness (47) have demonstrated that ^{35}S-methionine is incorporated into rat diaphragm acetylcholine receptor by purifying a crude solubilized membrane extract--first by cobratoxin-Sepharose which specifically binds acetylcholine receptor and then (to further reduce background radioactivity) by concanavalin A-Sepharose which recognizes the carbohydrate residues of the receptor. Isoelectric focussing and immunoprecipitation with specific antisera have also been used to isolate newly synthesized acetylcholine receptor (48). Very recently Mendez et al. (49) have translated polyadenylated RNA (mRNA) from Torpedo electric organ in a cell-free system and have immunoprecipitated newly synthesized receptor peptides. The authors project that the eventual purification of acetylcholine receptor mRNA and complementary DNA will facilitate investigation of the receptor gene and permit detailed analysis of the primary structure of receptor peptides via DNA sequencing.

Because membrane receptors often comprise a minute fraction of total cellular protein, the purification procedures required to confirm amino acid incorporation become very laborious particularly if kinetic analysis is attempted. In an effort to simplify the demonstration of newly synthesized

acetylcholine receptor Devreotes and Fambrough (50) have applied a density labeling technique, first used to detect de novo biosynthesis of enzymes (51,52), to the field of receptor research. Briefly, the technique requires the culture of cells in a medium containing metabolic precursors (usually amino acids) labeled with heavy stable isotopes of hydrogen, carbon or nitrogen. Newly synthesized receptors, enriched in these amino acids, are denser than native receptors and can be separated during isopycnic centrifugation by a density-shift. Fambrough and his colleagues have elaborated on the density-labeling method to measure the turnover rate and factors affecting the turnover rate of the acetylcholine receptor (53,54). The recent application of density-labeling to the investigation of insulin receptor regulation (55) confirms the value of this technique for studying receptor control in any cell-culture system.

Little is known of PRL receptor biosynthesis. Although various studies have suggested that the PRL receptor is newly synthesized, the approaches thus far have been indirect. Kelly et al. (56) have shown that cycloheximide, a potent inhibitor of translation, rapidly lowers hepatic PRL binding in estrogenized male rats. More recently cycloheximide has been shown to reduce PRL binding in vitro in rabbit mammary gland organ culture (57). Upon removal of the drug, binding activity increases, implying a need for de novo protein synthesis in the maintenance of PRL receptors. While the use of translation

inhibitors may confirm that protein synthesis is necessary for an increase in measurable binding activity, such agents do not demonstrate conclusively that the PRL receptor (per se) is newly synthesized. Short-lived regulatory proteins (as yet unidentified) which may control receptor turnover, activation, or stabilization in the cell membrane may be more sensitive to the effects of antibiotics than the receptor itself. Consequently, variable estimates of receptor half-life are often reported when the rates of receptor biosynthesis or degradation are measured by different methods. The half-life of insulin receptors in 3T3-L1 adipocytes, for example, has been estimated at 20 - 25 hours in the presence of cycloheximide (58,59), 9 hours in the presence of an inhibitor of phosphoryldolichol-mediated protein glycosylation, tunicamycin (59), and at 6.7 hours by the density-shift method (55). The longer half-life in the presence of cycloheximide suggests that the synthesis of a short-lived protein(s) is required for the normal turnover of the receptor (55). Unlike insulin binding activity, PRL binding is depleted very rapidly in the presence of cycloheximide (56,57). Rather than a PRL receptor with a rapid turnover rate, however, one can propose a short-lived regulatory protein (i.e., quickly depleted in the presence of translation inhibitors) whose loss rapidly lowers the number of assayable PRL-binding sites. The receptor itself may have a relatively long half-life. Increased binding following cycloheximide clearance in vivo or removal

from the culture medium may therefore represent the reappearance of the activating protein--not de novo receptor biosynthesis.

Inhibitors of translation, including cycloheximide, have additional effects on cellular function that may be undesirable. Anomalous actions of cycloheximide have been observed on RNA synthesis (60,61), enzyme activity (62), protein degradation (63), glycogenolysis (64), gluconeogenesis (65) and phosphorylation of ribosomes (66). Although antimetabolic agents such as cycloheximide should continue to be used in the study of protein biosynthesis, the diverse nature of their action should be considered in the interpretation of results.

A second experimental approach has indicated that PRL receptors may be synthesized in rat liver. The discovery of lactogenic binding sites in purified fractions of female rat liver Golgi membranes (67,68) suggests that intracellular sites may represent a precursor pool of receptor awaiting transport and incorporation into the plasma membrane (69). Consistent with this hypothesis is the finding by Posner et al. (68) that estrogen-stimulated induction of PRL receptors in rat liver occurs very rapidly in Golgi membranes but slowly in plasma membranes, lactogenic binding being undetectable in the latter until nearly four days after estrogen treatment. The demonstration of newly synthesized acetylcholine receptors in the Golgi apparatus of skeletal muscle cells (70) also strengthens the possibility that lactogenic binding sites on Golgi membranes

represent PRL receptors synthesized de novo.

However, alternative explanations for the Golgi receptors also exist. Josefsberg et al. (71) have reported that intravenously injected ^{125}I-ovine PRL is taken up by the female rat liver and is concentrated, intact, in the Golgi subfractions particularly at the points of maximum PRL receptor concentration. The authors propose that the arrival of bindable PRL at Golgi binding sites suggests that intracellular PRL receptors may not only be on route to the plasma membrane but may have a functional role within the cell, in the mediation of hormone action. Alternatively, the intracellular accumulation of PRL may represent the mechanism by which bound PRL is cleared from the hepatocyte surface. Rather than possessing a functional role, internalized PRL may merely be degraded and/or excreted into the bile (71). It is possible, too, that a substantial portion of the intracellular lactogenic receptors are not newly synthesized, but are recycled or internalized receptors sequestered in Golgi elements prior to lysosomal association and proteolytic breakdown.

While it is obvious that once PRL receptors are detectable within a cell they have undergone synthesis at some point, previous studies have demonstrated only changes in receptor activity rather than the biosynthesis of PRL receptors. As with other membrane receptors, endogenous labeling with metabolic precursors, and sensitive detection systems will be required to make a demonstration of PRL receptor biosynthesis possible.

CURRENT INVESTIGATION

The purpose of the current study is to examine and demonstrate definitively the biosynthesis of PRL receptor by the incorporation of labeled amino acids into receptor protein.

An important consideration in the study of receptor biosynthesis is selecting a target tissue which is rich in binding activity and in which the 'apparent' turnover rate of the receptor is rapid to maximize the incorporation of labeled amino acids. Late pregnant rat liver satisfies both of these criteria. First, on a 'per μg protein' basis it contains an abundance of assayable PRL receptors, compared to most other tissues (72). The high level of binding appears to be caused by elevated concentrations of lactogenic and estrogenic hormones during gestation; lactogenic hormones in particular are known to be potent inducers of hepatic PRL receptors in rats (26,27). Secondly, as mentioned above, the inhibition of protein synthesis causes a rapid drop in hepatic PRL binding (56) 'suggesting' a fast turnover rate for the liver PRL receptor.

Practical considerations, as well as theoretical ones, also favor the use of rat liver. The organ is large and can easily and quickly be removed after sacrifice, thus preserving the integrity of labile cellular components such as polyribosomes or mRNA. Liver is also virtually free of fatty and connective tissue (unlike mammary gland)--an appreciable property during the preparative steps of homogenization and fractionation.

Preliminary work in our lab by Dr. Richard Carlson and myself has shown that polyribosomes isolated from late pregnant rat liver by the method of Palmiter (73) contain specific binding sites for hGH. Some of the properties of these sites have been reported (74).

An exciting possibility (and one I have chosen to investigate) is that lactogenic hormones can interact with and bind to nascent receptor chains still associated with polyribosomes. To test this hypothesis I translated hepatic mRNA in a cell-free system derived from rabbit reticulocytes. In other studies, evidence for PRL receptor biosynthesis was examined in liver minces incubated in the presence of a radioactive amino acid.

Lastly, density-labeling techniques were applied to the study of receptor synthesis in the Nb2 node lymphoma cell line. This unique suspension-grown line has been established from the node lymphoma tumors of male rats (Nb strain). In vivo, growth of the tumors is accelerated by estrogen pellet implants and while the serum of estrogenized rats possesses 'lymphoma growth-promoting activity', the serum of estrogenized, hypophysectomized rats does not (75). This suggests that pituitary hormones or factors are needed to stimulate tumor growth. In vitro, Gout et al. (76) have demonstrated that PRL is the specific mitogenic agent from the pituitary. Stationary lymphoma cell cultures can be stimulated to divide by extremely low levels (10 pg/ml) of PRL in the medium. Because other lac-

togenic hormones (hGH and placental lactogens) are also mitogenic, the Nb2 lymphoma cell has provided a specific and highly sensitive bioassay system for this family of hormones (77).

The PRL-responsiveness of the Nb2 lymphoma cells has also made the cell line a useful model in the study of PRL receptors and the mechanism of PRL action. In this thesis, the development of the methodology for density-labeling PRL receptor is described; the approach should be applicable to the study of the rates of PRL receptor biosynthesis and degradation.

MATERIALS AND METHODS

GENERAL METHODS

Animals

For studies utilizing the cell-free translational system and liver mince system, timed pregnant Sprague-Dawley rats were killed by decapitation at day 15 - 21 of gestation. Livers were removed quickly and used immediately in the preparation of minces. Liver used for RNA purification was stored frozen at -20°C.

Hormones

Purified hGH (2019G, 2.2 IU/mg; HS 19340, 2.6 IU/mg), oGH (AFP 2924-B) and oPRL (PS-12, 35 IU/mg) were obtained from the NIAMDD (NIH), Bethesda, Maryland.

Radioiodination

hGH was radioiodinated by the lactoperoxidase method (79). Five µg hGH in 25 µl 50 mM phosphate buffer was mixed with 25 µl $Na^{125}I$; 5 µl of 0.002% H_2O_2 was added and the tube contents were mixed by shaking. After 2 min another 5 µl H_2O_2 (0.002%) was added to the mixture for 2 more minutes. (In oGH iodinations the reaction times were 2 x 5 min.) The reaction was stopped by the addition of 1 ml Tris buffer. An aliquot (10 µl) of the reaction mixture was TCA-precipitated to determine the per cent incorporation of ^{125}I. The remainder

was applied to a Sephadex G-100 column and 3 ml fractions were collected. Tracer quality in the peak fractions was assessed using female rat or rabbit liver membrane radioreceptor assays.

Tracer evaluation

By incubating tracer with an excess number of membrane binding sites it is possible to determine what fraction of the tracer is capable of interacting with its receptor. A 100 µl aliquot of ^{125}I-hGH (70,000 cpm) was incubated with increasing amounts of membrane protein. Duplicate tubes received 1 µg cold hGH or assay buffer. The final volume was adjusted to 0.5 ml/tube with assay buffer. After an overnight incubation at 22°C, 3 ml cold assay buffer was added to each tube and membranes were centrifuged for 20 - 30 min at 3000 rpm (PR 6000). Radioactivity in the pellets was determined.

Protein determination

Membrane protein concentrations were measured by the method of Lowry (78). Aliquots (0.1 ml) of membrane suspensions were mixed with 0.1 N NaOH (0.3 ml), boiled for 30 min and allowed to cool; 0.6 ml distilled water was added to each sample. BSA standards (0.1 ml) in concentrations ranging from 0.05 - 2.5 mg/ml were mixed with 0.3 ml 0.1 N NaOH; water was added bringing the final volume to 1.0 ml. Reagent #1 (a 1:1

v/v mixture of 1% $CuSO_4$ and 2% NaK-tartrate) was then prepared and used in the preparation of reagent #2 (a 50:1 v/v mixture of 2% Na_2CO_3 and reagent #1). Four ml of reagent #2 were added to each sample; after 10 min 0.5 ml of 1.0 N phenal reagent was added, tubes were vortexed and allowed to stand for 30 min. The absorbance at 660 nm of each sample was then determined.

Buffers (listed in order of use)

1. TMN buffer: 50 mM Trizma base (Sigma), 25 mM NaCl, 5 mM $MgCl_2$, pH 7.5.
2. Homogenization buffer: TMN buffer containing 2% (v/v) TX-100 (J. T. Baker), 500 µg/ml heparin (Sigma).
3. Polysome precipitation buffer: 50 mM Trizma base, 25 mM NaCl, 200 mM $MgCl_2$, pH 7.5.
4. Hepes buffer: 20 mM, pH 7.5.
5. RNA buffer A (ribonucleoprotein dissociation): 100 mM Trizma base, 4% (w/v) Sarkosyl (Schwarz/Mann), pH 8.0.
6. RNA buffer B (ethanol precipitation of RNA): 20 mM Trizma base, 120 mM NaCl, 0.5% SDS (Biorad), pH 7.5.
7. RNA buffer C (RNA disaggregation): 10 mM Trizma base, 0.5 M NaCl, 0.5% SDS, 1 mM EDTA, pH 7.5.
8. High-salt buffer: 10 mM Trizma base, 0.5 M NaCl, 1 mM EDTA, pH 7.5.
9. Low-salt buffer: 10 mM Trizma base, 0.1 M NaCl, 1 mM EDTA, pH 7.5.
10. Elution buffer: 10 mM Trizma base, 1 mM EDTA, pH 7.5.
11. Immunoprecipitation buffer: 15 mM Trizma base,

150 mM NaCl, 0.5% TX-100, 0.1% SDS, 0.5% DOC, 2 mM PMSF, pH 7.6.

12. Phosphate-buffered saline: 150 mM NaCl, 8.7 mM Na_2HPO_4 (dibasic), 1.3 mM NaH_2PO_4 (monobasic), 0.01% sodium azide, pH 7.4.
13. Solubilization buffer: 25 mM Trizma base, 10 mM $MgCl_2$, 1% TX-100, pH 7.6.
14. Assay buffer: 25 mM Trizma base, 10 mM $MgCl_2$, 0.1% BSA, pH 7.6.

STUDIES ON PRL RECEPTOR BIOSYNTHESIS USING A CELL-FREE TRANSLATION SYSTEM

Ribonuclease inhibition

Gloves were worn during preparative procedures and all solutions and glassware were autoclaved to minimize ribonuclease contamination. Detergents and ribonuclease inhibitors (heparin) were added to the homogenization buffer after autoclaving.

Polyribosome isolation by magnesium-precipitation

The methodology was adapted from Palmiter (73). For each 2 g wet weight of finely minced tissue (previously frozen at -20^{o}C), 10 ml of homogenization buffer was added. Tissue was homogenized on ice slush in a glass-glass Duall #23 homogenizer by motor-driven pestle. Four 30 sec burst were separated by 30 sec pauses. The homogenate was centrifuged for 15 min at 27,000 x g (Beckman 60 Ti rotor), the supernatant was decanted into a second 60 Ti tube and an equal volume of polysome precipitation buffer was added. After a 60 min incubation on ice, aliquots (8 ml) were layered over 4 ml pads of sucrose (0.25 M in TMN buffer) in polyallomer tubes and centrifuged for 15 min at 27,000 x g (Beckman SW-40 Ti rotor). The supernatant was removed by aspiration part way into the sucrose pad; the upper portion of the tube was washed with Hepes buffer and the wash removed by aspiration. The tube was

then inverted to decant the remaining sucrose. The tube was cut about 1 cm above the base; the polysomal pellet was resuspended and extracted.

Extraction of total polysomal RNA

RNA was extracted according to Glisen et al. (80) with minor modifications. Polysomal pellets from 2 g liver were resuspended in 6.0 ml RNA buffer A using several strokes of a Duall #22 homogenizer. CsCl was added to the resuspension (.67 g/ml) and stirred on ice until dissolved. The resuspension (3.5 ml) was then layered onto 1.4 ml of a 6.2 M CsCl, 100 mM EDTA pad in a cellulose nitrate centrifuge tube. RNA buffer A (0.2 ml) was finally layered on top and the tube was centrifuged in a Beckman SW-50.1 rotor at 35,000 rpm for 12 hr at 4°C. After centrifugation the supernatant was aspirated leaving about 0.5 ml CsCl above the RNA pellet. The tube was then inverted and all but the bottom 1 cm was sheared off. RNA was then ethanol-precipitated overnight.

Ethanol-precipitation of RNA

The RNA pellet was resuspended in 1 ml RNA buffer B using a Pasteur pipette, then transferred to a SW-40 cellulose nitrate tube. Solubilization was aided by smearing the RNA against the sides of the centrifuge tube with a glass rod and by vortexing the tube vigorously. Two volumes of ethanol were added, the tubes were vortexed and stored for at least 8 hr

at -20°C. Precipitated RNA was pelleted by centrifugation at 15,000 x g for 20 min (SW-40 rotor).

Salt-wash treatment of RNA

To effectively remove heparin, glycogen, DNA, and low molecular weight RNA (tRNA, 5S rRNA) (81), the RNA was washed in high concentrations of salt. RNA pellets were smeared against the centrifuge tube walls with a glass rod and resuspended (by vortexing) in 5 ml 2M LiCl, then pelleted by centrifugation at 30,000 x g for 5 min (SW-40 rotor). This procedure was repeated 2x more with 2M LiCl, then 3x with 3M sodium acetate pH 5.5, 5 mM EDTA. After the final wash the RNA pellet was solubilized and ethanol-precipitated as before.

Affinity chromatography of poly(A) RNA

Oligo(dT)-cellulose which selectively binds the poly(A) segment of mRNA was used to separate mRNA from other cellular RNAs. One-half gram of oligo(dT)-cellulose (T-3, Collaborative Research Inc.) was mixed in high-salt buffer and poured as a slurry into a sterile 5 ml plastic syringe attached to a stand. An autoclaved glass wool plug at the bottom of the column (syringe) retained the cellulose. Prior to sample application, several bed volumes of RNA buffer C were passed through the oligo(dT) column.

