A STUDY OF THE BIOSYNTHESIS OF GROWTH HORMONE AND PROLACTIN IN BOVINE PITUITARY SLICES AND CELL-FREE SYSTEMS

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ABSTRACT

The biosynthesis of growth hormone and prolactin was studied in both slice and cell-free systems from bovine anterior pituitary tissue. Slice preparations were highly active in incorporating radioactive amino acids into prolactin, growth hormone and protein. Modification of existing procedures were employed for the isolation of the labeled hormones in a relatively purified state. A number of physical, chemical, and biological criteria were used to demonstrate that the radioactive proteins were indistinguishable from reference standards. These included amino acid composition, N- and C-terminal analysis, gel filtration, ion-exchange chromatography, polyacrylamide gel electrophoresis, and Ouchterlony immunodiffusion.

Using the same procedure, the relationship of growth hormone releasing factor (GRF) to the synthesis and release of growth hormone in vitro with both bovine and rat pituitaries was studied. Although no effect on synthesis and only a slight effect on release were noted, these results must be considered inconclusive because of the small number of samples studied.

With a cell-free system, consisting of ribosomes plus pH 5 enzyme fraction, and fortified with ATP, GTP, and Mg^{++}, the optimal conditions for biosynthesis of growth hormone and general protein were established. When a polysome-enriched preparation was resolved into fractions of discrete particle size on sucrose density gradients, incorporation of radioactive amino acids into the hormone was most active with polysomes containing six to seven ribosomal units. The isolated radioactive product was indistinguishable from the authentic growth hormone

standard by four physical criteria; ion-exchange chromatography, Sephadex gel filtration, polyacrylamide disc gel electrophoresis and sucrose density gradient centrifugation. In addition, it displayed specific antibody binding.

Examination by similar physical criteria, of the radioactive polypeptide associated with the prolactin fraction isolated from the pituitary ribosomal system, revealed no evidence of incorporation of labeled amino acids into the monomer form of prolactin. Several explanations have been offered for the failure to demonstrate synthesis of prolactin with this cell-free system.

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ABBREVIATIONS

ACTH	adenocorticotrophin
BSA	bovine serum albumin
Calbiochem	California Corporation for Biochemical Research
CG-50	Amberlite CG-50
DEAE	diethylaminoethyl cellulose (DEAE-cellulose)
DNP	dinitrophenyl
DOC	deoxycholate
FSH	follicle-stimulating hormone
GH	growth hormone
GRF	growth hormone releasing factor
LH	luteinizing hormone
LTH	prolactin, lactogenic hormone, mammotrophic hormone
NIH	National Institutes of Health
PEP	phosphoenolpyruvic acid
PK	pyruvic kinase
RNAse	ribonuclease
r-RNA (s-RNA, m-RNA)	ribosomal (soluble, messenger) RNA
TCA	trichloroacetic acid
TSH	thyroid-stimulating hormone
UV	ultraviolet

I. Introduction

A. Statement of the problem

Although extensive studies have been undertaken on the problems of protein synthesis few investigations have dealt with the biosynthesis of specific proteins.

Earlier work in this laboratory was concerned with the biosynthesis of protein and ACTH in both slice (Adiga, et al., 1965) and ribosomal-pH 5 enzyme (Adiga, et al., 1966) preparations derived from bovine pituitary glands. It was of interest to extend these experiments to larger protein hormones. Prolactin and growth hormone appeared particularly attractive.

Both of these substances are of relatively low molecular weight: 20,000 to 22,000 for prolactin and 21,000 to 26,000 for growth hormone. Neither molecule contains constituents other than amino acids. The two proteins can be isolated from anterior pituitary tissue by a single series of fractionation steps. The advantages of simultaneously investigating the mechanisms of synthesis of two proteins isolated in the same system appeared considerable. Any difference noted in the mode of synthesis of these two very similar proteins would be significant. At the onset of these studies, it appeared that different types of regulatory mechanisms operated at the hypothalamic level for the release of these hormones from the hypophysis. It may also be mentioned that in the human, the growth hormone molecule has been shown to possess both growth hormone and prolactin activities; the existence of a separate human prolactin molecule being still in doubt.

The first section of this dissertation describes such experiments in slices of bovine anterior pituitary tissue. The step-wise

fractionation method of Ellis (1961) and Kwa, et al. (1965) was modified to a small scale procedure for the consecutive isolation of growth hormone and prolactin in a relatively purified state. The relative rates of synthesis and release of the two hormones were investigated, and several criteria were employed to demonstrate that the isolated labeled proteins were indistinguishable in their physical, chemical and immunological properties from authentic hormone standards. Using this same technique with rat pituitaries, the effect of growth hormone releasing factor (GRF) on the synthesis and release of growth hormone was also examined.

The second section of this dissertation describes the optimal conditions for synthesis of growth hormone and prolactin in a cell-free system. It was shown that the incorporation of radioactive amino acids into the hormones was most active with polysomes containing six to seven ribosomal units. The isolated growth hormone fraction was indistinguishable by physical and immunological criteria from an authentic hormone standard. However similar studies with the radioactive polypeptide fraction associated with prolactin were less convincing and raised questions regarding the identify of this fraction.

B. Previous studies on the biosynthesis of protein hormones

In recent years an increasing number of reports have appeared on the biosynthesis of the polypeptide and protein hormones. These studies are of value first, because these well-defined molecules which range widely in molecular size, provide attractive model systems for investigation of the basic mechanisms of general protein synthesis. In addition, there is great inherent interest in the biogenesis of these

hormones, in view of their unique activity as regulators of important physiological processes.

Biosynthetic studies based on the use of surviving tissue slices have included insulin (Humble, 1963; Taylor, et al., 1964; Wagle, 1965b; Steiner and Oyer, 1966), thyro globulin (Seed and Goldberg, 1963; Vecchio, et al., 1966), Vasopressin (Haller, et al., 1964), adrenocorticotropin (Jacobowitz, et al., 1963; Adiga, et al., 1965), placental lactogen (Friesen, 1968), rat prolactin (Catt and Moffat, 1967), luteinizing hormone (Wakabayashi and Tamaoki, 1965; Samli and Geschwind, 1967; Winnick and Winnick, 1968) and parathyroid hormone (Hamilton and Cohn, 1969). An example of the valuable information derived from such experiments is the discovery by Steiner and Oyer (1966) of an insulin precursor molecule.

Studies of the biosynthesis of protein hormones in cell-free systems have been less numerous. Wagle (1965a) described the synthesis of insulin by microsome-pH5 enzyme preparation from fetal dog pancreas. The labeled product was characterized by its binding to anti-insulin serum, followed by precipitation with sodium sulphite.