Ethanol-precipitated RNA was dried by a N_2 stream or lyophilized, then dissolved in 1.0 ml RNA buffer C by breaking

up the pellet with a Pasteur pipette and vigorously vortexing. RNA was disaggregated by heating to 65 - 70°C (water bath) for 3 min and cooling quickly to room temperature in ice slush. The RNA sample was applied to the column and non-specifically adsorbed RNA was removed by washing the column with at least 5 ml high-salt and 5 ml low-salt buffer until no A_{260} absorbing material could be detected. Poly(A) RNA was then collected in 8 - 10 ml elution buffer; 1.0 M NaCl (in 10 mM Tris, 1 mM EDTA, pH 7.5) was added to the eluate to yield a NaCl concentration of 0.12 M, and poly(A) RNA was precipitated by the addition of two volumes ethanol.

A second oligo(dT) step was used to further purify mRNA since the poly(A) RNA preparation usually contains contaminating rRNA after a single column step (81). Preparative and chromatographic steps were essentially the same as before. After ethanol-precipitation and centrifugation of the purified mRNA, the pellet was lyophilized and dissolved in autoclaved, distilled, deionized water to yield a mRNA stock concentration of 4 - 5 µg/µl (1.0 A_{260} unit = 42 µg RNA). Aliquots of this preparation were stored at -70°C or were diluted and used directly in translation assays.

After use, oligo(dT) cellulose was stored in sterile 1.0 M NaCl-Tris, pH 7.5 at 4°C. Before reusing for the purification of a new batch of poly(A) RNA the cellulose was repacked, washed with 0.1 N NaOH, and pH-adjusted with 10 mM Tris pH 7.5.

Translation of poly(A) RNA in a cell-free system

A rabbit reticulocyte lysate kit (New England Nuclear) was used in all translation assays. The necessary ingredients for cell-free translation (except mRNA) were included in the kit. In preparation for the assay, potassium acetate, magnesium acetate, the 'cocktail' (a solution containing various components including spermidine, creatine phosphate and GTP) and a radioactive amino acid (^{3}H-leucine or ^{35}S-methionine) were combined into a 'premix'. Two µl mRNA (or water) were pipetted into 1.5 ml Eppendorf microtubes; next 13 µl 'premix' were added per tube; lastly, 10 µl lysate were added. Twenty µCi ^{3}H-leucine or ^{35}S-methionine were present in each assay tube (unless otherwise specified) and potassium and magnesium concentrations were 80 mM and 0.65 mM, respectively. A 25 µl reaction volume was typically used for testing the translating activity of various mRNA preparations. A 4-fold scale-up was used when immunoprecipitation of PRL receptor was attempted.

Immediately after adding the lysate, the tube contents were vortexed, then centrifuged for 15 sec in a Beckman microfuge to ensure complete mixture of all components at the bottom of each tube. Tubes were incubated for 1 hr in a 37^{o}C water bath and the reaction was stopped by placing tubes on ice.

Determination of labeled amino acid incorporation

Aliquots of each assay tube were spotted onto 1 cm wide strips of Whatman filter paper divided into 1 cm squares.

Strips were placed into boiling 10% TCA for 5 min (to wash off charged tRNA), transferred to cold 5% TCA for 10 - 15 min and then to cold acetone for 10 - 15 min. Squares were cut apart after the papers were dry, and placed into scintillation vials. Five ml ACS (Amersham) were added to each vial and the radioactivity per filter determined on a LKB Rack-beta β-counter.

Immunoprecipitation of cell-free products

Aliquots (100 µl) of the reaction mixture were mixed with a small volume (10 µl) of concentrated immunoprecipitation buffer yielding final concentrations of 8 mM Tris, 90 mM NaCl, 0.3% TX-100, 0.06% SDS, 0.3% DOC and 1 mM PMSF. Cold amino acid (leucine or methionine) was added (10 mM); guinea pig antiserum to rabbit mammary PRL receptor (anti PRL-Rc), normal guinea pig serum (NGPS), rabbit anti-rat serum albumin (anti-RSA) from Cappel Labs, or normal rabbit serum (NRS) were then combined with the mixture yielding a final dilution of 1:12 - 1:50. (All sera had been complement-inactivated by heating at 56^{o}C for 30 min.) Tubes were vortexed and incubated at least 24 hr at 4^{o}C.

Preparation of Staphylococcus aureus (Staph A)

The method of Kessler (82) was used in the isolation of immunoprecipitates. Staph A (Pansorbin, Calbiochem-Behring) was treated prior to use by resuspending in a 10% glycerol,

2% SDS solution, heating at 85 - 90°C for 5 min, and centrifuging the resuspension in a microfuge. The pellet was washed once with immunoprecipitation buffer containing 3 M urea (Schwartz/Mann), then twice with immunoprecipitation buffer only. Finally the Staph A was resuspended in fresh immunoprecipitation buffer.

Isolation of immunoprecipitates

For each µl serum added to the cell-free reaction mixture, 15 µl Staph A suspension was added (following the incubation period). Tubes were incubated on ice for 30 min with intermittent vortexing. Immunoprecipitates were then sedimented in Eppendorf microtubes through a discontinuous gradient (83) consisting of 200 µl of 0.5 M sucrose (PBS) overlayered on 800 µl 1.0 M sucrose, each containing 1% TX-100, 1% DOC, and 10 mM nonradioactive leucine (or methionine). Gradients were 'microfuged' for 15 min. The top 0.5 ml of supernatant was aspirated and discarded; 0.5 ml immunoprecipitation buffer was added, washing the upper portion of the tube; the wash was then removed. The remainder of the sucrose cushion was aspirated and the pellet was transferred to a new microtube after resuspension in 200 µl 3 M urea/immunoprecipitation buffer. Immunoprecipitates were washed twice in 1 ml immunoprecipitation buffer (first wash with 3 M urea, second wash without urea). Washed pellets were resuspended in 10% glycerol, 2% SDS, 5% β-mercaptoethanol (Biorad), heated in a 95°C water

bath for 5 min and centrifuged. Aliquots (5 µl) of the supernatant were mixed with scintillation fluid and radioactivity determined on a β-counter; larger aliquots were analyzed by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

Equipment and reagents were purchased from Biorad. Resolving gels were 7 - 10% acrylamide:bis and were overlayered by 4% stacking gels; slab gel thickness was 0.75 mm. Twenty µl samples were applied with a Hamilton syringe and electrophoresis was carried out for 3 - 4 hr using a 20 - 30 mAmp current. Gels were fixed for 30 min in 50% TCA and soaked in a 7% acetic acid solution overnight.

Fluorography

To reduce autoradiography exposure time, gels were impregnated with the fluor PPO (84). Gels were placed in a DMSO-bath for 30 min; this step was repeated with fresh DMSO. After dehydration gels were bathed in a 20% PPO-DMSO (w/v) solution (under gentle shaking) for 3 hr, then placed in distilled water for 1 hr to reswell. Gels were heat-dried under vacuum and exposed to Kodak film at -70^{o}C.

STUDIES ON PRL RECEPTOR BIOSYNTHESIS USING A MINCED TISSUE SYSTEM

Feasibility of the indirect immunoprecipitation method

Sucrose gradients were used to analyze the interaction of guinea pig anti-hGH antiserum (anti-hGH) with ^{125}I-hGH-receptor complexes. ^{125}I-PRL was not used because it forms aggregates in the presence of TX-100 (85). Microsomal membranes (100,000 x g pellet) of male or late pregnant female rat liver were resuspended in solubilization buffer and stirred for 30 min at 22^{o}C. The mixture was centrifuged for 90 min at 92,000 x g in a Beckman Type 25 rotor. The supernatant was diluted 1:10 with assay buffer to yield a final TX-100 concentration of 0.1%. Aliquots (0.5 ml) were then incubated with ^{125}I-hGH (200,000 cpm/tube) overnight at 22^{o}C. Each tube contained the solubilized extract of 400 µg membrane protein.

An excess of anti-hGH (5 µl) was added to tubes containing ^{125}I-hGH and solubilized membrane (0.5 ml total volume) and to tubes containing ^{125}I-hGH (200,000 cpm) only in 0.5 ml 0.1% TX-100-assay buffer. Tubes were then incubated for 24 hr at 4^{o}C.

Each 0.5 ml sample was layered onto a linear 2 - 30% (w/w) sucrose (in TMN buffer) gradient in a cellulose nitrate tube and centrifuged for 15 hr at 40,000 rpm in a SW-40 rotor.

Gradients were fractionated with an Isco density gradient fractionator; forty-five 6-drop fractions were collected

per gradient and the radioactivity in each fraction was determined. Also, the bottom 0.5 cm of the centrifuge tube was sheared off, placed into an assay tube and counted.

^{35}S-methionine-labeling of liver mince proteins

The liver of freshly killed late pregnant rats was removed and perfused with saline to rid the tissue of as much blood as possible. One gram pieces were finely cut with scissors into 1 - 2 mm cubes in either Krebs-bicarbonate medium containing 0.2% glucose and 100 µg/ml soybean trypsin inhibitor, or Eagles-Hela medium (diluted 1:10 with Earl's balanced salt solution). After low-speed centrifugation the 'wash' medium was decanted and minces were resuspended in 5.0 ml fresh medium in 25 ml Erlenmeyer flasks. ^{35}S-methionine (100 - 200 µCi/ml/flask) was added; the flasks were covered with perforated parafilm and incubated in a 37^{o}C shaking water bath (95% O_2, 5% CO_2 atmosphere) for 6 hr. Small samples of medium (50 - 100 µl) were withdrawn at various time intervals to determine the incorporation of label during the incubation period. Minces were centrifuged after incubation, the pellets were washed twice with 10 ml fresh medium and then frozen at -70^{o}C.

Preparation of solubilized extract

Minces were thawed, washed with 10 ml medium, mixed with 1 ml 25 mM Trizma, 150 mM NaCl, 1.0% TX-100 (pH 7.5) and homogenized as described above for polysome preparation.

Homogenates were stirred slowly at room temperature for 30 min, sonicated for 1 min (Bransonic) and centrifuged for 90 min at 92,000 x g (Type 25 rotor). The supernatant was removed and analyzed for acid-insoluble radioactivity, and labeled immunoprecipitable rat serum albumin and PRL receptor, using direct methods of immunoprecipitation (see below).

To prepare solubilized microsomal fractions, the labeled minces were homogenized in 5 ml TMN buffer (20 mM methionine) and centrifuged for 10 min at 20,000 x g. The supernatant was centrifuged for 1 hr at 105,000 x g (Beckman Ti 50 rotor) and the pellet was resuspended in 1 ml solubilization buffer, stirred for 30 min at 22^{o}C, sonicated for 2 min and centrifuged for 90 min at 92,000 x g (Type 25 rotor). The supernatant was analyzed for acid precipitability and immunoprecipitable PRL receptor (via direct and indirect methods).

TCA precipitation

Aliquots (5 µl) of the homogenate, solubilized extract and incubation medium were spotted onto 1 cm squares of filter paper and TCA-treated as described above.

Direct immunoprecipitation

Antisera (anti-RSA and anti-PRL-Rc) or normal sera were added to solubilized extract (100 µl and 250 µl aliquots) containing 10 mM nonradioactive methionine to yield a final serum dilution of 1:20. Incubation was carried out for 48 hr

at 4°C and immunoprecipitates were isolated as described below.

Indirect immunoprecipitation

Aliquots (250 µl) of solubilized microsomal extract were diluted 1:10 with assay buffer reducing the TX-100 concentration to 0.1%. Each tube contained the solubilized extract of 0.23 g equivalents of liver minces. Unlabeled hGH (75 ng in 15 µl) was added and tubes were incubated overnight at 22°C. Twenty-five µl anti-hGH (or NGPS) were then added yielding a final serum dilution of 1:100. Following a 24 hr incubation at 4°C immunoprecipitates were isolated as described above.

Gel slicing

Immunoprecipitates were prepared for scintillation counting and SDS-polyacrylamide gel electrophoresis as described in the section on cell-free translation studies. To shorten the time required for gel analysis, scintillation counting of gel slices (rather than fluorography) was performed. After electrophoresis, slab gels were placed onto a grid and the sample lanes were cut apart in 2 cm wide strips. Gel strips (placed onto plexiglass sheets and frozen on dry ice) were cut into 2 mm slices with a Biorad model 190 gel slices, and placed into scintillation vials. Slices were treated with NCS (Amersham) and hyamine hydroxide (New England Nuclear) by the method of Aloyo (86) and the radioactivity was determined.

STUDIES ON PRL RECEPTOR BIOSYNTHESIS USING DENSITY-LABELING TECHNIQUES

Cell culture conditions

Fischer's medium, FCS, HS and penicillin/streptomycin were obtained from Gibco. Nb2 node lymphoma cells (Nb2 cells) were cultured in Fischer's medium containing 10% FCS, 10% HS, penicillin (50 units/ml), streptomycin (50 μg/ml), β-mercaptoethanol (10^{-4} M) and $NaHCO_3$ (0.075%). Cell suspensions were incubated at 37°C in plastic culture flasks in a 5% CO_2 - 95% air atmosphere. Cell growth was monitored with a Coulter counter. Cells were routinely cultured to a density of 1.5 x 10^6 cells/ml and then passaged.

Preparation of solubilized extract

Nb2 cells (about 10^8) were pelleted by centrifuging for 5 min at 1000 rpm and stored at -70°C. Cells were washed once with 10 ml 0.3 M sucrose, resuspended in sucrose solution (12 ml) and homogenized by the method described for rat liver. The homogenate was sonicated for 1 min and centrifuged for 30 min at 100,000 x g (SW-40 rotor). The clear sucrose supernatant was decanted; solubilization buffer (0.5 ml) was added and the pellet (about 100 mg) was resuspended with a glass-glass homogenizer. After stirring the mixture for 30 min, it was sonicated for 1 min and centrifuged for 90 min at 92,000 x g (Type 25 rotor). The supernatant which was assayed

for binding activity was stored at -22°C.

Binding assays

^{125}I-hGH was routinely used in binding assays because of the aggregating effect of TX-100 on ^{125}I-PRL (85). Solubilized extracts were diluted 1:2 - 1:32 and ^{125}I-hGH binding activity was measured in 100 µl aliquots of each dilution. Duplicate tubes received in order: 100 µl ^{125}I-hGH (60,000 cpm), 100 µl cold hGH (1 µg) or 100 µl assay buffer, 200 µl assay buffer and 100 µl extract. The final concentration of TX-100 in each tube was about 0.2%. After overnight incubation at 22°C, 0.5 ml of 0.1% bovine λ-globulin (fraction II) in PBS buffer was added to each tube followed by 1 ml of 25% PEG in PBS. Tubes were vortexed, refrigerated (4°C) for 15 - 20 min and centrifuged at 3000 rpm (PR 6000) for 30 min. The supernatant was aspirated and the radioactivity in the pellets was determined.

To measure specific binding of ^{125}I-hGH to intact Nb2 cells, cells were centrifuged at 1000 rpm for 5 min, washed once with Fischer's medium (10% HS) and concentrated in a small volume such that cell density was about 10^7 cells/ml. Duplicate assay tubes received 100 µl ^{125}I-hGH (10^6 cpm), and 100 µl cold hGH (1 µg) or 100 µl assay buffer. Cells (0.5 ml) were then aliquotted into the assay tubes and incubated for 1 hr at 37°C in a 5% CO_2 - 95% air atmosphere. The reaction was stopped by the addition of 3 ml cold buffer and cells were

centrifuged for 15 min at 3000 rpm (PR 6000). The supernatant was aspirated and radioactivity in the cell pellets was determined.

Specificity of hGH binding

Cells cultured in 10% FCS - 10% HS-medium were transferred to 10% HS-medium for 24 hr. After centrifugation, cells were concentrated in 10% HS-medium (8.8×10^6 cells/ml) and 500 µl was aliquotted into duplicate assay tubes containing ^{125}I-hGH (4.5×10^6 cpm) and increasing concentrations of cold hGH, oPRL and oGH. Tubes containing ^{125}I-oGH, plus or minus cold hGH or oGH (1 µg), also received 500 µl of the cell suspension. Incubation conditions and bound/free separation of tracer were described above.

The solubilized extract of Nb2 cells cultured in 10% HS-medium was incubated with ^{125}I-hGH or ^{125}I-oGH (60 - 70,000 cpm/tube) in the presence or absence of excess (1 µg) cold hormone. Tubes were incubated overnight at 22°C, PEG-treated, centrifuged, and the pellets were counted.

Effect of HS and FCS on ^{125}I-hGH binding activity

Three groups of Nb2 cells were cultured in medium containing 10% FCS, 10% HS until the cell density was 1.5×10^6 cells/ml. All the cells were centrifuged (1000 rpm for 5 min) and one group was frozen at -70°C. The other two groups were resuspended in the original volume (150 ml) of medium, but

containing HS (10%) only--no FCS. The second group of cells was collected and frozen after 12 hr incubation, and the third group after 29 hr incubation.

Cells were washed in 0.3 M sucrose and processed as described above. The absorbance (A_{210}) of each of the three extracts (diluted 1:64) was measured against a solubilization buffer reference. The same buffer was then added to each extract to equalize their concentrations of solubilized material. Serial dilutions were made and duplicate 100 µl aliquots of each extract dilution were assayed for binding activity as described.

Effect of cycloheximide on ^{125}I-hGH binding activity

Cells cultured in Fischer's medium (10% FCS, 10% HS) were transferred to 10% HS-medium at a concentration of 1.4 x 10^6 cells/ml. The cells were plated out (15 ml/culture dish) and increasing concentrations of cycloheximide (in 0.6 ml sterile water) were added to the dishes. After a 12 hr incubation the contents of each dish were centrifuged and resuspended in 2.5 ml of fresh Fischer's medium (10% HS), a 6-fold concentration. Control cells grown in FCS-containing medium (no cycloheximide) were centrifuged, washed once with 10% HS-medium, then resuspended in the same medium and at the same density (8 x 10^6 cells/ml) as cycloheximide-treated cells. Duplicate aliquots (500 µl) of each group were assayed for ^{125}I-hGH binding as described above.

An aliquot of each group of cells was centrifuged and resuspended in isotonic saline (Hematall). Trypan blue (about one drop per 0.25 ml cells) was added and cells were incubated at 37°C for 20 - 30 min. Dye exclusion was examined by light microscopy.