Morais and Goldberg (1967) investigated the cell-free synthesis of thyroglobulin with microsomal and polyribosomal fractions, prepared from calf thyroid glands. A microsomal system incorporated radioactive amino acids into a protein which resembled thyroglobulin in sedimentation and immunological properties; however, their ribosomal system was unsuccessful.

The first study of the incorporation of a labeled amino acid (35 S-methionine) into pituitary proteins by subcellular fractions

prepared from rat pituitary tissue are those of Ziegler and Melchior (1956). Mitochondria were required to supply energy to the "small granules" (microsomes) and the supernatant phase. Kraicer (1964) was able to correlate an increase in the production of ACTH with an increase in the polysomal units of a profile of rat pituitary ribosomes. Adiga, et al. (1966) described the preparation and general properties of a ribosomal-pH 5 enzyme system from bovine adenohypophysis, which was highly active in the incorporation of radioactive proline into ACTH and proteins. Hussa (1968) characterized the radioactive product by demonstrating that the labeling was coincident with reference ACTH carrier during chromatography on ion exchange resins, and after gel filtration on Sephadex G-75. After carboxypeptidase treatment, 60% of the radioactivity still remained associated with ACTH, suggesting that the amino acids had been mostly incorporated into the central region of the polypeptide. Todorov, et al. (1966, 1967) also reported a cell-free synthesis of ACTH in an E. coli ribosomal system to which an RNA fraction from bovine pituitary gland had been added. They concluded that ribosomes were required for the synthesis of ACTH.

Adiga, et al. (1968a, 1968b) carried out the first detailed investigations of the physicochemical and biosynthetic characteristics of bovine pituitary ribosomal subunits, ribosomes, and polysomes. A similar study was later reported by Gospodarowicz and Laporte (1968). Salaman and Kirby (1968) have also characterized the rapidly labeled RNA of rat pituitaries.

Electron microscopy, differential centrifugation (McShan, 1965; Kwa, et al., 1965) and fluorescent antibody techniques (Nayak, et al.,

1968) have helped to identify the types of cells of the pituitary gland which synthesize, store, and secrete the various hormones. Thus Herlant, 1965, identified a cell-type which, stained with orange G and showed little variation in a variety of physiological states, was believed to be the source of growth hormone. A second variety which stained with erythrosin, showed marked variation in relation to the female reproduction cycle, was thought to be the site of prolactin biogenesis.

The first studies of prolactin synthesis in tissue explants appear to be those of Robboy and Kahn (1966). Homogenates of rat adenohypophysis, both prior to and after cultivation in vitro, and the media in which these explants had been maintained were assayed for protein content and for prolactin activity and were subjected to zone electrophoresis in polyacrylamide gels. Eluates of the latter were assayed for prolactin and the activity was correlated with the protein bands. These experiments indicated synthesis of both prolactin and protein. The explant released approximately 110% of its original prolactin content during each day of cultivation, while the corresponding release of protein was only 15%.

Catt and Moffat (1967) incubated rat pituitary glands in vitro in the presence of isotopic amino acids, and were able to prepare prolactin labeled internally with ^{3}H- and ^{14}C-lysine. A smaller amount of radioactive growth hormone was also isolated. The products were identified by analytical disc electrophoresis on polyacrylamide gel. The radioactivity present in the prolactin peak migrated with the same mobility as rat prolactin. In the same year, 1967, Rao, Robertson,

Winnick and Winnick published their study on the biosynthesis of prolactin and growth hormone in slices of bovine anterior pituitary tissue. These results have been incorporated into the first portion of this dissertation.

McLeod and Abad (1968) demonstrated that rat pituitary glands incubated in vitro incorporate radioactive amino acids into prolactin and growth hormone at comparable rates. However prolactin was largely recovered in the medium, while most of the growth hormone was retained by the tissue cells.

To date, no studies have been reported on the biosynthesis of either hormone in a cell-free system.

C. Heterogeneity of growth hormone and prolactin

In early studies on the isolation of protein hormones from pituitary glands, the preparations appeared homogeneous by criteria then available: chromatography on ion-exchange resins (Li, 1957) countercurrent distribution (Li, 1957), and ultracentrifugal analysis (Li, Cole and Corval, 1957). However, in recent years, the advent of techniques of high resolving power, such as gel electrophoresis, led to the detection of multiple components in most protein hormone preparations.

The heterogeneity of bovine growth hormone has been shown by starch gel electrophoresis (Barret, et al., 1962; Ferguson and Wallace, 1963; Ferguson, 1964) polyacrylamide gel electrophoresis (Lewis, 1963; Lewis, et al., 1967; Reusser, 1964) and gel filtration (Dellacha and Sonenberg, 1963; Free and Sonenberg, 1966). Ferguson and Wallace (1963) demonstrated the presence of hormone activity in the various components

separated by chromatography on DEAE-cellulose. Reusser (1964) submitted similar evidence of immunological activity in each component of a series of components separated from a bovine growth hormone preparation by electrophoresis on a basic polyacrylamide gel. The three components of bovine growth hormone, detectable upon starch gel electrophoresis, could be separated by chromatography on diethylaminoethyl Sephadex (Free and Sonenberg, 1966). All three components were biologically active, and displayed similar amino acid compositions.

Similarly prolactin was resolved into a number of components each with biological activity, by the methods of counter-current distribution (Cole and Li, 1958), zone electrophoresis of starch (Cole and Li, 1959), chromatography on Amberlite IRC-50 in urea-containing buffers (Cole, 1961), starch gel electrophoresis (Ferguson and Wallace, 1963; Barret, Friesen and Astwood, 1962), paper electrophoresis (Reisfield, et al., 1961), and disc electrophoresis on polyacrylamide gel (Reisfield, et al., 1964; Lewis, et al., 1968).

Heterogenity may arise in a variety of ways. The experiments of Pierce and Carsten (1958) with selective permeable membranes first suggested that the multiple biologically active components of prolactin might differ in molecular weight. Squire, Starman and Li (1963), in studying sheep prolactin in the ultracentrifuge through a wide range of pH, found their preparation to contain appreciable amounts of a high molecular weight material. This material could be separated from the monomer by gel filtration on Sephadex, but was not in equilibrium with the monomer under the conditions of the experiment. Further studies have indicated that freezing (Lewis and Cheever, 1965), dialysis, and

lyophilization (Squire, et al., 1963) may all result in irreversible polymer formation. Ultracentrifugal experiments with the monomer at pH 1.3 to 8.5 (Squire, et al., 1963) and gel filtration have revealed reversible polymer formation from the monomer.