Effect of CsCl on the binding of ^{125}I-hGH

Microsomal membranes (20.7 mg protein) of pregnant rat liver were solubilized in 6 ml solubilization buffer as described in the section on minced tissue systems. The 100,000 x g supernatant contained the solubilized extract of about 350 µg membrane protein per 100 µl.

A 7.355 M stock solution of CsCl (in assay buffer) was serially diluted to 1:50. Dilutions (300 µl) were then added to assay tubes containing 100 µl ^{125}I-hGH (60,000 cpm), and 100 µl cold hGH (1 µg) or 100 µl assay buffer. Finally 100 µl extract was added to each tube. Assay tubes were shaken to ensure proper mixing of the CsCl, and incubated overnight at 22°C. Bound and free tracer were separated by PEG as described above and the pellets were counted.

Detection of ^{125}I-hGH binding activity on CsCl gradients

Nb2 cells (about 2 x 10^8) were solubilized and extracted as described above. The method of Reed and Lane (55) was applied to the study of PRL receptor on CsCl gradients but the SW-40 rotor (13 ml capacity tubes) was used so that larger

fractions could be collected. An aliquot (0.7 ml) of Nb2 cell solubilized extract was mixed uniformly with 12.3 ml CsCl (0.5 g/ml) in 25 mM Tris buffer, pH 7.6. The mixture was poured into a SW-40 cellulose nitrate tube and centrifuged for 48 hr at 35,000 rpm.

In order to obtain fractions of equal size (in the presence of TX-100) timed fractions were collected (1.3 min fractions; flow rate = 0.375 ml/min). Twenty-five fractions (about 0.5 ml each) were obtained from each gradient. The shape and density (refractive index) of the gradients were determined with a Zeiss refractometer.

Duplicate aliquots (2 x 100 µl) were assayed for ^{125}I-hGH binding activity in the presence or absence of cold hGH (1 µg). Assay buffer was added to each tube (0.5 ml final volume) and tubes were incubated overnight at 22^{o}C. Following PEG-treatment the assay tubes were centrifuged and radioactivity in the pellets was determined.

CsCl gradient improvement

In order to improve gradient linearity and to 'centre' the peak of lactogenic binding activity, discontinuous CsCl gradients were formed prior to centrifugation. A dense CsCl solution (0.5 g/ml) was overlayered by a lighter (0.25 g/ml) solution and Tris buffer was layered on top. Each 'step' had a volume of 4.3 ml. After 14 and 40 hr centrifugation, gradients were fractionated and the linearity determined with a

refractometer.

Solubilized Nb2 extract was mixed with 0.25 g/ml CsCl, the centre 'step'; the gradient was layered as before and centrifuged for 38 hr at 40,000 rpm. Gradients were fractionated and duplicate aliquots (100 µl) were assayed for specific ^{125}I-hGH binding activity. Because nonspecific binding (i.e., binding in the presence of excess cold hGH) was identical to background binding (i.e., binding to gradient fractions in the absence of solubilized membrane protein), only total binding rather than specific binding was measured in subsequent experiments.

The solubilized extract of female rat liver membranes was also centrifuged on CsCl gradients (prepared by the 'step' method) and ^{125}I-hGH binding activity was analyzed.

^{2}H,^{13}C-labeled amino acids

^{2}H,^{13}C-labeled ('dense') amino acids were purchased as algal hydrolysates from Merck, Sharpe and Dohme (Pointe Claire, Quebec). Hydrolysates (250 mg) were dissolved in 50 ml Fischer's medium (containing no amino acids except glutamine) and sterilized by Millipore filtration. A 5-fold dilution of the stock solution was made during cell culture so that the final concentration of hydrolysates was 1 mg/ml. A comparison of amino acid analyses for normal Fischer's medium and Fischer's medium containing dense amino acids is presented in this section.

Amino acids which were destroyed during acid hydrolysis or were present in very low concentrations in algae, were supplemented during cell culture. The stock solution was concentrated 10-fold in amino acid-deficient Fischer's medium. A list of the supplementary amino acids and the concentrations present in the culture medium is included in this section (p. 41).

To increase the specific activity of the dense amino acids in the culture medium, HS (100 ml) was dialyzed against one litre 0.9% NaCl for 18 hr and against 15 litres of fresh saline for 28 hr, then filter-sterilized (Nalgene).

During cell culture the concentrations of HS, penicillin/streptomycin, β-mercaptoethanol and $NaHCO_3$ in the medium containing dense amino acids were identical to the concentrations in control medium.

Detection of newly synthesized proteins

Nb2 cells were grown in Fischer's medium (10% FCS, 10% HS) to a density of 1.2×10^6 cells/ml. Cells were then centrifuged (25 ml/tube) and pellets were resuspended in Fischer's (10% HS) medium (25 ml) containing either normal or dense amino acids. ^{3}H-leucine was added to each flask; the final concentration of radioactivity was 15 μCi/ml. After a 24 hr incubation (5% CO_2, 95% air), cells were collected, washed with 0.3 M sucrose and homogenized in 12 ml 0.3 M sucrose. After centrifugation, the 100,000 x g crude pellet was resuspended with 1.0 ml solubilization buffer stirred for 30

Amino acid composition of Fischer's medium (mg/100 ml)

Amino acid	'Normal' med.	'Dense' med.	Supplement
Alanine	-	9.17	-
Arginine	1.5	5.78	-
Asparagine	1.0	-	0.5
Aspartic acid	-	8.91	-
Cysteine	-	0.47	-
Cystine	2.0	-	2.0
Glutamic acid	-	11.41	-
Glutamine	20.4	20.40	-
Glycine	-	4.53	-
Histidine	6.6	0.84	3.0
Isoleucine	7.5	4.34	-
Leucine	3.0	7.80	-
Lysine	5.0	3.84	-
Methionine	10.0	1.28	5.0
Phenylalanine	6.0	3.64	-
Proline	-	3.60	-
Serine	1.5	4.52	-
Threonine	3.0	5.34	-
Tryptophan	1.0	-	1.0
Tyrosine	6.0	2.59	-
Valine	7.0	5.83	-

min and centrifuged at 92,000 x g. The supernatant (about 1 ml) was applied to a discontinuous CsCl gradient as described above and centrifuged for 90 hr at 35,000 rpm (SW 40 rotor).

Gradients were fractionated (46 fractions) and the refractive index measured. Aliquots (50 µl) of each fraction were mixed with Aquasol-2 (New England Nuclear) in scintillation vials and radioactivity was determined.

Detection of density-labeled PRL receptor

Cells were cultured in normal or 'heavy' Fischer's medium containing 10% HS (as described above) at a concentration of 1.4×10^6 cells/ml and in a total medium volume of 60 ml/flask. After a 12 hr incubation cells were collected and a solubilized extract was prepared as before.

A shorter column of CsCl was used. One ml of normal or 'heavy' Nb2 extract was mixed with 3.5 ml CsCl (0.25 g/ml) and poured into a centrifuge tube. Using a Pasteur pipette, 2 ml of dense CsCl (0.5 g/ml) was placed under the mixture. The upper half of the tube was filled with mineral oil and gradients were centrifuged for 65 hr at 40,000 rpm (SW 40 rotor).

Mineral oil was removed by aspiration and gradients were fractionated (31 x 0.5 min fractions; flow rate = 0.375 ml/min). The volume of each fraction was about 0.18 ml; 150 µl was assayed for ^{125}I-hGH binding activity and a small aliquot was read on the refractometer.

RESULTS

STUDIES ON PRL RECEPTOR BIOSYNTHESIS USING A CELL-FREE TRANSLATION SYSTEM

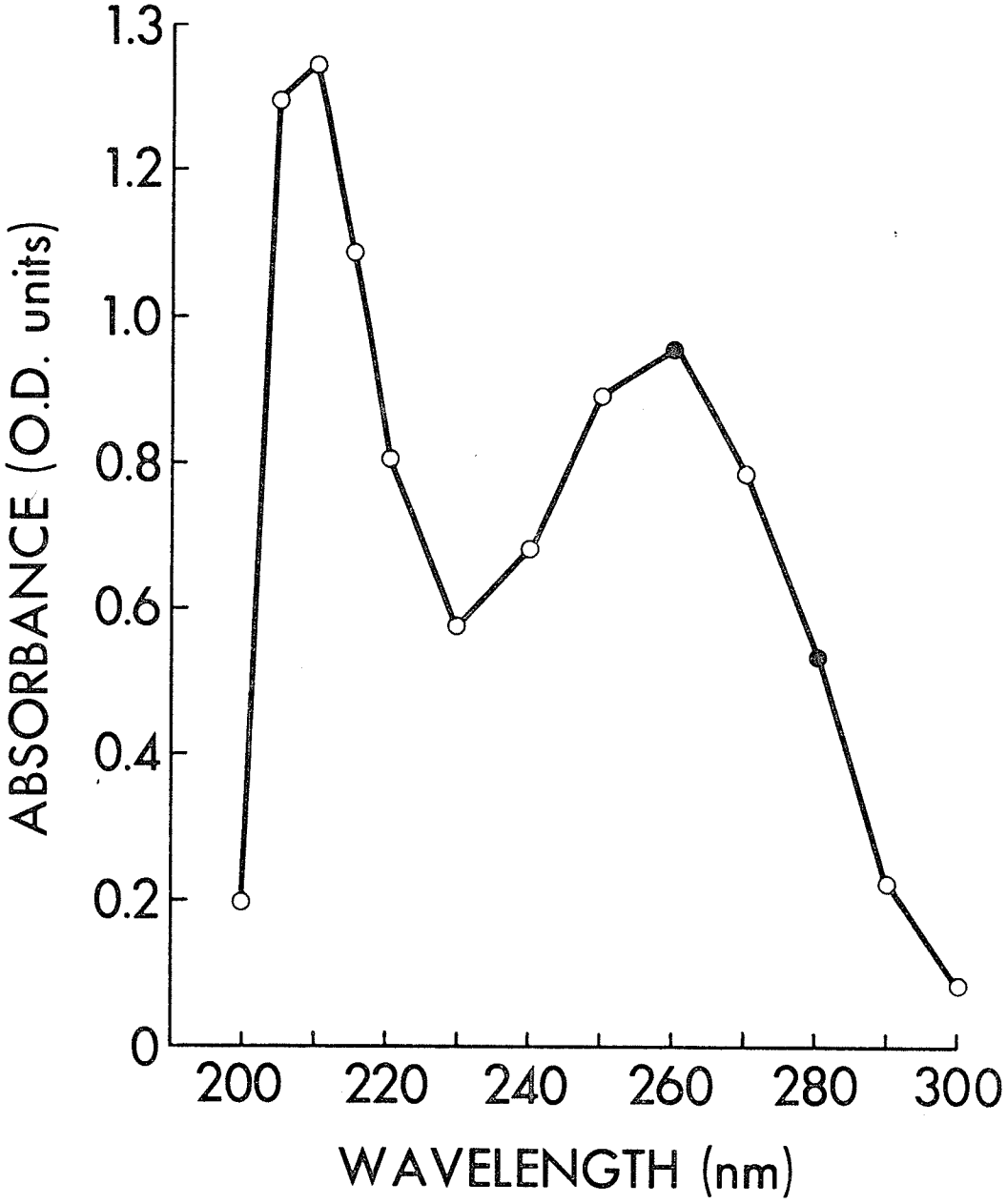
Purification of poly(A) RNA

The absorbance profile of late pregnant rat liver RNA purified on CsCl gradients (80) is shown in figure 1. Total RNA prepared by the CsCl method typically had an $A_{260}:A_{280}$ ratio of 1.7 - 1.8 (occasionally > 1.9), demonstrating a successful dissociation of RNA from contaminating cellular proteins. Washing procedures and oligo(dT) chromatography further increased the $A_{260}:A_{280}$ ratio (data not shown) yielding a final ratio of 2.0 - 2.2 for the RNA tested for translational activity in a cell-free system.

The affinity chromatography of total liver RNA on oligo(dT) cellulose is demonstrated in figure 2. The bulk of the cellular RNA (> 90%), particularly ribosomal RNA was removed from the column by Tris buffer containing 0.5 M NaCl. Additional A_{260}-absorbing material was eluted from the column with low-salt (0.1 M NaCl) Tris buffer. Bantle et al. (87) have indicated that this fraction is mostly 18S and 28S RNA but contains, in addition, a third, rapidly sedimenting component which is capable of hybridizing with oligo(dT) cellulose after disaggregation with DMSO; the proportion of oligo(dT)-hybridizable RNA in the 0.1 M NaCl fraction, however, is very

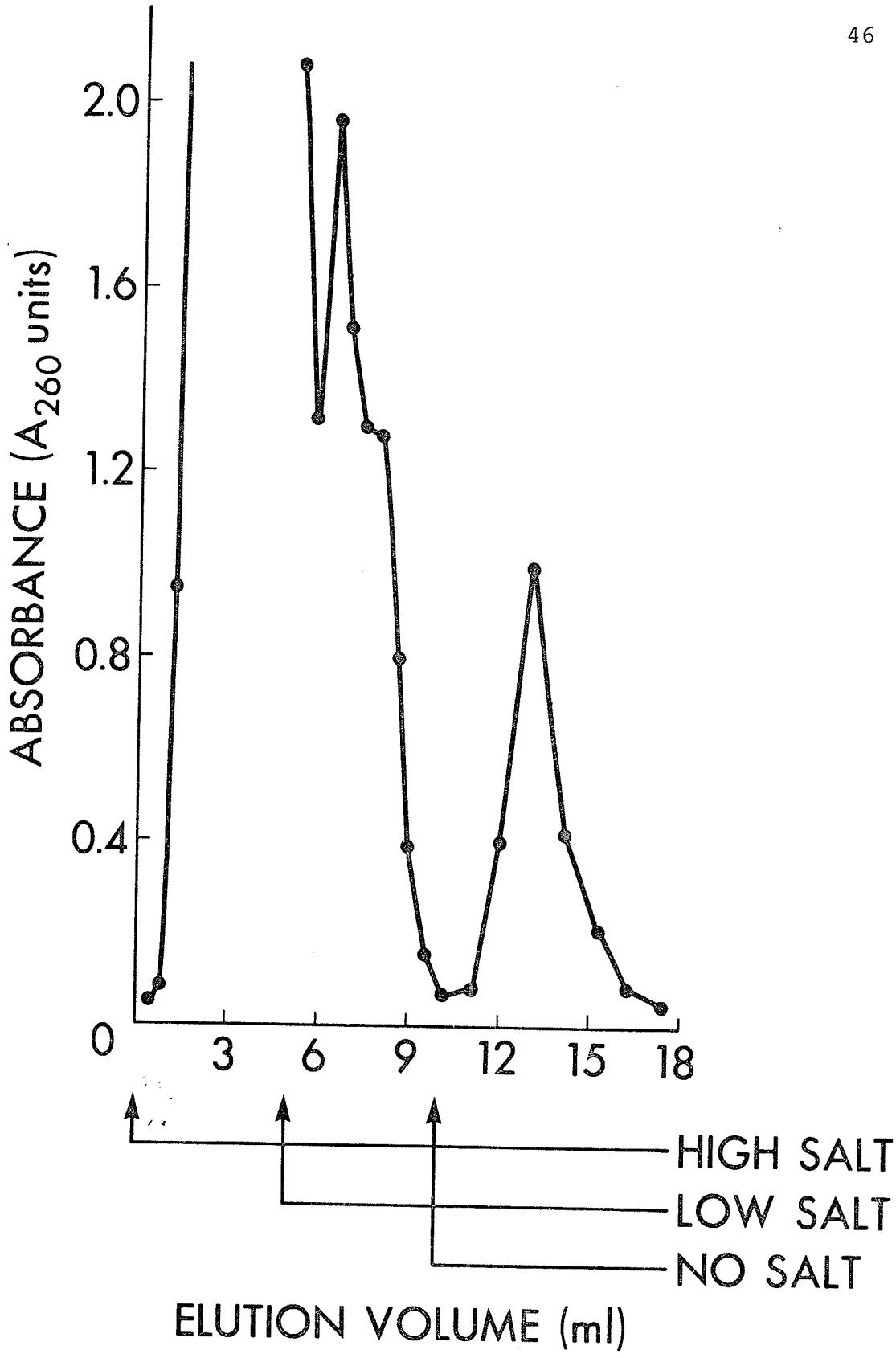
Figure 1. Absorbance profile of CsCl-purified RNA: Rat liver RNA isolated by CsCl gradient centrifugation was dissolved in 0.5% SDS. The absorbance ratio $A_{260}:A_{280}$ was used as an index of purification.

Table 1. Recovery of poly(A) RNA by oligo(dT) chromatography: RNA from approximately 2 g of liver was adsorbed to oligo(dT) cellulose and poly(A) RNA was eluted at low ionic strength. Absorbance at 260 nm was used to quantitate recoveries. Run #1 shows the poly(A) RNA eluted from the column after removal of non-specifically bound RNA. Run #2 demonstrates the chromatography of the initial poly(A) RNA preparation.



	TOTAL RNA	POLY (A) RUN #1	POLY (A) RUN #2
A_{260} UNITS	66.0	2.0	0.8
% RECOVERY	100.0	3.0	1.2

Figure 2. Purification of poly(A) RNA by oligo(dT) chromatography: Crude RNA dissolved in RNA buffer C was heated, quick-cooled and applied to the oligo(dT) column (0.5 g cellulose). Five ml high salt buffer (0.5 M NaCl), 5 ml low-salt buffer (0.1 M NaCl) and 8 ml elution buffer (10 mM Tris, pH 7.5) were added sequentially.


2.0
1.6
1.2
0.8
0.4
0
ABSORBANCE (A_{260} units)
3
6
9
12
15
18
HIGH SALT
LOW SALT
NO SALT
ELUTION VOLUME (ml)

small (87) and further purification of this fraction was therefore not carried out in our laboratory.

The poly(A) RNA was eluted in 8 - 10 ml 10 mM Tris buffer (pH 7.5) containing no NaCl (figure 2). Table 1 (which corresponds to the experiment illustrated in figure 2) quantitates the recovery of poly(A) RNA. In most experiments the recovery of poly(A) RNA represented 2 - 5% of the total amount of RNA applied to the column. In two separate experiments, however, (data not shown) 13.0 and 13.4%, respectively, of the total RNA were recovered in the NaCl-free fraction. The reason for such variations in poly(A) RNA recovery are not clear; Bantle et al. (87) have suggested that incomplete disaggregation of crude RNA prior to column application leads to the elution of rRNA (bound to column-adsorbed poly(A) RNA) in the NaCl-free fraction. In this way, the percentage of total RNA represented by poly(A) RNA can be overestimated.

Rechromatography of 'crude' poly(A) RNA was performed to remove contaminating RNA which had co-eluted with the poly(A) RNA during the initial purification. This second chromatography step was conducted similarly to the first (see Materials and Methods). Only 40 - 45% of the applied RNA was adsorbed by oligo(dT) cellulose (table 1), suggesting that approximately one-half the RNA in the preparation was of a non-poly(A) nature. The twice-purified poly(A) RNA was used in cell-free translation studies; additional chromatography steps are probably not necessary since virtually all of this RNA is

oligo(dT)-adsorbable (87).

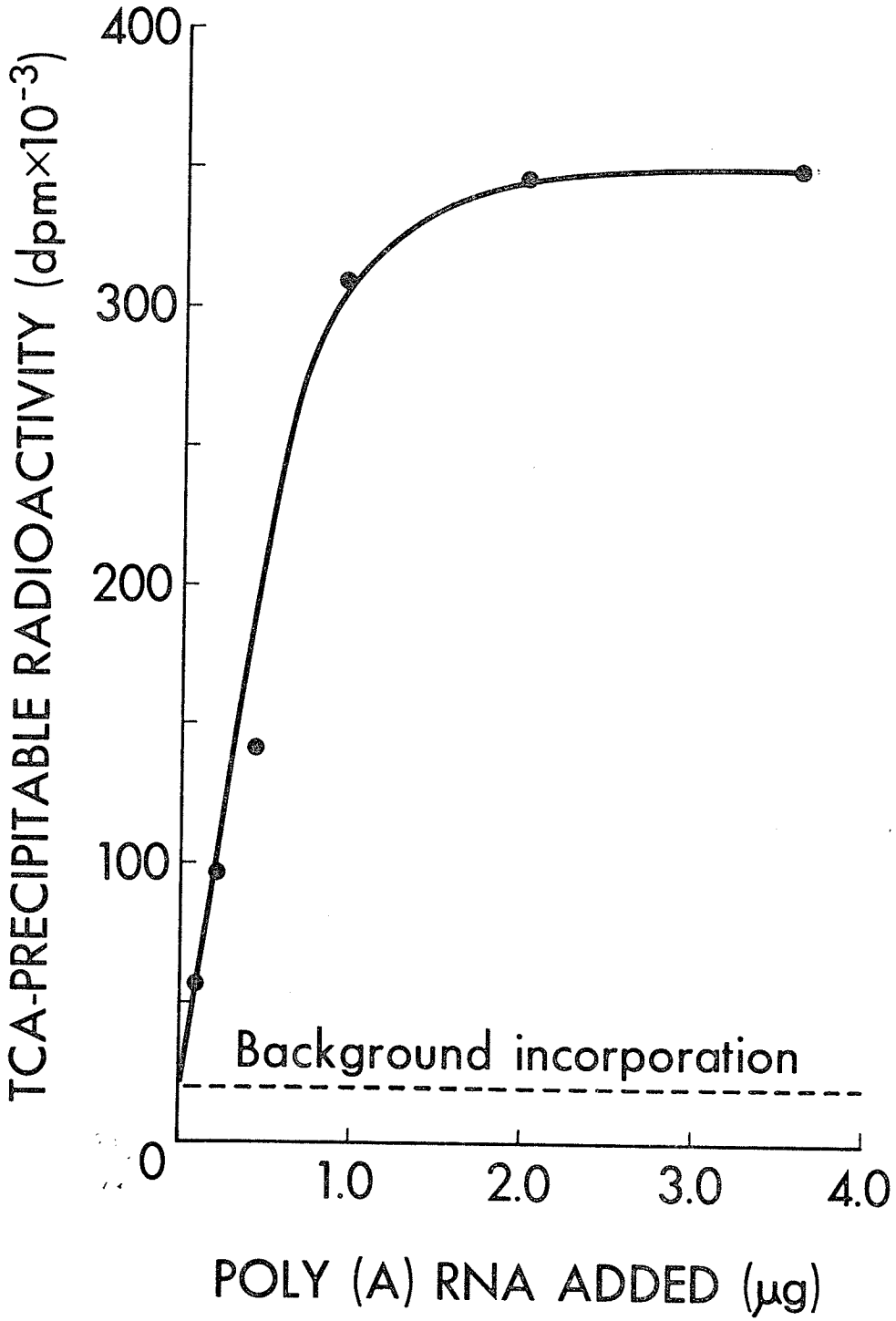
Translation assay of poly(A) RNA

Poly(A) RNA from pregnant rat liver was translated in a cell-free rabbit reticulocyte system as described in Materials and Methods. Figure 3 shows a typical dose-response curve for the poly(A) RNA. Maximum incorporation (about 8 x 10^5 dpm above background/25 µl cell-free reaction volume) was observed at 2 - 3 µg poly(A) RNA per 25 µl assay volume. The added RNA produced about a 17-fold stimulation of ^{3}H-leucine incorporation above background.

Background incorporation in the reticulocyte lysate system is due to non-specific trapping of radioactivity on filters and to protein synthesis by the incompletely inactivated endogenous mRNA. The micrococcal nuclease-lysate reaction, which is designed to increase the sensitivity of the reticulocyte cell-free system to exogenous mRNA (88), cannot be carried out to completion since RNA's in the lysate (other than mRNA) which are necessary for protein synthesis would be damaged by the non-specific enzyme. Consequently, about 5 x 10^4 dpm/25 µl cell-free reaction volume were incorporated by residual mRNA activity in the system (figure 3).

In one experiment (data not shown) maximum tracer incorporation (10-fold above background) occurred at a much lower poly(A) RNA concentration (0.6 µg/25 µl assay volume). Higher concentrations of RNA greatly inhibited ^{3}H-leucine incorpora-

Figure 3. Incorporation of ^{3}H-leucine in a rabbit reticulocyte cell-free translation system: Rat liver poly(A) RNA was mixed with the kit 'premix' and lysate; each tube contained 20 µCi radioactivity/25 µl reaction volume. After incubation (1 hr at 37°C) an aliquot (10 µl) of each cell-free mixture was spotted onto Whatman GF-C filters (or strips of filter paper) and TCA-precipitated. Background incorporation was measured in tubes receiving 2 µl water/25 µl reaction volume. Each point represents the mean of duplicate reaction mixtures. One A_{260} unit = 42 µg RNA.


400
300
200
100
0
TCA-PRECIPITABLE RADIOACTIVITY (dpm×10⁻³)
Background incorporation
1.0
2.0
3.0
4.0
POLY (A) RNA ADDED (μg)

tion; at 4.0 µg poly(A) RNA/25 µl assay volume, tracer incorporation had fallen to 4-fold above background. The reason for the dose-related inhibitory effect is uncertain but may involve the introduction (via a contaminated poly(A) RNA preparation) of either ribonucleases or calcium-containing salts into the cell-free reaction mixture. While ribonucleases would directly damage the lysate's protein-synthesizing machinery, calcium would cause the reactivation of the micrococcal nuclease present in the lysate (88), thereby lowering significantly the cell-free system's ability to synthesize proteins.

Analysis of cell-free translation products

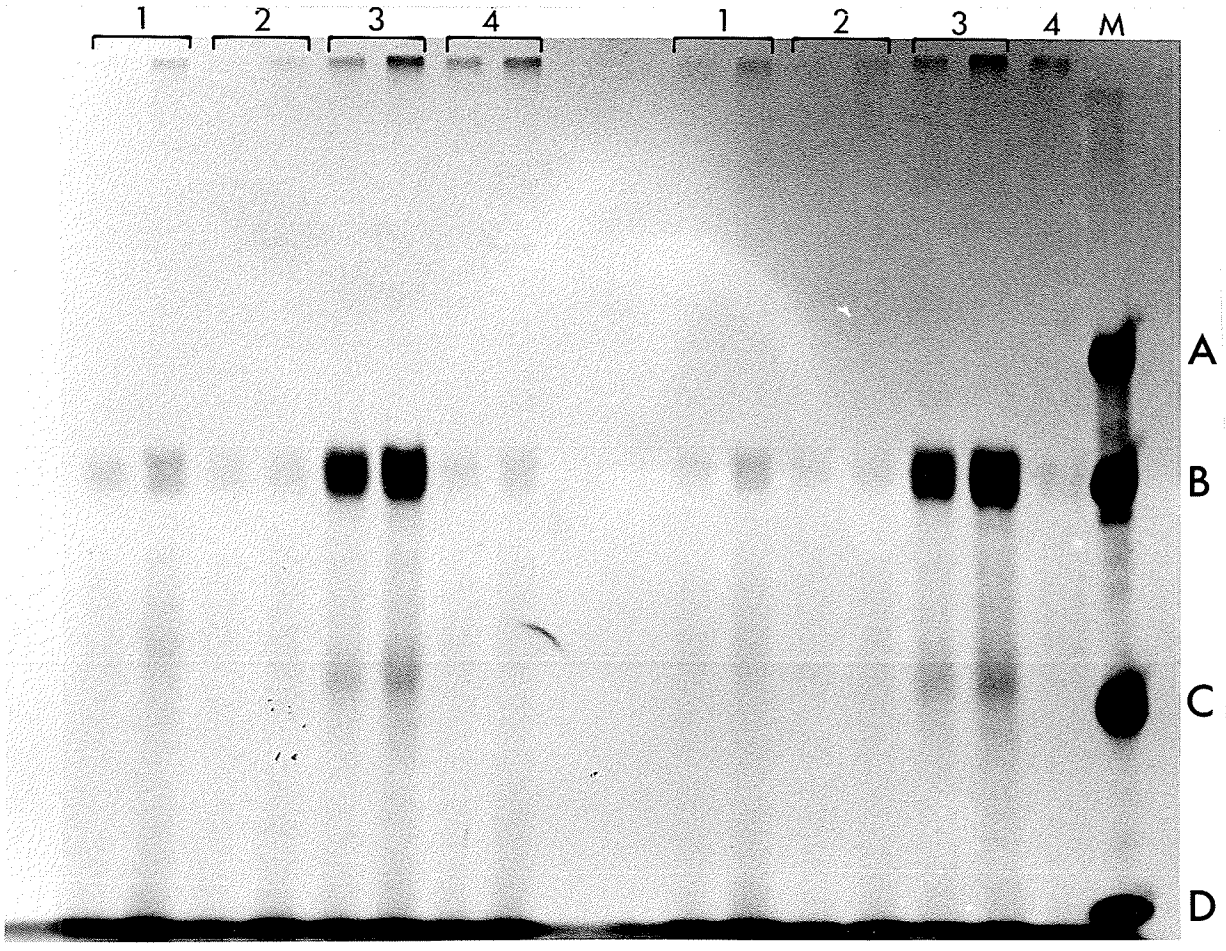
1) Rat serum albumin immunoprecipitation

Cell-free reaction mixtures were incubated with antiserum or normal serum and immunoprecipitates were isolated as described in Materials and Methods. The biosynthesis of RSA in the cell-free system was examined to verify that a specific and relatively abundant rat liver protein could be translated by the poly(A) RNA preparation. Table 2 shows that rabbit antiserum against RSA immunoprecipitated 2300 cpm ^{3}H above the normal rabbit serum background. The specifically immunoprecipitated cpm represented about 6% of the TCA-precipitable cpm. Electrophoresis of the sample on SDS-polyacrylamide gels (figure 4) revealed a major band with a molecular

Table 2. Immunoprecipitation of ^{3}H-labeled cell-free translation products: Cell-free mixtures (100 µl) were mixed with immunoprecipitation buffer (described in Materials and Methods) and antisera or normal sera were added yielding a final serum dilution of 1:20. Staph A adsorbed immunoprecipitates were dissolved in 100 µl 10% glycerol, 2% SDS, 5% β-mercaptoethanol, heated in a 95°C water bath and centrifuged (Beckman microfuge). Supernatant aliquots (5 µl) were mixed with 5 ml toluene-Omnifluor (New England Nuclear) and radioactivity was analyzed. Tracer incorporation was measured by TCA-precipitation of aliquots of the cell-free translation mixture.

Figure 4. SDS-PAGE of ^{3}H-labeled cell-free translation immunoprecipitates: Samples were electrophoresed on 7% polyacrylamide gels and fluorography was performed. Samples: (1) anti-PRL-Rc (2) NGPS (3) anti-RSA (4) NRS. For immunoprecipitation, each antiserum or normal serum was used at a 1:50 dilution (left lane of each pair) and a 1:20 dilution (right lane of each pair). (M) Marker proteins: (A) Phosphorylase B; MW = 92,500 (B) BSA; MW = 69,000 (C) Ovalbumin; MW = 46,000 (D) Carbonic anhydrase; MW = 30,000.

TCA-PPT. CPM (5μl)	ANTI-RSA	NRS	ANTI-PRL-RC	NGPS
4.0×10^4	4527	2225	1861	1678



weight (68,000) of RSA. The band may therefore represent the RSA precursor molecule.

2) Studies with guinea pig anti-PRL receptor antiserum

a) Characterization of antiserum

The guinea pig antiserum against purified PRL receptor from rabbit mammary gland was provided by Dr. R. P. C. Shiu. The antiserum had been characterized previously (6) and was known to specifically block ^{125}I-oPRL binding to rat liver membranes. In addition, the antiserum could precipitate ^{125}I-labeled PRL-receptor complexes but not ^{125}I-PRL alone, demonstrating a specific interaction with the PRL receptor. Maximum precipitation of PRL-receptor complexes occurred at serum dilutions less than 1:100. A final serum dilution of 1:20 was therefore used to analyze the cell-free reaction mixture.

b) Immunoprecipitation

The cpm ^{3}H immunoprecipitated from the cell-free translation mixture by guinea pig anti-PRL receptor and normal serum are shown in table 2. The antiserum precipitated about 200 cpm/5 µl more than the normal serum. The samples were subjected to SDS-polyacrylamide gel electrophoresis as shown in figure 4. While the lane containing the antiserum-precipitated sample appears darker than the control lane, particularly in the regions of molecular weight 45 - 50,000 and 70,000, no

specific unique band is present in this lane.

c) Effect of increased specific radioactivity

In an attempt to precipitate a greater number of cpm with the anti-receptor antiserum, 90 μCi/100 μl reaction volume was used. ^{35}S-methionine, a β-emitter of higher energy level, was substituted for ^{3}H-leucine.

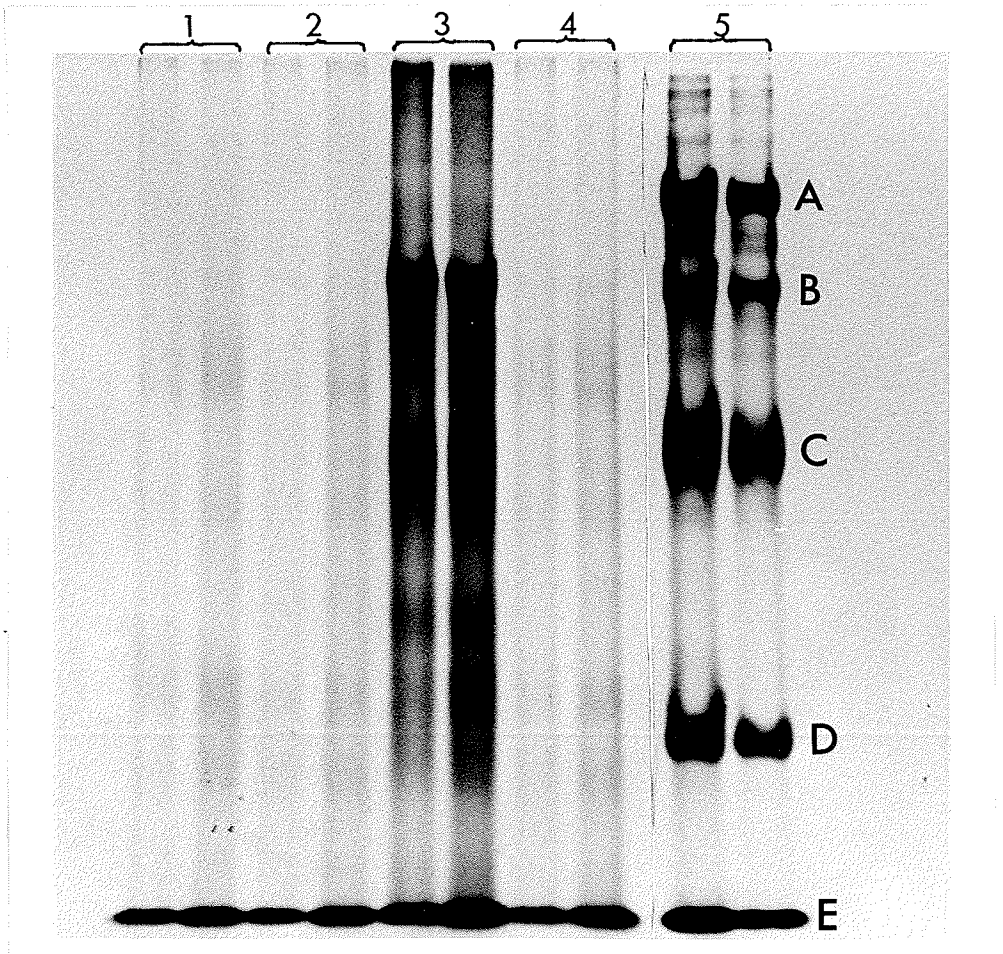
Table 3 shows the ^{35}S-labeled TCA precipitates and immunoprecipitates (in dpm). Despite a 4.5-fold increase in added radioactivity only a 2-fold increase in TCA-precipitated radioactivity was observed (comparing tables 2 and 3; the counting efficiency of ^{3}H in table 2 was about 30%).