By gel filtration Andrews (1966) has shown that aggregation of growth hormone and prolactin is both concentration and pH dependent. A probable change in conformation in the growth hormone molecule has also been detected by Burger, Edelhoch and Condliffe (1966) by fluorescent studies when the pH was reduced from 5 to 2. Dellacha, Santome, and Paladini (1968) found evidence of an interaction of growth hormone with the anions of the buffer, leading to aggregation. The following anionic series, in order of increasing aggregating power, was obtained: $Cl^- < SCN < Br < NO_3$ (Dellacha, et al., 1967).

It has been a consistent finding in the experiments of Li and co-workers that fractions of prolactin consisting largely of monomer, dimer and polymer show no significant differences in amino acid composition, amide content, terminal amino acids, peptide maps of digested samples, or biological activity. However other workers have disagreed. Components separated by disc electrophoresis, although not differing in biological activity may differ in amide content. Lewis and Cheever (1965) reported two types of conversion reactions for prolactin and growth hormone. One which occurred when the pH of the medium was raised to pH 10.5 was attributed to the loss of ammonia, without a change in terminal groups. This treatment resulted in the formation of faster moving bands. Another transformation was observed in certain preparation of sheep prolactin which were allowed to stand at neutral

pH (Lewis and Cheever, 1965). This reaction involved the formation of new end groups, and was attributed to the action of contaminating proteolytic enzymes. Ellis, Nuenke and Grindeland (1968) have recently reported the conversion of native rat and bovine growth hormone into a faster-moving biologically active electrophoretic form, by a trypsin-like peptidase present in pituitary extracts. This enzyme was found to be identical with plasmin.

Other sources of heterogeneity may include the chemical treatment during preparation and also genetic variations. As stated by Rohde and Dorner (1969) probably the first step in the isolation procedure determines the extent of heterogeneity.

D. Hypothalamic releasing factors

The concept of hypothalamic control of anterior pituitary function was first postulated by Marshall (1936, 1942) and Hinsey and Markee (1933). Studies of the anatomical relationship between the hypothalamus and adenohypophysis revealed little innervation between the hypothalamus and adenohypophysis. However, the anterior pituitary was amply supplied by blood vessels. The discovery of the hypophyseal portal venous system, vessels originating in median eminence and stalk region of the hypothalamus that have direct access to the anterior lobe, prompted the suggestion that humoral agents secreted into these hypophyseal portal veins might trigger secretion of anterior lobe hormones (Harris, 1964). Several groups of investigators soon demonstrated that crude acidic extracts of the hypothalamus or stalk median eminence (SME) region evoked release of all anterior pituitary hormones with the exception of prolactin, the secretion of which is inhibited. However recent studies

have reported a prolactin releasing activity in the hypothalamus of post partum rats (Mishkinsky, et al., 1968), and an inhibiting factor for growth hormone (Krulich, et al., 1968). Subsequent studies have been concerned with the purification of these factors.

The presence of a growth hormone releasing factor (GRF) in crude or purified hypothalamic extracts from several animal species has been well documented. These extracts are active both in vivo, where they cause depletion of growth hormone from the pituitary of the rat (Pecile, et al., 1965; Krulich, et al., 1965) or increased plasma levels of growth hormone as detected by radioimmunoassay in the sheep (Machlin, et al., 1967) or monkey (Knobil, 1966; Garcia and Geschwind, 1966; Smith, et al., 1967). In vitro, they increase output of growth hormone from incubated rat pituitaries (Deuben and Meites, 1964; Schally, et al., 1965) and the growth hormone content in pituitary tissue cultures (Deuben and Meites, 1965). Recently, Krulich, et al. (1968) reported the detection of hypothalamic inhibitor which blocked the release of growth hormone from rat pituitaries in vitro.

In addition to this effect on discharge of hormone from the gland, there have been claims that the factors influence synthesis of hormones. For example, prolactin inhibiting factor (PIF) inhibited synthesis of prolactin (Talwalker, et al., 1963), whereas corticotrophin-releasing factor (CRF) (Vernikos-Danellis, 1964) and GH-releasing factor (GRF) (Deuben and Meites, 1964; Krulich, et al., 1967; Schally, et al., 1968) stimulated synthesis of ACTH and GH, respectively. Thyrotropin-releasing factor has been reported by Florshein, et al. (1963) to exert its effect only on release of TSH, whereas Sinha and Meites (1966) claim an effect

on both the synthesis and release of the hormone.

Little attention has been paid to the mechanism of action of these hypothalamic hormones. It is known that all of the factor can act on pituitaries incubated in vitro, but it is not certain whether release of hormones is primary, and is followed secondarily by synthesis of the hormone depleted from the gland or whether effects on synthesis are an integral part of the mode of action of the factors. Schally, et al., 1968, claimed a stimulation of growth hormone synthesis in vitro after addition of GRF. However, stimulation could be demonstrated only when the pituitaries of rats were depleted of growth hormone by a prior exposure to cold in vivo. Actinomycin D inhibited the GRF-induced stimulation of release of growth hormone in vitro.

Using incorporation of radioactive amino acids into hormones as an index of hormone synthesis, MacLeod and Abad (1968) found that SME extracts did not alter the rate of growth hormone or prolactin synthesis in normal rat pituitary-glands in vitro. Samli and Geschwind (1968) concluded that synthesis and release of luteinizing hormone (LH) were separate phenomena. The release of luteinizing hormone from rat pituitaries incubated in vitro in the presence of hypothalamic extract was found to be dependent upon the presence of Ca^{++} in the medium, and could be stimulated by excess K^{+} in the medium. These ions did not inhibit the incorporation of ^{14}C-leucine into LH, while dinitrophenol and oligomycin inhibited the incorporation of ^{14}C-leucine into LH, but did not affect the release of LH. After studying the effects of puromycin and actinomycin, on the follicle-stimulating hormone (FSH) releasing action of hypothalamic extracts in vitro, Watanabe, et al.

(1968), suggested that protein synthesis may be required for the action of the FSH-releasing factor in inducing release of FSH from the anterior pituitary. However, Jutisz, et al. (1968) propose that the principal action of the releasing factors for LH and FSH is on the release of the corresponding hormone, which in the second stage, are resynthesized in the tissues. Wilber, et al. (1969) have suggested that cyclic AMP may act as a mediator of the action of TRF, on TSH secretion. Obviously there is need for additional research to clarify the mode of action of hypothalamic factors on the various pituitary hormones.

II. Materials and Methods

A. Materials

1. Radiochemicals

The following tritiated amino acids (specific activity per m mole): L-leucine-4,5-^{3}H (5c), L-phenylalanine-^{3}H (4.25c), L-proline-3,4-^{3}H (5c), DL-lysine-4,5-^{3}H (4c), DL-glutamic-^{3}H (3c), and DL-valine-3,4-^{3}H (100mc), were obtained from New England Nuclear Corp.