RSA again served as a control for cell-free synthesis by the liver mRNA; rabbit anti-RSA antiserum precipitated about 3 - 4 x the number of dpm precipitated by normal serum. Although the total number of antiserum-precipitated dpms increased in table 3 (compared to table 2), the percentage of total TCA-precipitable material which was specifically immunoprecipitated by anti-RSA antiserum remained very constant at about 6%. Figure 5, the fluorography of the samples listed in table 3, shows that anti-RSA antiserum (as in figure 4) immunoprecipitated a major band with a MW ≈70,000, corresponding to RSA or perhaps its precursor form. Additional smaller molecular weight bands are also present. While these may imply cross-reaction of the antiserum with other cell-free products or immunoprecipitation of proteolytically generated RSA fragments,

Table 3. Immunoprecipitation of ^{35}S-labeled cell-free translation products: Cell-free reactions (100 µl/tube) were carried out in the presence of ^{35}S-methionine (90 µCi/tube). The final dilution of normal sera and antisera was 1:20. Immunoprecipitates were dissolved in 10% glycerol, 2% SDS, 5% β-mercaptoethanol (100 µl) and the radioactivity in duplicate 5 µl aliquots was determined. Duplicate aliquots of the cell-free mixture were also tested for acid precipitability.

Figure 5. SDS-PAGE of ^{35}S-labeled cell-free translation immunoprecipitates: Samples were electrophoresed on 10% polyacrylamide gels and fluorographs were prepared. Samples: (1) anti-PRL-Rc (2) NGPS (3) anti-RSA (4) NRS. For immunoprecipitation, each antiserum or normal serum was used at a 1:50 dilution (left lane of each pair) and a 1:20 dilution (right lane of each pair). (5) Markers: (A) Phosphorylase B; MW = 92,500 (B) BSA; MW = 69,000 (C) Ovalbumin; MW = 46,000 (D) Carbonic anhydrase; MW = 30,000 (E) Cytochrome C; MW = 12,300.

TCA-PPT. DPM (5μl)	ANTI-RSA	NRS	ANTI-PRL-RC	NGPS
2.75×10^5	21,953	6038	4598	5799



the rapidly migrating bands may be nascent RSA peptides of varying lengths. Unlike previous studies (89), polyribosomes were not removed from the cell-free reaction mixture prior to addition of antisera in our experiments. Nascent polyribosome-attached albumin chains are known to interact with antibodies against albumin (90,91); such peptides may therefore have been immunoprecipitated by the anti-RSA antiserum.

Despite the use of ^{35}S-methionine of high specific radioactivity in the cell-free translation studies, the anti-receptor antiserum failed to precipitate dpm in excess of the control serum (table 3). In other experiments (data not shown), the dpm precipitated by the antireceptor antiserum and the control serum were similar to each other.

SDS-polyacrylamide gel electrophoresis of the ^{35}S-labeled immunoprecipitates (table 3) is shown in figure 5. Again, as in figure 4, no distinct and unique bands were visible in the lane in which the anti-receptor antiserum precipitates were analyzed.

STUDIES ON PRL RECEPTOR BIOSYNTHESIS USING A MINCED TISSUE SYSTEM

Liver minces were tested for the presence of newly synthesized PRL receptor. In addition to attempting direct immunoprecipitation with the anti-receptor antiserum, indirect precipitation using hGH and anti-hGH antiserum to isolate new receptor was attempted. The feasibility of the indirect method is confirmed below.

Interaction of anti-hormone antiserum with ^{125}I-hormone receptor complexes

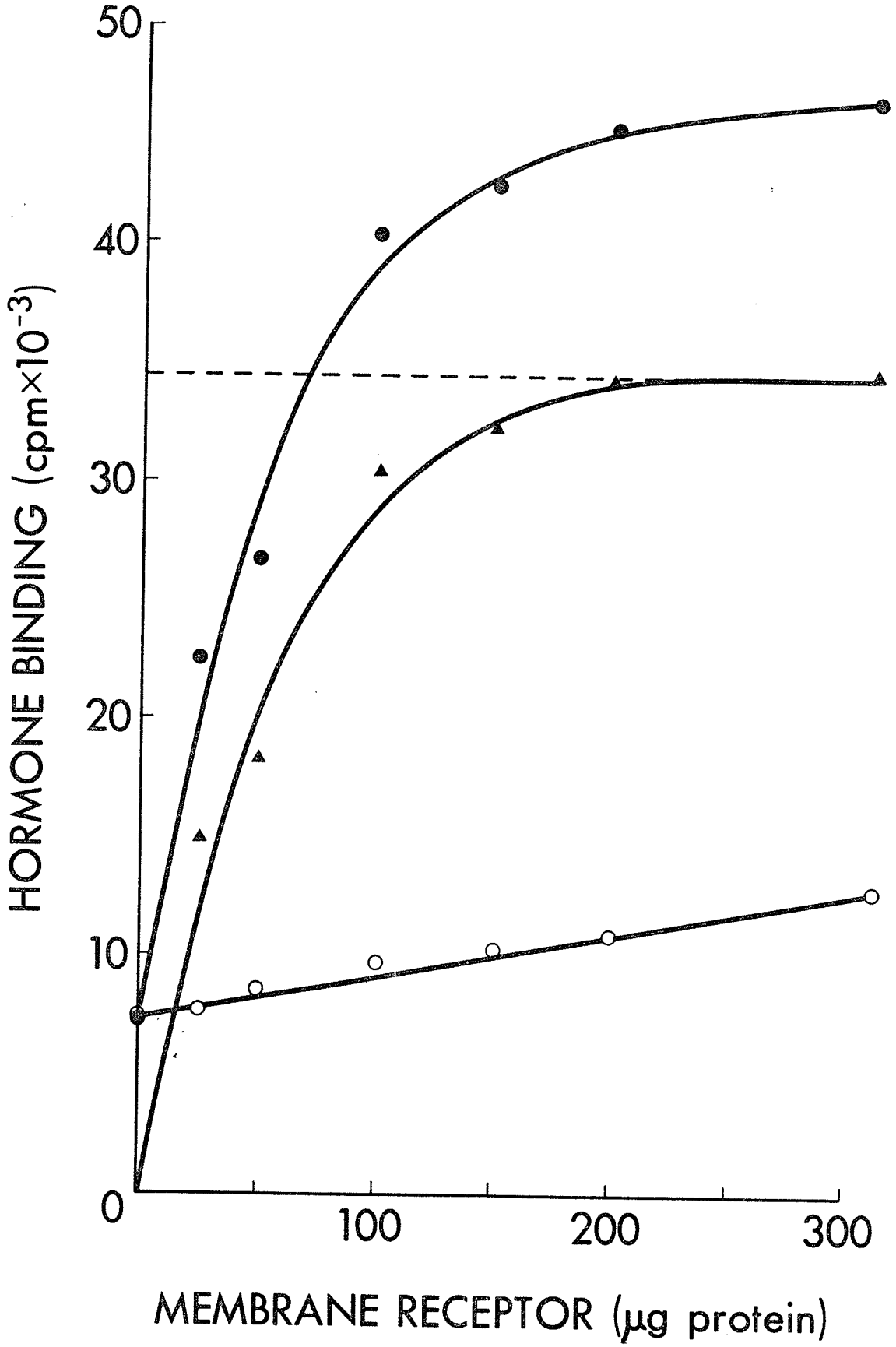
1) Tracer evaluation

Figure 6 depicts the results of the binding of ^{125}I-hGH to liver membranes. Both total and non-specific binding increased as a function of membrane protein whereas specific binding began to plateau at about 200 µg protein per assay tube. Approximately 47% of the added tracer was capable of binding when rat liver membrane binding sites were present in excess. In general, about 50% of the tracer was able to bind to membranes, but depending on the iodination, specific binding could range from 45 - 55% of the ^{125}I-hGH added.

2) Sucrose gradient analysis of ^{125}I-hGH interaction with solubilized membranes and anti-hGH antiserum

The formation of ^{125}I-hGH-receptor complexes was

Figure 6. Binding of hGH tracer to liver membranes: ^{125}I-hGH (73,000 cpm) was incubated with 100,000 x g membranes from pregnant rat liver in the presence or absence of cold hGH (1 μg). Each point represents the mean of duplicate determinations. Specific binding (▲—▲) = Total binding (●—●) - Non-specific binding (o—o). Extrapolating from the curve, the maximum specifically bound cpm ÷ (73,000 x 100) = the percentage of tracer capable of binding to membrane sites.


50
40
30
20
10
0
HORMONE BINDING (cpm×10⁻³)
100
200
300
MEMBRANE RECEPTOR (µg protein)

examined on gradients. Prior to the experiment, a binding study had shown that female rat liver membranes bound 27% of the tracer per 100 µg membrane protein while less than 1% specific binding was observed for the same quantity of male rat liver membranes (data not shown). Tracer incubated with TX-100 extracted male rat liver membranes and centrifuged on 2 - 30% gradients gave rise to one peak in fractions 4 - 5 (figure 7). This peak had the same migration rate as free ^{125}I-hGH. After the initial peak, radioactivity along the gradient decreased rapidly and the pelleted cpm at the centrifuge tube bottom were very low--less than 1% of the radioactivity applied to the gradient. Figure 7 demonstrates that in the presence of solubilized male membranes ^{125}I-hGH-receptor complexes were not formed. Also, the incubation of ^{125}I-hGH with solubilized membrane extracts did not produce high-molecular weight aggregates of a non-specific nature since no radioactivity had sedimented rapidly (figure 7).

The gradient profile of ^{125}I-hGH incubated with solubilized female rat liver membranes is also shown in figure 7. In contrast to male membranes, solubilized female membranes gave rise to two peaks; the first, found near the top of the gradient, represented unbound tracer. The second peak, which showed a maximum at fraction 27, represented ^{125}I-hGH-receptor complex. The tracer-receptor complex peak was broader than the free tracer peak, probably the result of size heterogeneity among the complexes.

Figure 7. Gradient analysis of ^{125}I-hGH interaction with solubilized rat liver membranes: Incubation mixtures (0.5 ml) of ^{125}I-hGH and solubilized male (●—●) or pregnant female (o—o) liver membranes (400 µg protein were applied to linear 2 - 30% sucrose gradients and centrifuged for 15 hr at 40,000 rpm (SW 40 rotor). The ^{125}I in 46 x 6-drop fractions was determined. Gradient density increased linearly with fraction number. Pelleted cpm were determined by removing and counting the bottom 0.5 cm of the centrifuge tube. Male liver: pellet = 1847 cpm. Female liver: pellet = 13,505 cpm.

Figure 8. Gradient analysis of anti-hGH antiserum interaction with ^{125}I-hGH or ^{125}I-hGH-receptor complexes: Incubation mixtures (0.5 ml) of guinea pig anti-hGH antiserum and ^{125}I-hGH (●—●) or ^{125}I-hGH previously incubated with solubilized pregnant female liver membranes (400 µg protein) (o—o) were centrifuged on 2 - 30% sucrose gradients. Conditions for centrifugation, fractionation and determination of radioactivity were identical to those of figure 7. Gradient density increased linearly with fraction number. Solubilized receptor absent: pellet = 5315 cpm. Solubilized receptor present: pellet = 27,384 cpm.

Fig. 8

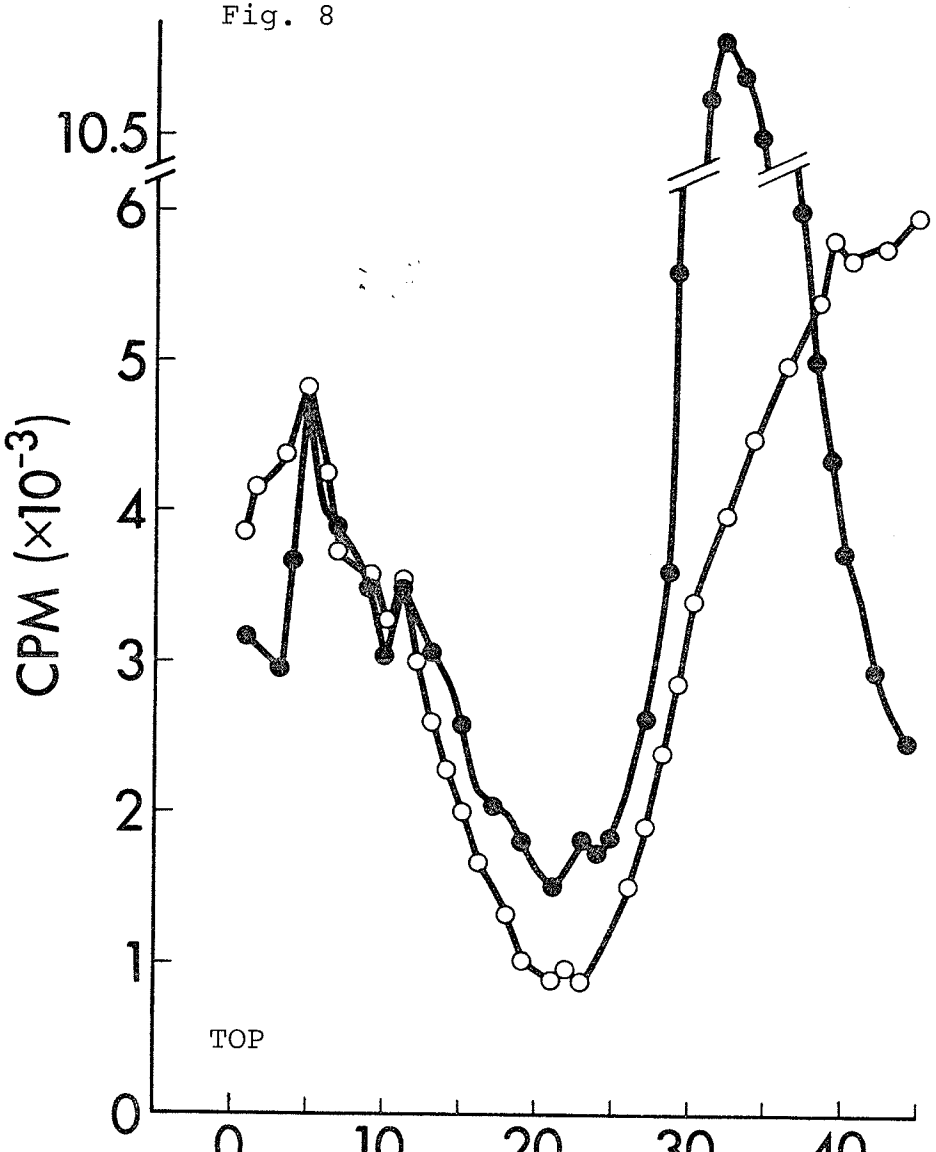


Fig. 7

FRACTION NUMBER

Figure 8 demonstrates the gradient profile of ^{125}I-hGH incubated with anti-hGH antiserum. Again two peaks were evident--free tracer and a much larger peak (fractions 31 - 34) near the bottom of the gradient. The second peak (^{125}I-hGH-antibody complex) was much sharper (less heterogeneous in size) and migrated faster than the hormone-receptor complex (figure 7). Far fewer cpm were present in the pellet of the hGH-antibody gradient than in the hGH-receptor gradient pellet, indicating a relative absence of very high molecular weight complexes in the hGH-antibody gradient.

Anti-hGH antiserum was added to assay tubes containing ^{125}I-hGH-receptor complexes as described in Materials and Methods. The gradient profile of the incubation mixture is shown in figure 8. Similarly to the two previously described gradients, this gradient contained two peaks of radioactivity, including the one in fraction 5. The second peak however merged with the bottom of the gradient tube indicating a higher molecular weight complex than was found in the other gradients. In two additional experiments (data not shown) the nature of this 'very large' complex was studied. If normal guinea pig serum was substituted for the guinea pig antiserum during incubation with ^{125}I-hGH-receptor, the gradient profile was identical to the profile illustrated in figure 7 (^{125}I-hGH plus solubilized female membranes). If antiserum was added to assay tubes containing ^{125}I-hGH and solubilized male (rather than female) liver membranes a profile which was superimposable

on the figure 8 profile (^{125}I-hGH plus anti-hGH antiserum) resulted.

The gradient experiments, therefore, strongly supported the hypothesis that anti-hGH antibodies could form a complex (or triplex) with hGH-receptor and suggested that indirect methods of immunoprecipitation would be useful, as a complement to direct precipitation methods, in the isolation of newly labeled PRL receptor.

Protein synthesis with different incubation media

To maximize the incorporation of tracers into PRL receptors two incubation conditions were tested--Krebs-bicarbonate and Eagles-Hela media.

Table 4 shows the number of TCA-precipitable ^{35}S-cpm in an aliquot of liver mince homogenate and solubilized extract as well as in the medium. Both in the homogenate and supernatant incorporation was about 2-fold greater when using Krebs-bicarbonate medium during the incubation. In the homogenate and supernatant of liver minces incubated in Krebs medium, 46% and 27% of the total cpm, respectively, were precipitated by TCA. In the incubation media the differences between Krebs and Eagles-Hela were less pronounced (table 4). Krebs-bicarbonate medium was subsequently used in most experiments for the labeling of rat liver proteins with ^{35}S-methionine.

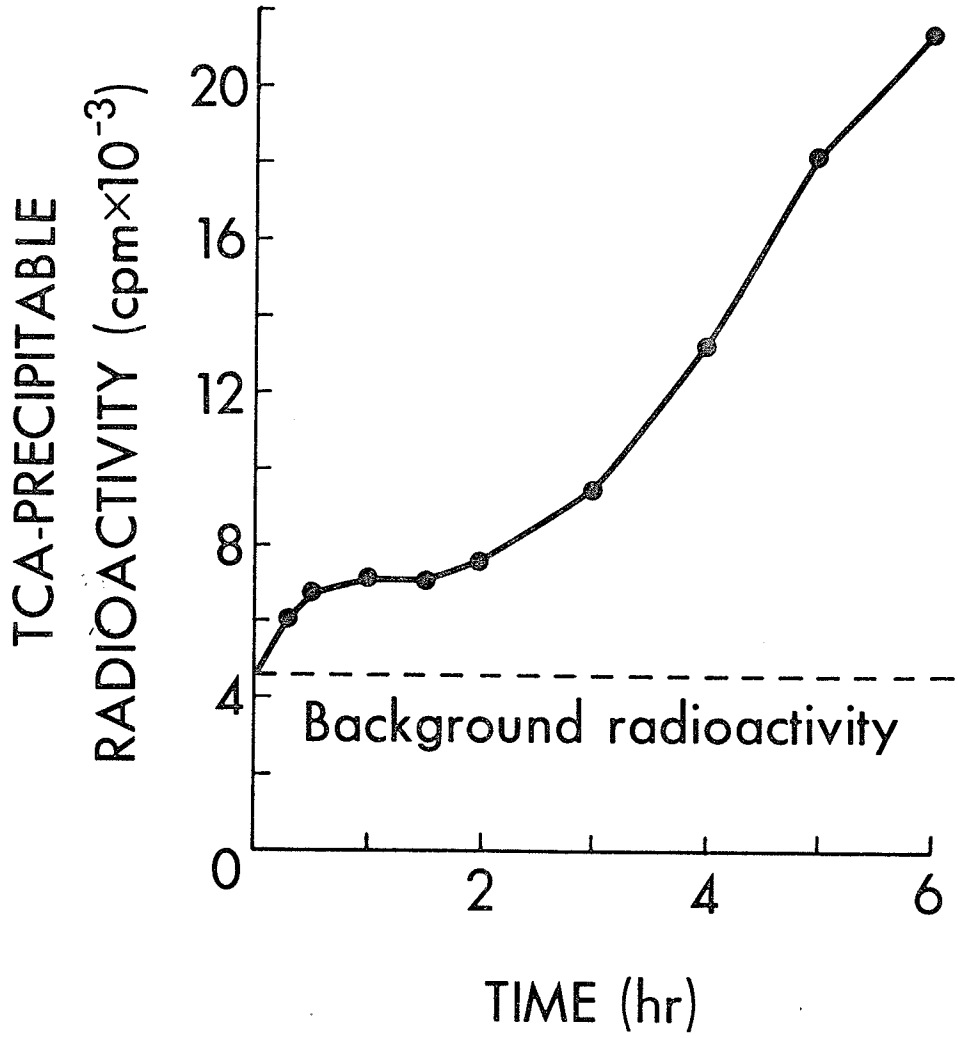
Kinetics of tracer incorporation

TCA-precipitable ^{35}S-cpm were measured in aliquots of

Table 4. Effect of media on ^{35}S-methionine incorporation into rat liver mince proteins: Liver minces (1 g wet weight) were incubated in Krebs-bicarbonate medium or Eagles-Hela medium (diluted 1:10 with EBSS) for 6 hr. Each flask contained 5 ml medium and 110 µCi/ml ^{35}S-methionine. Minces were homogenized in solubilization buffer (1 ml buffer/g) and centrifuged (100,000 x g). Quadruplicate aliquots (5 µl) of the homogenate, solubilized supernatant and medium were spotted onto filter paper; radioactivity was measured before or after TCA-precipitation in duplicate determinations.

Figure 9. Incorporation of ^{35}S-methionine into proteins released into the incubation medium by rat liver minces: Liver minces (1 g wet weight) were incubated in 5 ml Krebs-bicarbonate medium containing 200 µCi/ml ^{35}S-methionine. Samples (50 - 100 µl) of medium removed throughout the incubation period were centrifuged (Beckman microfuge) for 1 min to remove cellular debris. Aliquots of the supernatant (2 x 5 µl) were TCA-precipitated on filter paper and radioactivity was determined.

	TOTAL CPM (5μl)	TCA-PRECIPITABLE CPM	
		EAGLES-HELA	KREBS
HOMOGENATE	77,536	18,041	36,028
SUPERNATANT	69,673	9565	19,010
MEDIUM	573,512	6300	9746



Krebs medium (incubated with liver minces) as a function of incubation time. Figure 9 demonstrates that except for a lag period between 0.5 and 2 hr, ^{35}S-methionine incorporation increased steadily throughout a 6 hr incubation period.

Immunoprecipitation

Rabbit anti-RSA antiserum was used to examine the incorporation of ^{35}S-methionine into a specific protein by the liver minces. Table 5a shows that rat albumin was synthesized by the liver minces when incubations were performed in Krebs-bicarbonate or Eagles-Hela media. In minces incubated in Eagles-Hela medium albumin constituted about 1% of the TCA-precipitated cpm in the supernatant and 2 - 3% of the precipitated cpm in the media. Corresponding values for Krebs media were 0.5 - 1% and about 2%, respectively. Incubation in Krebs medium, rather than Eagles-Hela, yielded a 35% improvement in the amount of specifically immunoprecipitated rat albumin.

In both Eagles-Hela-incubated and Krebs-incubated minces, anti-receptor antiserum precipitated radioactivity above background (table 5a). The differences in cpm precipitated by normal serum and antiserum were small, but about a 2.5-fold greater difference was seen in minces incubated in Krebs medium compared to those in Eagles-Hela medium. The last column (table 5a) shows cpm precipitated by the indirect method (described in Materials and Methods). Although in this experiment parallel assay tubes receiving normal serum (rather than

anti-hGH) were not included it was obvious that the absolute number of cpm precipitated by this method was low.

Table 5b shows a second experiment in which solubilized proteins from liver were labeled to a higher specific activity in Krebs medium. While the TCA-precipitable cpm in the medium had doubled, those in the solubilized supernatant remained similar to the values shown in table 5a. A less efficient solubilization of the radioactivity incorporated by the minces (data not shown) was the major reason for the low TCA-precipitable cpm in the supernatant. However, the radioactivity precipitated by the anti-RSA antiserum was again greater than controls in both the solubilized extract of the liver minces and the media. The percentage of TCA-precipitable cpm accounted for by RSA was similar to that seen in table 5a--about 1% and 2%, in the supernatant and medium respectively.

Anti-receptor antiserum precipitated 90 cpm above background (table 5b), a slight improvement over the results in table 5a.

In table 6 the direct and indirect immunoprecipitation methods were tested on the microsomal membranes from liver minces. The rationale was to remove many of the labeled, non-membrane proteins which might be masking the actual number of cpm of newly synthesized PRL receptor being precipitated. As shown in the table, however, only 100 cpm above the background (normal guinea pig serum) were precipitated by anti-receptor serum. In the indirect immunoprecipitation, the incubations

Table 5. Immunoprecipitation of ^{35}S-labeled proteins from rat liver:

a) Minces of day 21-pregnant rat liver were incubated with ^{35}S-methionine followed by solubilization as described in the Materials and Methods and in the legend for table 4. Aliquots (100 µl) of the extract and the media were mixed with anti-RSA and NRS (final serum dilution = 1:20); the supernatant (extract) (100 µl aliquots) was incubated with anti-PRL-Rc and NGPS (final serum dilution = 1:20). hGH (5 ng/ml) was incubated with 100 µl solubilized extract overnight at 22°C; anti-hGH was then combined with the mixture (final serum dilution = 1:20) and incubated 24 hr at 4°C. All immunoprecipitates were isolated with Staph A and dissolved in 50 µl 10% glycerol, 2% SDS, 5% β-mercaptoethanol. Duplicate aliquots (5 µl) were counted to determine the immunoprecipitated radioactivity.

b) Minces (wet weight = 1 g) from day 15-pregnant rat liver were incubated in Krebs-bicarbonate medium containing 200 µCi/ml ^{35}S-methionine for 6 hr. The solubilized extract was prepared as described in the Materials and Methods. Conditions for immunoprecipitation of tissue extracts with antiserum were as described in table 5a. Radioactivity was determined in aliquots (2 x 5 µl) of the immunoprecipitates.

Table 6. Analysis of the ^{35}S-labeled 100,000 x g rat liver membrane fraction using direct and indirect immunoprecipitation techniques: Minces (wet weight = 1 g) from day 17-pregnant rat liver were labeled in Krebs-bicarbonate medium (200 µCi/ml ^{35}S-methionine) for 6 hr. A solubilized extract of the microsomal (72) membrane fraction was prepared as described in the Materials and Methods. Aliquots (250 µl) of the supernatant were immunoprecipitated directly with anti-PRL-Rc or NGPS or indirectly by techniques described in Materials and Methods. Immunoprecipitates were dissolved in 50 µl of 10% glycerol, 2% SDS, 5% β-mercaptoethanol and the radioactivity in duplicate aliquots (5 µl) was determined.

a)

	TCA-PPT CPM (5µl)		ANTI-RSA		NRS		ANTI-PRL-RC	NGPS	hGH+ ANTI-hGH
	SUP	MED	SUP	MED	SUP	MED	SUP	SUP	SUP
EAGLES-HELA	9565	6300	252	412	42	102	79	64	52
KREBS	19,010	9746	351	552	64	135	144	106	87

b)

TCA-PPT CPM (5µl)		ANTI-RSA		NRS		ANTI-PRL-RC	NGPS
SUP	MED	SUP	MED	SUP	MED	SUP	SUP
18,842	19,463	469	1142	101	457	220	130

TCA-PPT CPM (5µl)	ANTI-PRL-RC	NGPS	hGH+ ANTI-hGH	NGPS
8746	298	196	139	182

were carried out as they had been in the sucrose gradient experiments; the amount of hGH (ng) per μg equivalents of membrane protein (solubilized) was the same, as was the final dilution of anti-hGH antiserum or normal serum. Table 6, however, demonstrates that the indirect method was unable to precipitate any radioactivity above the background (normal guinea pig serum).

SDS-polyacrylamide gel electrophoresis

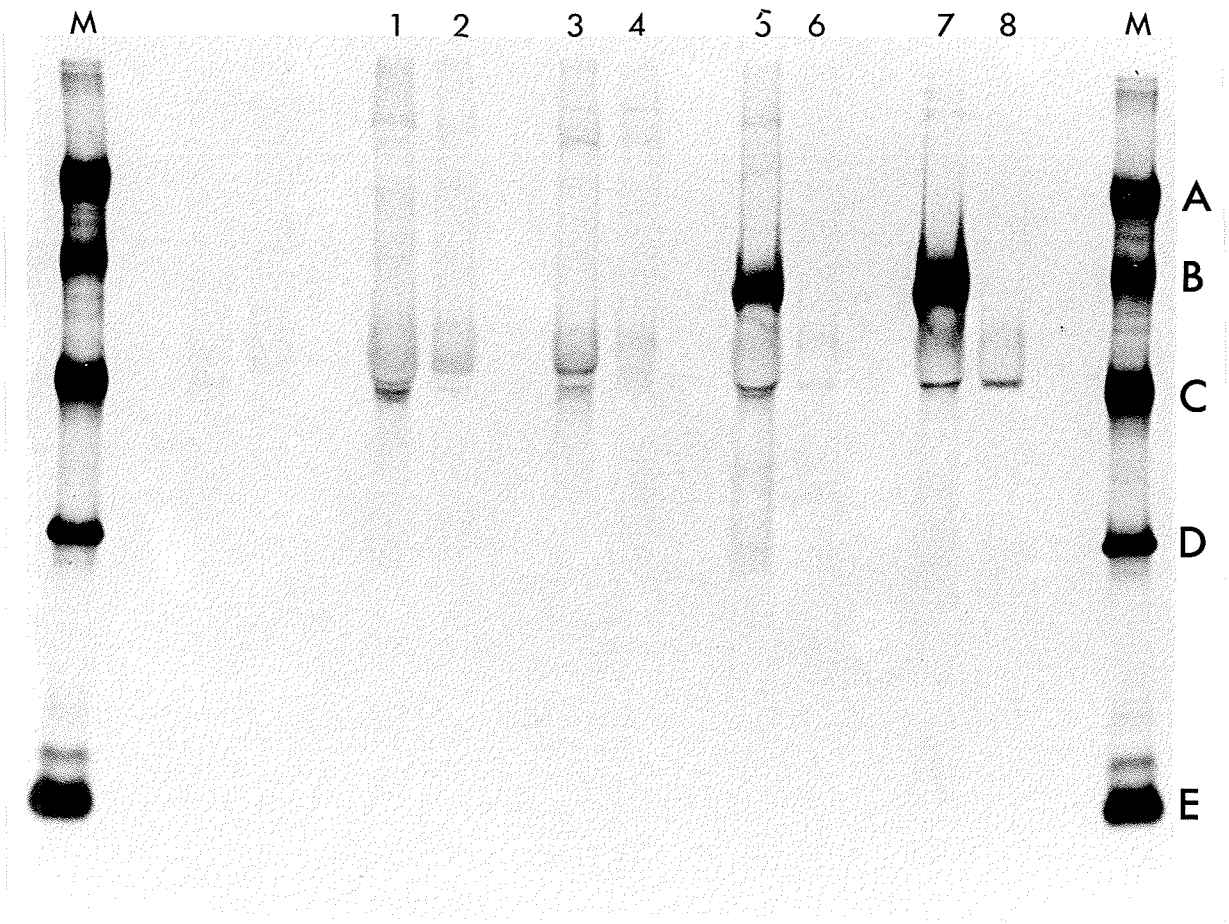
Figure 10 shows a fluorograph of the immunoprecipitates presented in table 5b. Rat albumin which appeared to be of slightly lower molecular weight than the BSA marker (in contrast to figures 4 and 5) was a very prominent band in both the medium and supernatant. Bands near the 46,000 MW marker were apparent when antiserum to the PRL receptor was used (figure 10, lanes 1 and 3), but were also present when other sera were used (figure 10, lanes 5, 7, and 8).

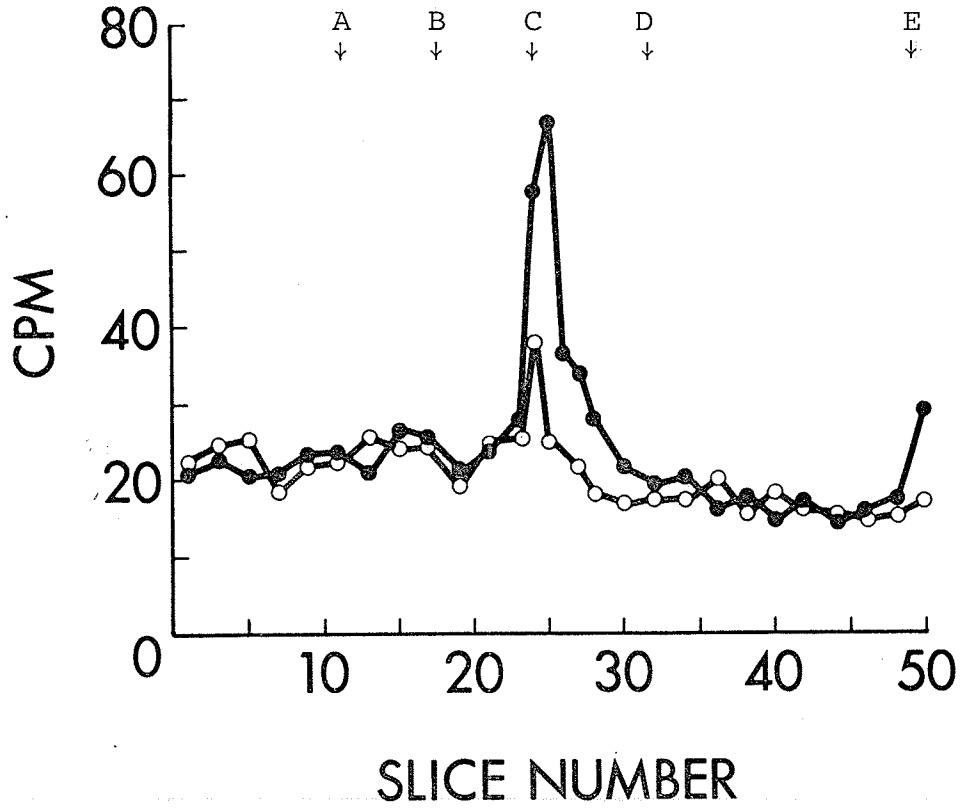
The distribution of radioactivity after electrophoresis of the samples listed in table 6 was determined as described in Materials and Methods. Figure 11 shows a sharp peak of radioactivity at MW = 45 - 46,000 which was precipitated by the anti-PRL receptor antiserum. A much smaller peak of radioactivity at the same molecular weight was precipitated non-specifically by the normal serum.

While it was thought initially that the peak at MW = 45,000 might represent a small component or subunit of the PRL

Figure 10. SDS-PAGE of ^{35}S-labeled proteins immunoprecipitated from extracts from rat liver minces: Aliquots (20 µl) of the samples described in table 5b were electrophoresed on 10% polyacrylamide gels. Fluorographs were prepared. Samples: (1) anti-PRL-Rc (2) NGPS {1 and 2, only, represent samples listed in table 5a. A larger (300 µl) aliquot of the supernatant of liver minces incubated in Krebs medium was precipitated with antiserum or normal serum (1:50 dilution) and treated as described in table 5a} (3) anti-PRL-Rc (4) NGPS (5) anti-RSA: supernatant (6) NRS: supernatant (7) anti-RSA: medium (8) NRS: medium. (M) Markers: (A) Phosphorylase B (B) BSA (C) Ovalbumin (D) Carbonic anhydrase (E) Cytochrome C.

Figure 11. SDS-PAGE of ^{35}S-labeled rat liver mince (solubilized microsomal fraction) immunoprecipitates: Aliquots (20 µl) of the samples described in table 6 were electrophoresed on 10% polyacrylamide gels. Gels were fixed and then processed by the 'One-step method' (86) as described in the Materials and Methods. Radioactivity in the 2 mm slices was determined. Anti-PRL-Rc (●—●), NGPS (o—o); (A) Phosphorylase B (B) BSA (C) Ovalbumin (D) Carbonic anhydrase (E) Cytochrome C.


M
1
2
3
4
5
6
7
8
M
A
B
C
D
E


A
B
C
D
E
80
60
40
20
0
CPM
10
20
30
40
50
SLICE NUMBER

receptor which had been endogenously labeled by ^{35}S-methionine, further investigation casts some doubt on the idea. In a follow-up experiment (data not shown) in which solubilized membrane extract was incubated with not only guinea pig anti-receptor antiserum and normal serum but also with guinea pig antiserum to hGH, hPRL and purified rabbit liver GH receptor, a peak of radioactivity at MW = 45,000 was seen with each antiserum tested. Indeed, some of the 'non-specific' antisera (against hGH and hPRL) precipitated a greater number of cpm at MW = 45,000 than did the anti-PRL receptor serum! It appeared therefore that the peak in figure 11 might represent radioactivity which was non-specifically bound to heavy immunoglobulin chains (MW ≈ 45,000) present perhaps in larger quantities in the various antisera than in normal guinea pig serum.