2. Proteins

a. Hormones - Bovine growth hormone (NIH-GH-B10, NIH-GH-B13) and prolactin (NIH-P-B-1, NIH-P-B-2) were gifts from the Endocrine Study Section, National Institutes of Health. Bovine growth hormone (somatotropin) was also purchased from California Corporation for Biochemical research. Glucagon was a gift from Dr. O.K. Behrens, Eli Lilly Co.

b. Enzymes - Pyruvic kinase (rabbit muscle) was purchased from California Corporation for Biochemical Research, while crystalline pancreatic RNAse and carboxypeptidase were products of Worthington Biochemical Corp. Dr. K.T. Yasunobu kindly provided samples of α-amylase (B. subtilis).

c. Other proteins - Cytochrome c and bovine serum albumen (BSA) were products of Sigma Chemical Company and California Corporation for Biochemical Research, respectively. Pituitary powder, anterior, was supplied by Mann Research Laboratories, Inc. GRF (porcine growth hormone releasing factor) was a gift from Dr. A.V. Schally (Tulane University).

3. Miscellaneous biochemicals

2-mercaptoethanol, GTP, ATP, and PEP(2-phosphoenolpyruvic acid, tri sodium salt) were purchased from California Corporation for Biochemical Research. Trizma base, DOC(sodium salt) and puromycin dihydrochloride, are products of Sigma Chemical Company. POPOP, dimethyl POPOP, and PPO were obtained from Packard Instrument Co. and tetramethylammonium hydroxide and N,N'-methylenebisacrylamide from Eastman Organic. Acrylamide and N,N,N',N',-tetramethylethylenediamine were products of Matheson, Coleman and Bell.

4. Resins and gels

Amberlite CG-50(200-400 mesh), was obtained from Mallinckrodt Chemical Works; and Bentonite U.S.P., from Robinson Laboratory, Inc. (San Francisco). The Sephadex gels and DEAE-cellulose (DE 52-microgranular preswollen) were products of Pharmacia Fine Chemicals, Inc., and Reeve Angel Co., respectively.

B. Methods

1. Amino acid incorporation techniques using pituitary tissue slices

a. Experiments with bovine adenohypophysis

Glands were excised from fresh cattle heads. The glands were dissected free of adhering tissue, and washed in cold Krebs-Ringer bicarbonate buffer (pH 7.4) containing 0.216% glucose. Each gland was bisected longitudinally, and the posterior-intermediate region removed and discarded.

The anterior tissue was then cut into para-saggital slices about 0.5 mm in thickness. Usually nine to ten slices were obtained per gland.

Three to four slices (about 1.0 g) were placed in each of a series of 25 ml conical flasks containing 4.0 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) and the desired radioactive amino acid. The systems were incubated for 4 hr. (unless otherwise stated) at 37°C in a rotary shaker. At the end of the incubation period 0.8 ml of 0.1N HCl was added to each flask and the contents were frozen.

b. Bovine adenohypophysis with growth hormone releasing factor (GRF)

Slices prepared at the abattoir were bathed in ice-cold Krebs-Ringer solution for approximately 1 hr. At the laboratory, the Krebs solution was removed, and the slices were quickly blotted and weighed.

One gm quantities of tissue were added to flasks containing 5 ml of Krebs-Ringer solution plus 10 μc each of tritium labeled Phe, Leu, Val, and Pro, and 4 μc of Lys. GRF in 0.1N acetic acid, or an equivalent volume of the acid, was added as indicated in Table XII. After incubation at 37°C for 4 hr, the medium was removed and the slices were washed with 5 ml of a solution containing approximately 0.01M concentration of each of the above amino acids in ^{12}C-form. This washing was combined with the medium. The separate tissue and medium fractions were

frozen to stop the reaction. Subsequently, the tissues were homogenized in 5 ml of the above solution containing the "cold" amino acids. As a carrier during the isolation procedure, 5 mg of anterior pituitary powder (Mann) was added to both the tissue and medium samples. Growth hormone and prolactin were extracted by procedures to be described presently.

c. Rat pituitary incubations with GRF

These experiments were performed in collaboration with Dr. A.V. Schally, at the Endocrine and Polypeptide Laboratories, Veterans Administration Hospital, New Orleans, Louisiana.

Pituitary incubations were carried out by the method of Saffran and Schally (1955) with a few modifications. Female Sprague-Dawley rats (190-220 g Body weight) were used as pituitary donors. In experiment 1, normal rats were used while in experiments 2, 3 and 4 the rats were exposed to the cold (4°C for 55-75 min) before decapitation. After removal each anterior pituitary was cut in half. A total of 8 pituitary halves were placed in each of two beakers containing 2 ml Krebs-Ringer bicarbonate medium plus 0.2% glucose and 23.5 μc each of ^{3}H-Phe, Leu and Pro. One group served as a control, while the other was used to test the effect of GRF.

In experiments 1 and 2 designed to measure release only, the pituitary halves were pre-incubated for 2 hrs in

the presence of radioactive amino acids in order to label the hormones. The medium was removed, fresh medium containing GRF added, and the incubation continued. In experiments 3 and 4, designed to measure both synthesis and release the labeled amino acids and releasing factor were present from the beginning. The same batch of porcine GRF was used for experiments 1, 2 and 3, while a different sample of higher activity GRF was used in experiment 4.