DENSITY-LABELING STUDIES IN Nb2 LYMPHOMA CELLS

PRL receptor biosynthesis was examined in PRL-sensitive Nb2 lymphoma cells by the density shift method (50 - 55) as described in Materials and Methods. Initially, however, characterization of the Nb2 PRL receptor and of CsCl gradient techniques was necessary.

Specificity of hGH binding sites

^{125}I-hGH binding to intact Nb2 lymphoma cells was examined in the presence of increasing quantities of cold hormone (figure 12). Unlabeled hGH and oPRL were both able to compete with ^{125}I-hGH for binding sites (although not equally well), whereas oGH did not compete except at very high concentrations; tracer displacement by 1 µg/tube oGH was possibly due to the presence of contaminating oPRL in the GH preparation.

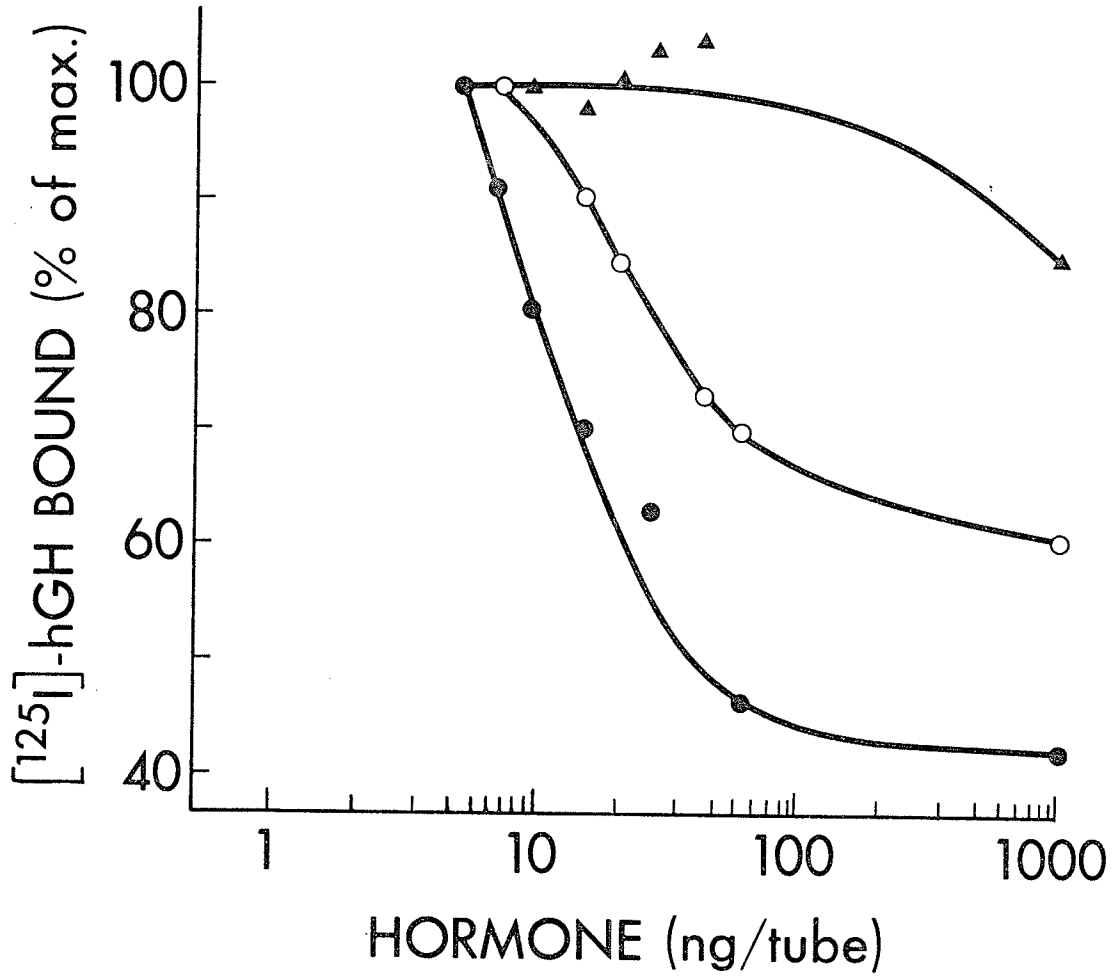
A similar result was obtained when ^{125}I-hGH binding to the solubilized Nb2 supernatant (100,000 x g) was examined (table 7). Again about 60% of the tracer was displaced by hGH; about 49% (instead of 40% as in figure 12) was displaced by oPRL; and only 7 - 8% was displaced by a large excess of oGH.

In addition, table 7 shows that ^{125}I-oGH binding is not detectable in either intact Nb2 lymphoma cells or in the solubilized extract. ^{125}I-oGH had been prepared as described in Materials and Methods, and the ability of the tracer to bind was examined in a rabbit liver membrane receptor assay. About 25% of the tracer added could bind to membranes (about

Figure 12. Effect of hGH, oGH and oPRL on the binding of ^{125}I-hGH to Nb2 lymphoma cells: Nb2 cells were added to duplicate assay tubes (4.4 x 10^6 cells/tube) containing ^{125}I-hGH (4.5 x 10^5 cpm/tube; 100,000 cpm ≈ 1 ng) and increasing concentrations of cold hormone. Tubes were incubated for 1 hr at 37°C, cold assay buffer (3 ml) was added to each tube and the cells were centrifuged. Radioactivity in the cell pellets was determined. hGH (●—●), oGH (▲—▲), oPRL (o—o).

Table 7. Effect of hGH, oGH and oPRL on the binding of (a) ^{125}I-hGH and ^{125}I-oGH to Nb2 lymphoma cell solubilized extract, and (b) ^{125}I-oGH to intact lymphoma cells: Nb2 lymphoma cells were cultured for 2 days in 10% HS-Fischer's medium and solubilized as described in Materials and Methods. A 1:2 dilution of the extract (100 µl) was incubated with ^{125}I-hGH (70,000 cpm/tube) or ^{125}I-oGH (65,000 cpm/tube) in the presence or absence of cold hormone (1 µg). Duplicate assay tubes were incubated overnight at 22°C and then PEG-treated and counted as described in Materials and Methods.

Nb2 cells (4.4 x 10^6 cells/tube) were incubated for 1 hr (37°C) with ^{125}I-oGH (7.6 x 10^5 cpm/tube) plus or minus cold hormone (1 µg). Cold assay buffer (3 ml) was added to the tubes and after centrifugation the radioactivity in the cell pellets was counted.



		TRACER ONLY	+hGH	+oGH	+oPRL
Nb2 SOLUB. EXTRACT	^{125}I-hGH	24,583	10,211	22,786	12,529
	^{125}I-oGH	17,333	17,492	17,612	—
CELLS	^{125}I-oGH	17,790	17,558	17,947	17,011

0.1 mg membrane protein) and nearly 60% of the bound tracer could be displaced by excess cold oGH. ^{125}I-hGH binding to Nb2 cells, therefore, appeared to be due primarily to the presence of lactogenic rather than somatogenic binding sites.

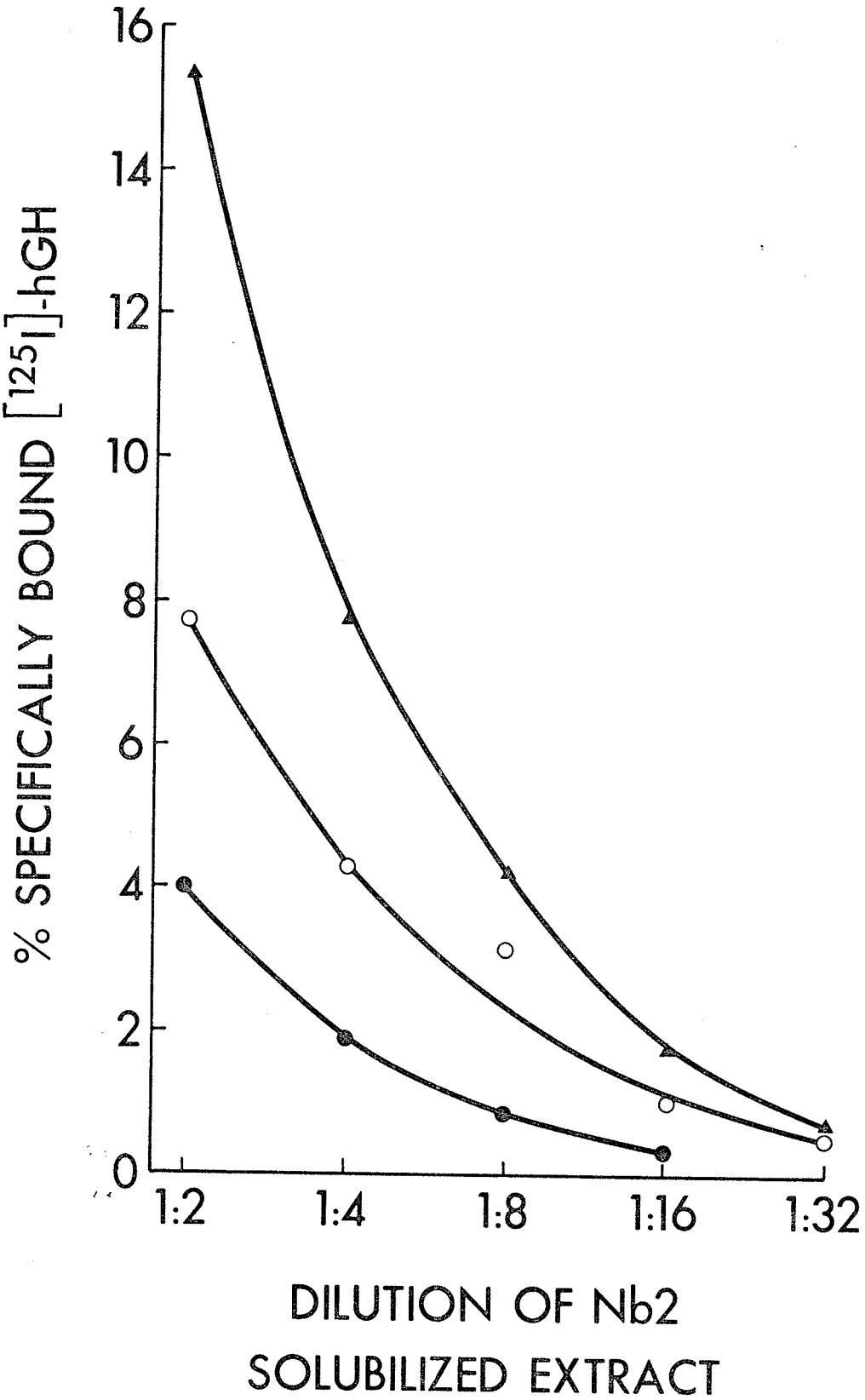
Effects of FCS and HS on hGH binding activity

Nb2 cells were cultured in growth medium (10% FCS, 10% HS) or in medium containing 10% HS only for different time periods; equivalent quantities of the solubilized extract were then assayed for hGH binding activity. Figure 13 demonstrates the stimulatory effect of HS on binding activity. Cells transferred from growth medium to HS-medium for 12 hr displayed an approximate 2-fold increase in binding activity whereas almost a 4-fold enhancement was seen in Nb2 cells cultured in HS-medium for 29 hr. A preliminary experiment (data not shown) had revealed that cells cultured in HS-medium for 27 and 45 hr, respectively, possessed similarly enhanced binding activities; a near-maximal increase in binding, therefore, seemed to occur by about one day after the transfer of cells from growth medium to HS-containing medium.

Effect of cycloheximide on hGH-binding activity

To test whether de novo protein synthesis was necessary for the increase in hGH binding which occurred in HS-containing medium, the effect of the protein synthesis inhibitor cycloheximide was investigated. Figure 14 demonstrates the enhanced

Figure 13. Effect of FCS and HS on ^{125}I-hGH binding in Nb2 lymphoma cells: Nb2 cells were cultured in Fischer's medium containing both FCS and HS, or HS only, as described in Materials and Methods. The relative concentrations of the solubilized extracts were equalized with solubilization buffer after reading the absorbance (A_{210}) of each extract against a solubilization buffer reference. Stock extract solutions were diluted serially and incubated with ^{125}I-hGH (60,000 cpm/tube) and with or without excess cold hGH. Duplicate assay tubes were incubated (overnight at 22°C), PEG-treated and radioactive pellets were counted. FCS-cultured (●—●); HS-cultured, 12 hr (o—o); HS-cultured, 29 hr (▲—▲).


16
14
12
10
8
6
4
2
0
% SPECIFICALLY BOUND [125I]-hGH
1:2
1:4
1:8
1:16
1:32
DILUTION OF Nb2
SOLUBILIZED EXTRACT

hGH-binding activity of Nb2 lymphoma cells after transfer from (FCS,HS)-medium to HS-medium for a 12 hr period. The increased binding in HS-medium was attenuated, however, by the presence of cycloheximide in the medium. The cycloheximide effect was dose-related and all but prevented the increase in binding activity entirely, at the highest concentration tested (0.1 µg/ml).

The viability of Nb2 cells after the incubation period was examined by light microscopy and by the trypan blue exclusion test as described in Materials and Methods. No differences between control and cycloheximide-treated cells were observed. Both groups of cells appeared round and clear under the microscope and uptake of the trypan blue dye was negligible. In an earlier experiment it was found that higher concentrations of cycloheximide (0.5 - 1.0 µg/ml) caused clumping or aggregation of the Nb2 cells; therefore these concentrations were avoided.

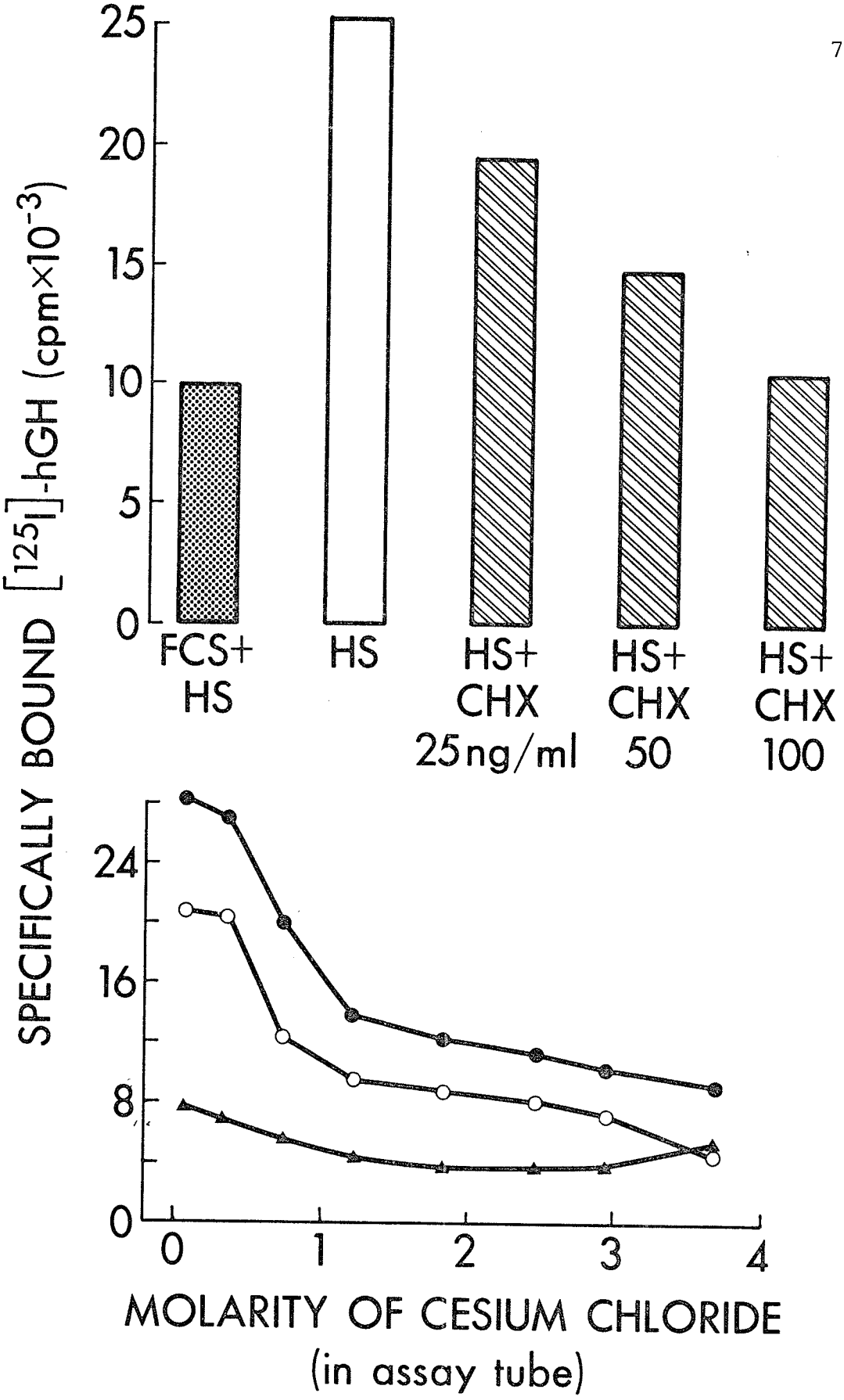
Although the action of cycloheximide did not demonstrate conclusively that the increase in hGH-binding activity represented newly synthesized PRL receptor, it strongly supported the hypothesis that new protein synthesis was required for increased hGH-binding activity to appear. The 'nature' of this new protein synthesis was investigated by the density-labeling approach.