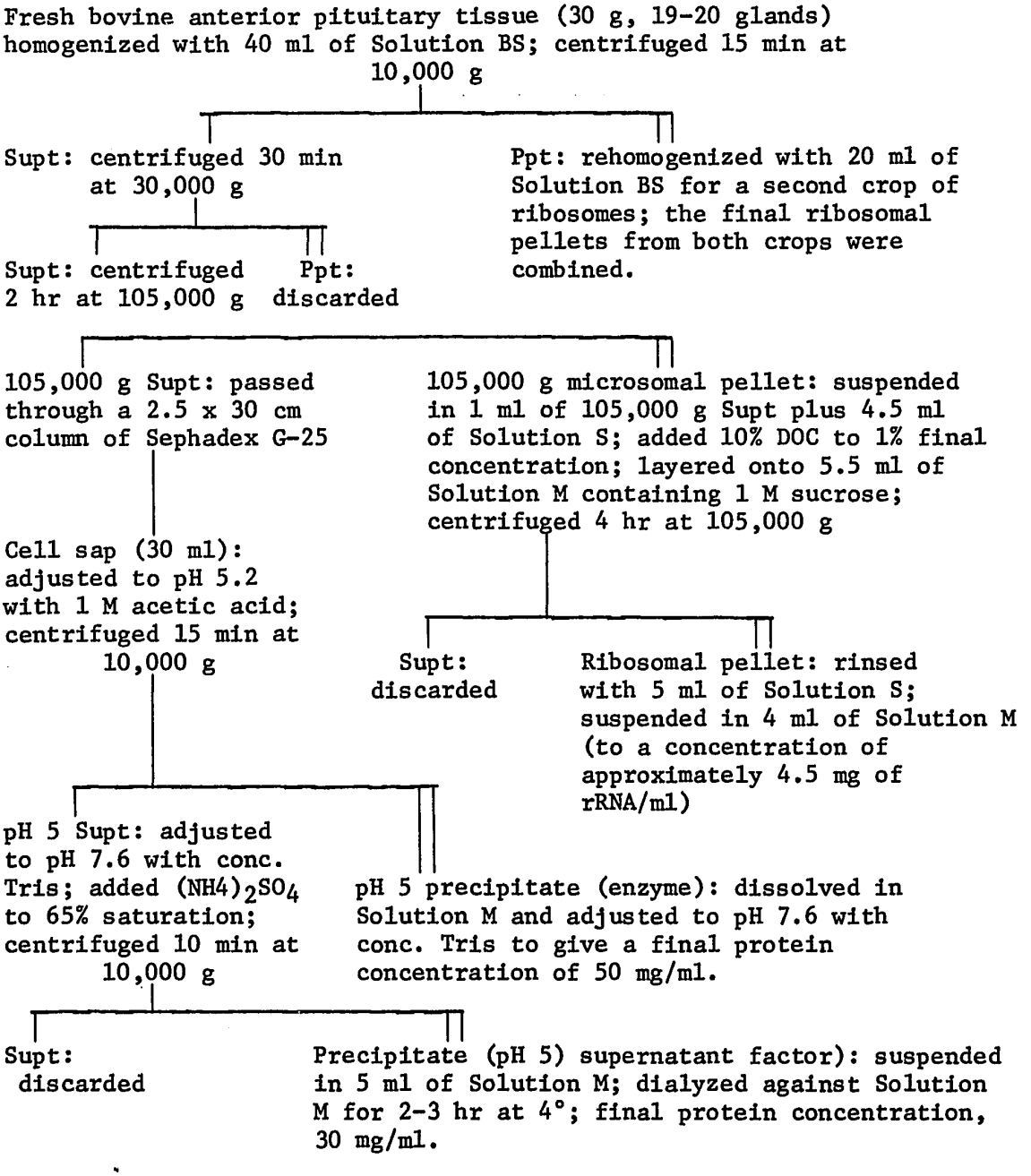
2. Procedures involving anterior pituitary cell-free systems

a. Preparation of components

At the abattoir the anterior tissue was washed with ice-cold Krebs-Ringer bicarbonate buffer and then immersed in this solution. At the laboratory, after removal of an adhering connective tissue capsule, the tissue was subjected, with minimum delay, to the fractionation scheme of Table I. Usually about 9 hrs were required to complete the entire procedure. The final preparation of ribosomes, pH 5 enzyme and pH 5 supernatant factor were generally used at once for incubation experiments. Otherwise they were stored at -20°C in medium S. The ribosomes lost approximately 50% of their activity after storage for one week at low temperature. The pH 5 fraction was more stable, retaining 90% of its biosynthetic activity (Adiga, et al., 1966).

When polysome-enriched fraction was desired, the procedure of Wettstein, et al. (1963) was followed). This involved sedimentation of the DOC-treated microsomes

TABLE I. ISOLATION OF ANTERIOR PITUITARY SUBCELLULAR COMPONENTS



through a discontinuous sucrose gradient (2.5 ml of 0.5 M sucrose in Solution M, layered over 3.0 ml of 2.0 M sucrose in Solution M). The pellet thus obtained was gently suspended in Solution S, clarified by brief low-speed centrifugation (30,000 g for 5 min at 0-4°C), and either used at once or stored overnight at -20°C in Solution S, and then resolved on sucrose gradients into fractions for use in biosynthetic experiments.

Medium M (Munro, et al., 1964) consisted of 0.02 M Tris-HCl buffer (pH 7.6) containing 0.1 M KCl, 0.04 M NaCl, 0.01 M Magnesium acetate, and 0.006 M mercaptoethanol. Medium S represented medium M fortified with 0.25 M sucrose, while medium BS contained, in addition, 4 mg of suspended Bentonite per ml.

b. Biosynthetic conditions

The composition of the standard reaction mixture used is shown in Table II. It was convenient to supply all of the components, except for the subcellular fractions (ribosomes, pH 5 enzyme and pH 5 supernatant factor) as 0.2 ml of a "cocktail" mixture. In some experiments certain of the constituents were varied. In all cases for the isolation of growth hormone GH and prolactin LTH, the final volume of the system was made up to 1.0 ml with Medium M. Separate incubation tubes of 0.25 ml final volume but with the same proportions of the above components, were set up to measure the incorporation of

TABLE II. STANDARD SYSTEM FOR ASSAY OF LABELED AMINO ACID INCORPORATION INTO GROWTH HORMONE, PROLACTIN AND TOTAL PROTEIN

Component	Quantity in 1 ml final volume
Amino acid mixture[a]	0.05 ml
ATP	5.0 μ moles
GTP	1.0 μ moles
Phospho(enol) pyruvate, sodium	2.5 μ moles
Pyruvate kinase	12.0 μg
Magnesium acetate	10.0 μ moles
B-mercaptoethanol	12.0 μ moles
Tris-HCl, pH 7.6	20.0 μ moles
3H-amino acid	10-20 μ curies
Ribosomal suspension	0.2 ml (r-RNA as indicated)
pH 5 enzyme	0.1 ml (5 mg of protein)
pH 5 supernatant factor	0.1 ml (protein as indicated)

[a]This mixture contained the following amino acids, micromoles per ml of solution; L-Glu, 4.9; L-Asp, 3.6; Cys-HCl, 0.8; L-Ser, 2.7; L-Thr, 2.5; Gly, 2.3; L-Ala, 2.7; L-Val, 1.4; L-Met, 0.8; L-Try, 1.2; L-Lys-HCl, 2.2; L-His, 0.7; L-Arg-HCl, 2.4; L-Ileu, 1.3; L-Try, 0.1.

the labeled amino acids into total protein. A mixture of the various component nonisotopic amino acids were employed in approximately the same relative proportions in which they occur in the bovine growth hormone molecule. ^{3}H-Phenylalanine and/or ^{3}H-Leucine were generally employed as the isotopic amino acids for incorporation into GH and LTH, because of their high abundance in the hormones, and because they are available at high specific activity.

Each tube was incubated for 1 hr at 37°C, unless otherwise indicated. With the 1.0 ml of reaction mixture used for studying the synthesis of GH and LTH, the reaction was stopped by the addition of 0.1 ml of 0.1 N HCl, followed by freezing. Labeled GH and LTH were isolated by the procedure outlined below.

With the 0.25 ml incubation mixture, used to measure total protein syntehsis, the reaction was stopped by adding 5% trichloroacetic acid (TCA) containing an excess of the ^{12}C amino acid corresponding to the isotopic amino acid used. The TCA-precipitated protein was processed successively with hot 5% TCA, cold 5% TCA and ethanol-ether (2:1), and dried in air (Adiga, et al., 1965). The dried material was dissolved in 0.2 ml of tetramethylammonium hydroxide, and diluted with ethanol. Aliquots of the resultant protein solution were counted in 10 ml of scintillation fluid.

In some cases, the residue remaining after GH and LTH isolation (residue IV) was processed by this same

procedure, and the amount of label incorporated into this fraction referred to as "residual protein."

c. Resolution of polysomes on sucrose density gradients

All operations were conducted at 0-4 C. The polysomal preparations were resolved into components of discrete particle size by sucrose density-gradient fractionation. Linear gradients (15-35% w/v in Medium M minus mercaptoethanol, 25 ml volume) were layered on cushions of 5 ml of 50% sucrose in Medium M, using 1X3 inch cellulose nitrate tubes. The polysomal preparation in 1-1.5 ml of Medium S was gently layered on top of the gradient. The tube was centrifuged for 270 min at 25,000 rpm in a Spinco model L ultracentrifuge equipped with an SW25.1 swinging-bucket V rotor.

After centrifugation, 50% sucrose in Solution M was injected slowly through a puncture near the bottom of the tube, using the ISCO model 180 Density Gradient Fractionator (Instrument Specialties Co.). From the top of the gradient, 24-drop fractions (0.72 ml) were collected. Ultraviolet (UV) absorbancies were either monitored continuously at 245 mμ with an ISCO model VA-2 UV analyzer equipped with a recorder, or measured at 260 mμ on an aliquot of each fraction (after dilution to 1 ml with water) in a Beckman DU spectrophotometer. Presumptive identification of the polysomal species separated on the gradient was made by direct electron microscopy examination, or by sedimentation analysis in a Spinco model E

ultracentrifuge (Adiga, et al., 1968).

d. Measurement of protein and hormone synthesis by isolated polysomal aggregates

Following centrifugation by the sucrose gradient procedure, each collected fraction was supplemented with pH 5 enzyme, pH supernatant factor, ^{3}H-leucine, ^{12}C-amino acid mixture, energy sources and various cofactors in a volume of 1 ml. After incubation for 1 hr. at 37°C, the reaction was stopped with 2 ml of 0.01 M-leu containing 2 mg BSA.

For isolation of the hormones tubes from 3 separate gradients corresponding to each polysome size were pooled. 0.5 mg of NIH-GH and 0.5 mg NIH-LTH were added to each pool as carriers. GH and LTH were isolated by the procedure to be described. Protein estimations were made on the residue remaining, and also on the total TCA-precipitable material from another corresponding gradient.

e. Determination of Protein and RNA

Protein was determined by the method of Lowry, et al. (1951). Ribosomal ribonucleic acid (r-RNA) was measured by the procedure of Scott, et al. (1956), as modified by Flick and Munro (1962), with crystalline bovine serum albumin added as carrier.

3. Procedures used for the isolation of growth hormone and prolactin

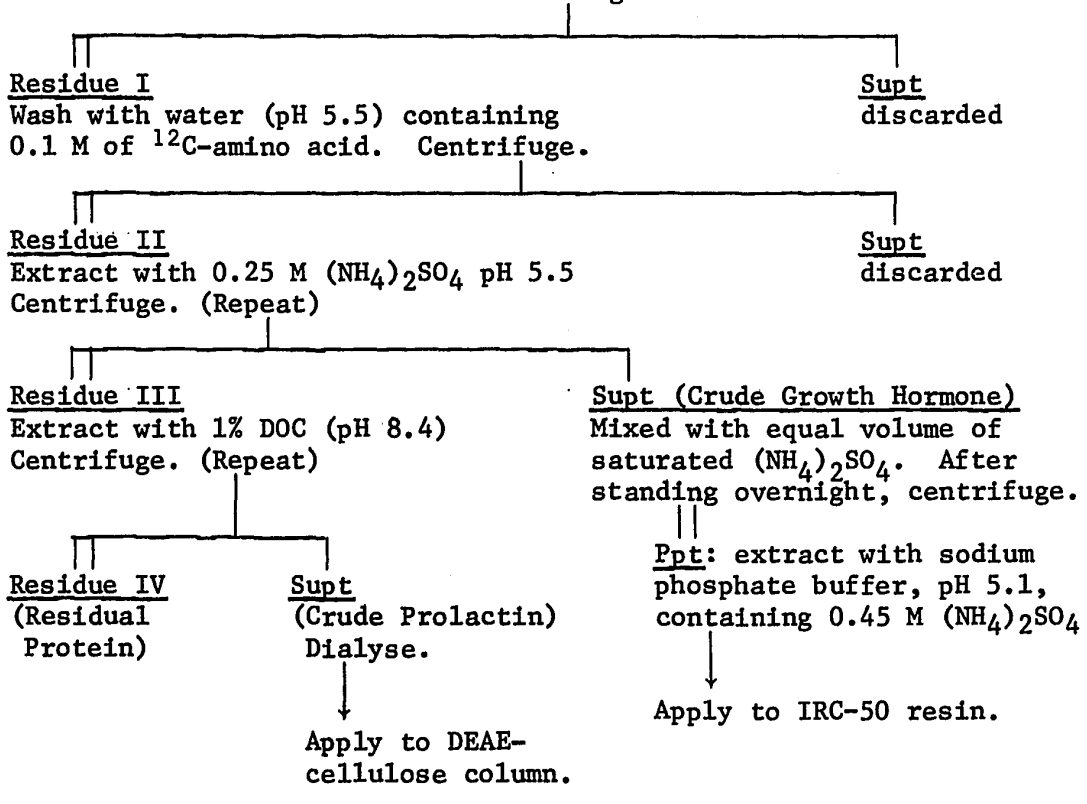
a. Initial extraction from the incubation mixtures

The reaction was stopped by adjusting to pH 5.5 with 0.1 N HCl and freezing. With slices the thawed tissue was homogenized together with the nutrient medium. The pH was adjusted to pH 5.5, and after standing for 1 hr, the precipitate was centrifuged. The residue was washed with 0.01 M solution of the ^{12}C amino acid corresponding to the isotopic compound. For extraction from tissue slices the extraction volumes were 10 ml, while for isolation from ribosomal incubation mixtures all extraction volumes were adjusted to 4 ml. Growth hormone was isolated by a modification of the method of Ellis (1961), involving overnight extractions by 0.25 M $(NH_4)_2SO_4$ (pH 5.5) followed by a second 1 hr extraction with the same salt solution. The two extracts were combined, mixed with an equal volume of saturated $(NH_4)_2SO_4$, allowed to stand overnight, and centrifuged.