Effect of CsCl on binding assays

To test whether isopycnic centrifugation of PRL

Figure 14. Effect of cycloheximide on ^{125}I-hGH binding activity in Nb2 lymphoma cells: Nb2 cells cultured in Fischer's growth medium (containing 10% FCS and 10% HS) were transferred to fresh growth medium or to 10% HS (no FCS) Fischer's medium. Increasing concentrations of cycloheximide were added to the culture dishes. After a 12 hr incubation cells were washed once with fresh medium (10% HS), concentrated and assayed for ^{125}I-hGH binding activity. Duplicate assay tubes received 4 x 10^6 cells, ^{125}I-hGH (1.1 x 10^6 cpm) ± cold hGH (1 µg). Tubes (final assay volume = 0.7 ml) were incubated for 1 hr (37°C), cold assay buffer was added and cells were centrifuged. Radioactivity in the cell pellets was determined.

Figure 15. Effect of CsCl on ^{125}I-hGH binding to solubilized rat liver microsomal membranes: A solubilized extract of the rat liver 100,000 x g membrane fraction (preparation described in Materials and Methods) was added to duplicate assay tubes containing ^{125}I-hGH (60,000 cpm), and excess cold hGH or assay buffer. Dilutions (Stock, 1:1.25, 1:1.5, 1:1.75, 1:2, 1:3, 1:5, 1:10, 1:50) of a 7.355 M CsCl solution were added to the tubes; each concentration of CsCl was further diluted 1:2 by the assay tube contents yielding a final range of dilutions of the stock CsCl solution from 1:2 to 1:100. Final assay volume was 0.6 ml. Tube contents were mixed well, incubated overnight (22°C) and PEG-treated. Radioactivity in the pellets was determined. Total binding (●—●), specific binding (o—o), non-specific binding (▲—▲).


SPECIFICALLY BOUND [125I]-hGH (cpm×10-3)
25
20
15
10
5
0
FCS+ HS
HS
HS+ CHX 25ng/ml
HS+ CHX 50
HS+ CHX 100
24
16
8
0
0
1
2
3
4
MOLARITY OF CESIUM CHLORIDE
(in assay tube)

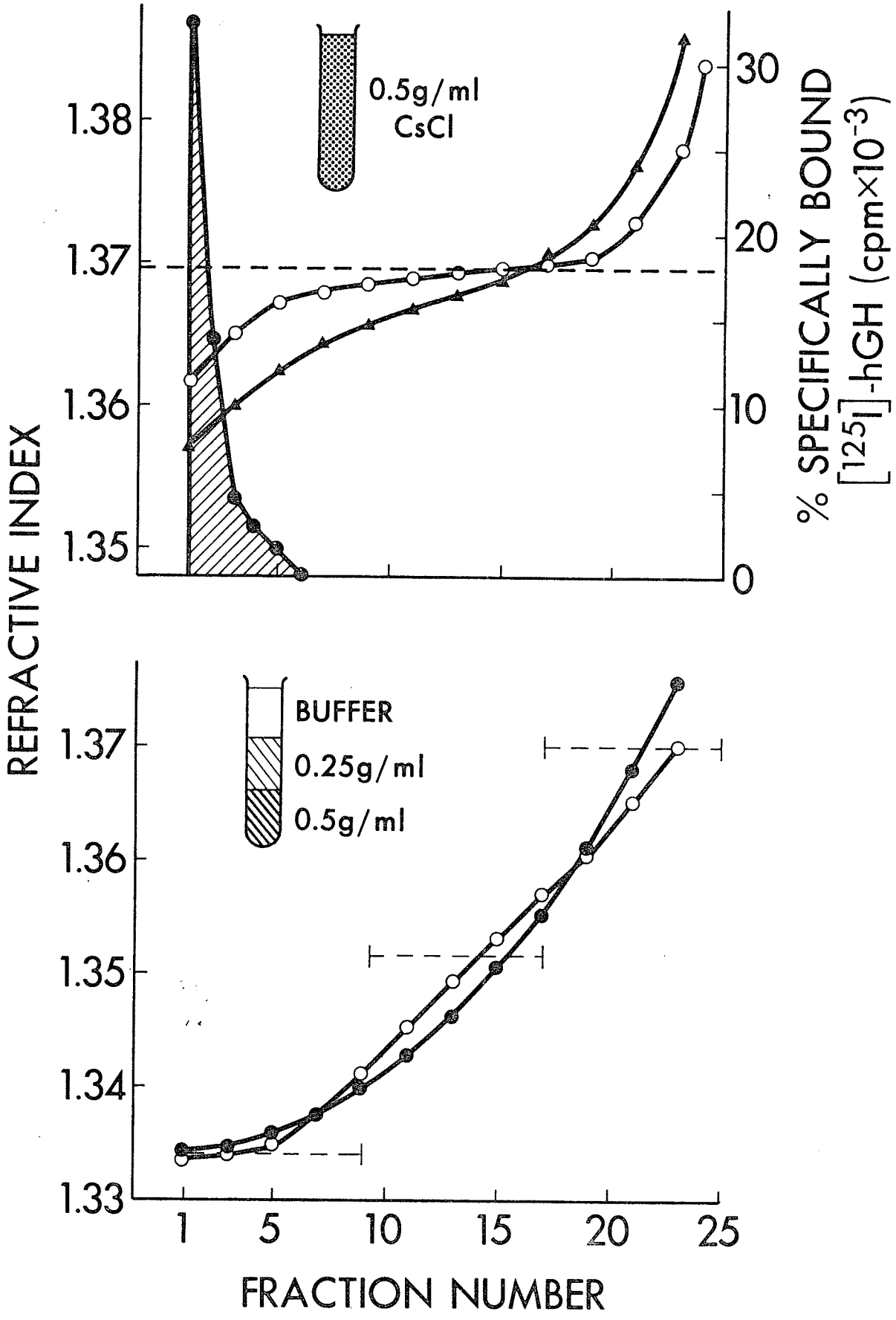
receptor on CsCl gradients was compatible with the subsequent assay of hGH binding activity, solubilized rat liver membranes were incubated with increasing concentrations of CsCl and ^{125}I-hGH binding was measured. Figure 15 shows that about 30% of the added tracer (60,000 cpm/tube) was specifically bound at CsCl concentration less than 0.4 M; thereafter binding dropped rapidly but stabilized at 10 - 15% specific binding over a wide range of concentrations (1.2 - 3.0 M). Even at the highest concentration of CsCl tested about 7% of tracer was able to bind specifically. When CsCl gradient fractions were assayed for hGH binding (data presented below) the CsCl concentration in the assay tube was usually below 0.5 M--the region of maximum binding (figure 15).

Isopycnic banding of the soluble PRL receptor in CsCl gradients

Solubilized extract from Nb2 lymphoma cells was centrifuged on CsCl gradients by the method of Reed and Lane (55) as described in the Materials and Methods and illustrated in figure 16 (inset). Specific ^{125}I-hGH binding was found only at the very top of the gradient (figure 16) indicating that the gradient was too dense to allow penetration and proper resolution of the PRL receptor. The curved gradient profiles in the same figure showed that extremely long centrifugation times would be required to form a linear gradient from a homogeneous CsCl solution if the SW-40 rotor was used.

Figure 16. Detection of Nb2 lymphoma cell ^{125}I-hGH binding activity on CsCl gradients: Nb2 cells were solubilized and the extract was mixed homogeneously with CsCl (0.5 g/ml) in a SW 40 cellulose nitrate tube (inset). Tubes were centrifuged and fractionated as described in Materials and Methods. Duplicate aliquots (100 µl) of each fraction were incubated with ^{125}I-hGH (80,000 cpm) plus or minus excess cold hGH and incubated overnight (22°C). PEG-treated assay tubes were centrifuged and the pellets counted. Percentage of total added cpm specifically bound (●—●); gradient profile prior to centrifugation (---) and after 24 hr (o—o) and 48 hr (▲—▲) of centrifugation.

Figure 17. Effect of centrifugation time on the density profiles of discontinuous CsCl gradients: Discontinuous CsCl gradients were prepared in SW 40 cellulose nitrate tubes as described in Materials and Methods. A gradient is illustrated (inset) prior to centrifugation. Density profiles are plotted prior to centrifugation (⊢--⊣) and after 14 hr (o—o) and 40 hr (●—●) of centrifugation.


0.5g/ml
CsCl
BUFFER
0.25g/ml
0.5g/ml
REFRACTIVE INDEX
1.38
1.37
1.36
1.35
1.37
1.36
1.35
1.34
1.33
% SPECIFICALLY BOUND
[125I]-hGH (cpm×10-3)
30
20
10
0
1
5
10
15
20
25
FRACTION NUMBER

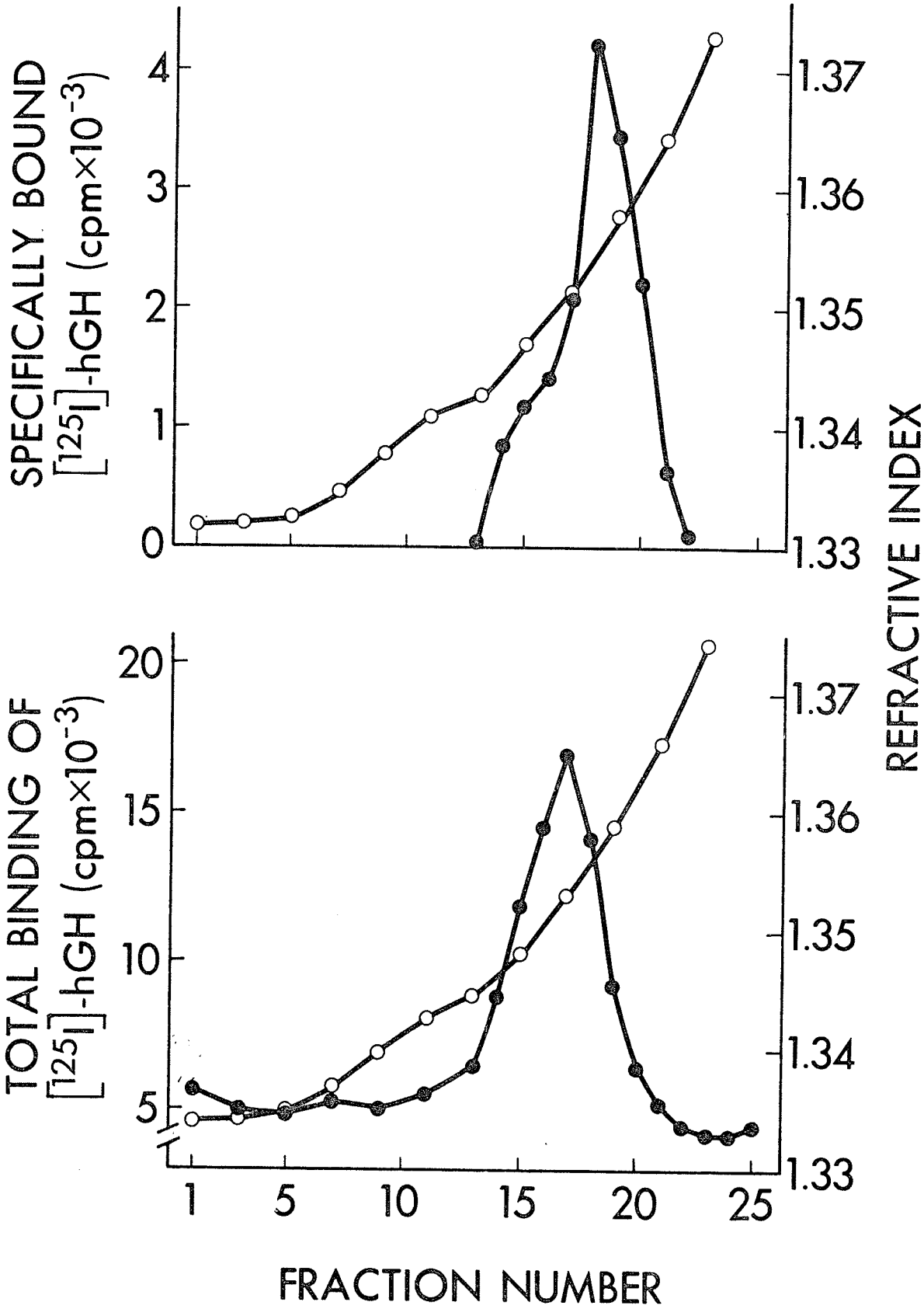
Both problems were corrected by performing discontinuous CsCl gradients as shown in figure 17 (inset) and as described in Materials and Methods. Figure 17 shows that the gradient was almost linear after a relatively short (14 hr) period of centrifugation. After a longer centrifugation time the gradient became gently curvilinear with an upward concavity. The top fractions of the gradients had a very low density of about 1.02 g/cm^3 whereas the lower fractions were much denser than the PRL receptor peak shown in figure 16.

The isopycnic banding of soluble Nb2 cell PRL receptor is shown in figure 18. The gradient was essentially linear in the region of binding activity; the small inflection in the gradient between fractions 9 and 11 was caused by TX-100 which bands at a low buoyant density but has a high refractive index. By extrapolating vertically downward from the tip of the binding peak (figure 18), the refractive index of CsCl at which PRL receptor banded was estimated at 1.355. The mean refractive index from five experiments was 1.356 {± 0.0015}.

Soluble receptor from rat liver membranes was also analyzed on CsCl gradients (figure 19). Whereas in figure 18 ^{125}I-hGH binding was assayed in the presence or absence of excess cold hGH to determine specific binding, in figure 19 only total binding was measured. (The two methods were equally useful in determining the position of the PRL receptor in the gradient.) From figure 19 the banding position of rat liver PRL receptor was estimated at $\eta D = 1.353$. In a second experiment

Figure 18. Detection of Nb2 lymphoma cell ^{125}I-hGH binding activity on 'improved', discontinuous CsCl gradients: Discontinuous CsCl gradients were prepared as illustrated (figure 17, inset) except that the centre step contained solubilized extract from Nb2 cells. Gradients were centrifuged, fractionated and assayed for ^{125}I-hGH (75,000 cpm/tube) binding activity as described in the Materials and Methods. Specifically bound cpm (●—●); gradient profile (o—o).

Figure 19. Detection of rat liver ^{125}I-hGH binding activity on CsCl gradients: A solubilized extract of pregnant rat liver membranes was prepared as described in Materials and Methods. An aliquot (250 µl) of the extract (containing 500 µg membrane protein equivalents) was mixed with 0.25 g/ml CsCl (final volume = 4.3 ml) and layered onto a discontinuous gradient as shown in figure 17 (inset). Gradients were centrifuged (42 hr) and fractionated as described previously; 2 x 200 µl aliquots were incubated with ^{125}I-hGH (62,000 cpm/tube). Assay volumes were increased to 0.7 ml/tube with assay buffer because of the greater sample volume (i.e., greater quantity of CsCl) added per tube. After overnight (22°C) incubation tubes received 0.5 ml 0.1% bovine γ-globulin (PBS) and 1.3 ml 25% PEG (PBS) so that the final PEG concentration was 12 - 13%. Tubes were centrifuged and radioactivity in the pellets was determined. Total cpm bound (●—●); gradient profile (o—o).


SPECIFICALLY BOUND
[125I]-hGH (cpm×10-3)
0
1
2
3
4
1.33
1.34
1.35
1.36
1.37
TOTAL BINDING OF
[125I]-hGH (cpm×10-3)
5
10
15
20
1.33
1.34
1.35
1.36
1.37
REFRACTIVE INDEX
1
5
10
15
20
25
FRACTION NUMBER

using rat liver membranes an identical measurement was obtained, suggesting that small differences in the buoyant density of PRL receptor from rat liver and Nb2 cells may exist.

Density-labeling of membrane proteins

Nb2 lymphoma cells cultured in FCS-HS medium were transferred to normal or heavy HS-medium containing ^{3}H-leucine, cell membranes (100,000 x g pellet) were solubilized with TX-100, and the extract was centrifuged on CsCl gradients as described in Materials and Methods. Figure 20 shows the tritiated proteins of normal Nb2 cells and those cultured in medium containing ^{2}H, ^{13}C-enriched amino acids. The centres of the protein peaks were separated by about 3 fractions. Normal proteins formed a distinct band at $\eta D \simeq 1.357$, a similar refractive index to that of the Nb2 PRL receptor (mean $\eta D = 1.356$). The density-labeled protein peak showed a definite higher density buoyancy at $\eta D \simeq 1.362$. In a second experiment (data not shown) these banding densities were confirmed {±0.0005 units}.

Density-labeling of PRL receptor

The solubilized extract of Nb2 lymphoma cells cultured in HS-medium containing normal or 'heavy' amino acids was centrifuged on CsCl gradients as described in Materials and Methods. The fractionated gradient was assayed for total ^{125}I-hGH binding activity and non-specific binding was subtracted to obtain specific binding (described in the legend

Figure 20. CsCl gradient centrifugation of normal and $^{2}H,^{13}C$-labeled protein from Nb2 lymphoma cells: Nb2 cells were cultured in Fischer's medium (10% HS) containing either normal amino acids or $^{2}H,^{13}C$-enriched amino acids. Culture medium also contained ^{3}H-leucine (15 µCi/ml). Normal and density-labeled cells were incubated for 24 hr, solubilized and extracts centrifuged (separately) on CsCl gradients as described in Materials and Methods. ^{3}H was counted in aliquots (50 µl) of each fraction. Normal proteins (o—o), $^{2}H,^{13}C$-labeled proteins (●—●); gradient profile (---).

Figure 21. Centrifugation of normal and $^{2}H,^{13}C$-labeled PRL receptor from Nb2 lymphoma cells on CsCl gradients: Cells cultured in normal or 'heavy' Fischer's medium were extracted and centrifuged as described in Materials and Methods. Aliquots (150 µl) of each fraction were incubated with ^{125}I-hGH (80,000 cpm/tube) in a final assay volume of 0.75 ml. Following overnight (22°C) incubation tubes were PEG-treated as described (figure 19). Non-specific binding for the 'peak' fractions was determined from an estimated best-fit line (inset) drawn through points of non-specific binding in the upper and lower portions of the gradient where no binding activity was present. Normal PRL receptor (o—o), $^{2}H,^{13}C$-labeled PRL receptor (●—●). Inset: non-specific binding to the normal (o—o) and density-labeled (●—●) gradients; fractions 12 - 24 represent the region of specific binding.