Prolactin was recovered from the residue remaining after 0.25 M $(NH_4)_2SO_4$ treatment by taking advantage of the solubility of the hormone in 1% deoxycholate (DOC), pH 8.4 (Kwa, et al., 1965). The residue was extracted overnight with freshly prepared 1% DOC, followed by a second wash of approximately 1 hr. The DOC extracts were dialysed against 5 changes of water brought to pH 8.0 with NH_4OH.

Table III is a schematic representation of the methods used in the fractionation of the two hormones to a state of partial purity.

TABLE III. STANDARD PROCEDURE FOR THE ISOLATION OF GROWTH HORMONE, PROLACTIN AND RESIDUAL PROTEIN FROM TISSUE SLICES AFTER INCUBATION PROCEDURE



All operations were conducted at 4°. All centrifugations were performed at 30,000 x g for 30 min.

b. Chromatography of hormones on ion exchange resins

(1) Growth hormone

The crude GH preparation (Supt II of Table III) was further purified by ion exchange chromatography on Amberlite CG-50 according to the method of Papkoff and Li (1958). The resin was prewashed by the procedure described by Hirs, et al. (1953) before use. The $(NH_4)_2SO_4$ precipitate was extracted twice with 10 ml quantities of phosphate buffer (pH 5.1): 0.052 M NaH_2PO_4, 0.0025 M Na_2HPO_4, containing 0.45 M $(NH_4)_2SO_4$. The pooled clear extracts were applied to an Amberlite CG-50 ion exchange column (1X7 cm for slices, 1X4 cm for cell-free mixtures) which had been equilibrated with the same buffer. The column was washed with approximately 50 ml of the buffer, and the hormone was then eluted with 0.3 M phosphate buffer (pH 6.0) containing 0.45 M $(NH_4)_2SO_4$ (Papkoff and Li, 1962). The first 6-10 ml of the eluate were discarded, and the hormone in the following 75 ml was recovered by dialysis and lyophilization.

(2) Prolactin

For the isolation of LTH from the 1% DOC extract, the chromatographic technique of Kwa, et al. (1965) was used with suitable modifications. After dialysis with five changes of water and once against 0.025 M Borate buffer, pH 8.4, the prolactin solution was applied slowly to a DEAE cellulose column (1X8 cm) equilibrated with the same

buffer. The column was then washed with 50 ml of the borate buffer. In a number of cases, prolactin was eluted with a slow gradient of NaCl applied in 2 stages: (1) 50 ml of buffer plus 50 ml of buffer containing 0.4 M NaCl; and (2) 50 ml of buffer containing 0.4 ml NaCl and 50 ml of buffer containing 1.0 M NaCl, in the mixing and reservoir chambers, respectively. However, in routine isolations, prolactin was eluted by stepwise addition of 50 ml of buffer containing 0.4 ml NaCl. Prolactin was recovered from the eluate by dialysis and lyophilization.

In earlier experiments with slices, the crude preparation of prolactin (Supernatant III) was further purified by isoelectric precipitation (Cole and Li, 1955). After dialysis against water, the crude prolactin in the DOC extract (Supt III) was recovered by lyophilization. It was then dissolved in 5 ml of alkaline water (pH 9.0) and the pH of the solution was adjusted to pH 6.3 with 0.5 N HCl. After standing for 30 min at 0°C, the resulting precipitate was sedimented at 30,000 X g for 30 min, and discarded. The pH of the solution was lowered to 5.7, and the solution was kept at 0°C for 1 hr. The resulting prolactin precipitate was again sedimented. It was then dissolved in a known volume of alkaline water, and an aliquot was taken for radioactivity measurement. Because of the large variability between duplicates, especially with cell-free samples, this procedure was abandoned in

favor of the DEAE-cellulose procedure.

4. Techniques used to characterize the two hormones

a. Gel filtration on Sephadex

For estimation of molecular weight, an analytical Sephadex G-100 column (110X1.6 cm) was prepared as described by Andrews (1966), using 0.05 M tris-0.1 M KCl (pH 7.5) buffer. All operations were performed at 2-5°C, with flow rates of 10-20 ml/hr. The column was calibrated with the following standards of indicated molecular weights: sucrose (342), glucagon (3,850), cytochrome c (12,400), ovalbumin (44,000), α-amylase (48,700), BSA monomer (70,000) and dimer (140,000) and Blue dextran (2,000,000). NIH-bovine growth hormone (26,000) and prolactin (20,000) were also included as standards. Protein concentration was measured at 280 mμ, 412 mμ for cytochrome c, or 625 mμ for Blue dextran. In analysing the labeled hormone from slice preparations, 5 mg quantities were applied to the column, and 2 to 3 ml effluents were collected. Aliquots (0.4 ml) of each fraction were taken for measurement of radioactivity.

For growth hormone preparations, Sephadex G-100 columns (36X2.5 cm, or 54X2.0 cm) were equilibrated with the following buffers: 0.036 M sodium acetate, pH 4.0 (Free and Sonnenberg, 1966), 0.1 M glycine-HCl, pH 3.6 (Dellacha, et al., 1968), and 0.05 M and 0.15 M NH_4HCO_3, pH 8.1.

For prolactin, Sephadex G-75 columns (36X2.5 cm) were equilibrated with 0.05 M borate-KCl, pH 8.6 (Saxena and Henneman, 1966) and 5% acetic acid. Sephadex G-100 columns (54X2.0 cm) were equilibrated with 0.05 M NH_4HCO_3 (Lewis, et al., 1967) and 0.036 M sodium acetate, pH 4.0.

With all columns, the sample was initially suspended in 1.0 ml of the appropriate buffer. When necessary, growth hormone was brought into solution by the addition of a small amount of 0.5 N NaOH. The pH was then readjusted to that of the buffer and the solution made up to 3.0 ml before layering on the bed of Sephadex. Fractions of 2 to 3 ml were collected, the absorbance was measured at 280 mμ, and 0.4 ml aliquots were analyzed for radioactivity.

b. Polyacrylamide gel electrophoresis

Analysis were carried out at pH 9.5 by the method of Ornstein (1964) and Davis (1964). Small columns with an inside diameter of 0.5 cm and a length of 8 cm were employed. The lower gel comprised 6.0 cm of the column. The sample (200-500 μg), was dissolved in Tris-glycine buffer (pH 8.9); mixed 1:1 with the spacer gel, and applied to the column. A current of 2.5 ma/tube was applied. Migration was indicated by the marker dye, bromophenol blue, and was usually completed in 1.5 to 2 hrs. To locate the protein on the column, gels were stained for 1 hr, with 0.5% amido black in 7.5% acetic acid, and excess stain was removed by washing in the acid. To measure the

radioactivity present in the protein, corresponding unstained gels were sectioned into 0.4 cm segments. Each segment was dispersed with 1 to 2 ml of water made alkaline with NH_4OH. After standing overnight in the cold, the eluate was removed and transferred to a counting vial. This process was repeated 1 to 2 times and the combined washes were evaporated to dryness in vacuo. The residue was dissolved in 0.4 ml of water, and then mixed with 10 ml of scintillation mixture for radioactivity measurement.

c. Sucrose density gradients

This procedure served to reveal any low molecular weight peptides or high molecular aggregates in the labeled hormone preparations. Linear gradients of 5 to 20% sucrose in 0.1 M glycine-HCl (pH 3.6 buffer for growth hormone or 0.1 M NH_4HCO_3, pH 8.1 for prolactin) were layered on cushions of 0.6 ml of 50% sucrose in the appropriate buffer, using 9/16X3 1/2 inch cellulose nitrate tubes (11.5 ml). 0.5 to 1 mg samples were dissolved in 0.2 to 0.5 ml of the appropriate buffer and layered on the gradients. Centrifugation was carried out for 36 hrs, at 40,000 rpm in the SW-41 rotor at 5°C.

After centrifugation, 50% sucrose was injected slowly through a puncture near the bottom of the tube, using an ISCO model 180 Density Gradient Fractionator. From the top of the gradient 30 to 31 fractions of 0.4 ml were collected. Ultraviolet absorbancies were monitored continuously at

280 mμ with an ISCO model VA-2 UV analyzer equipped with a recorder. These fractions plus any precipitate at the bottom of the tubes were assayed first for optical density at 280 mμ in a Beckman DU spectrophotometer and then for radioactivity.

d. Amino acid composition

Samples were hydrolyzed for 24 hrs at 110°C with 6 N HCl in tubes sealed in vacuo. Subsequently the acid was removed under a stream of nitrogen. Genevieve Go operated the Technicon amino acid analyzer employed in these determinations. The number of amino acid residues per molecule of hormones was calculated by dividing the experimental μ moles of each amino acid by a constant. The constant was computed by dividing the total μ moles (determined experimentally) by the total (theoretical) number of residues of that amino acid present in the molecule. The constant was calculated, based on selected amino acids, stable during hydrolysis, and present in both bovine growth hormone and prolactin. These amino acids were: ala, val, gly, lys, glu, arg, leu and ileu.

e. C- and N-terminal analysis

DNP-bovine growth hormone was prepared according to the method of Li and Ash (1953). Its N-terminal residue was identified by paper chromatography of: (1) the DNP amino acid in water-saturated butanol (Mellon, et al., 1953); and (2) the free amino acid obtained upon hydrolysis of the DNP derivative (Lowther, 1951), using butanol:

acetic acid: water (12:3:5), for the solvent system.

DNP-prolactin was prepared as described by Cole, et al. (1957), and the N-terminal residue was identified as for growth hormone.

The C-terminal amino acids in the labeled hormones were determined using DFP-treated carboxypeptidase (Harris, et al., 1954), with a hormone to enzyme ratio of 50:1. The reaction was stopped after 6 hrs in the case of growth hormone, and 18 hrs for prolactin. The liberated amino acid was separated from high molecular weight material by absorbing the former on Amberlite IR 120. After centrifuging down the resin, the supernatant and washings were evaporated to dryness in vacuo. The residual protein was dissolved in a known volume of NH_4OH, and aliquots were taken for measurement of radioactivity and for paper chromatography. The free C-terminal amino acid was then recovered from the resin by elution with 5 N ammonium hydroxide. The eluate was evaporated to dryness in vacuo, the residue was dissolved in a known volume of water, and aliquots were analyzed as before.

f. Ouchterlony gel diffusion

This method (Outchterlony, 1949) was used for immunological characterization of the purified hormones, and also to detect trace contamination of these preparations by serum protein. NIH bovine growth hormone and bovine prolactin were used as reference standards.

g. Antibody binding

The growth hormone antiserum used for these experiments was prepared in rabbits by the procedure to be described presently and was capable of binding approximately 120 μg of bovine growth hormone ml antiserum as determined by radio-immunoassay.

Labeled growth hormone fraction isolated from cell-free incubation mixture containing 40 μc each of H-Phe, Leu, Pro, Lys, and Glu, was further purified by gel filtration on Sephadex C-100, equilibrated with 0.05 M NH_4HCO_3. After absorbance and radioactivity measurements the fractions corresponding to the growth hormone region were pooled (20 ml). The content of endogenous hormones was estimated by absorbance at 280 mμ. A solution of bovine growth hormone containing 1.0 mg per ml gave an optical density of 0.70 in a 1.00 cm cuvette at 280 mμ (Burger, et al., 1966).

To equal quantities of the radioactive hormone were then added one of the following: (a) 0.4 ml of anti-bovine growth hormone serum, (b) 0.4 ml of anti-bovine growth hormone serum plus 8.0 mg of NIH-growth hormone, (c) 0.4 ml of an anti-BSA-serum and (d) 0.4 ml of NH_4HCO_3 buffer (control). To ensure that excess antibody was present to bind the labeled hormone, a second sample of highly labeled growth hormone was similarly purified by gel filtration (25 ml). Quantities of labeled hormone corresponding to

those described above were used and treated with: 0.8 or 1.6 ml of anti-bovine growth hormone serum; and 0.8 or 1.6 ml of anti-bovine growth hormone serum plus 8.0 mg of NIH-growth hormone. A fraction receiving only NH_4HCO_3 buffer served as a control.

After incubation at 0-4°C for 4 days, the mixtures were separated by gel filtration on Sephadex C-100 (42 x 2.0 cm) in 0.05 M NH_4HCO_3. Fractions of 1.5 to 2.0 ml were collected, the absorbance was measured at 280 mμ, and 0.5 ml aliquots were analyzed for radioactivity.

5. Additional procedures

a. Preparation of antibodies

Antiserum to bovine growth hormone was prepared in rabbits by the procedure described by Trenkle and Li (1964) for guinea pigs, with some modifications. The required amount of antigen was dissolved in saline, emulsified with an equal volume of Freunds' adjuvant (complete) and injected intradermally into two foot pads. A total dose of 2.5 mg was administered according to the following schedule: three doses of 0.5, 0.5 and 1 mg at weekly intervals. After a 2 week rest period, a final booster dose of 0.5 mg was dissolved in saline and injected subcutaneously. The animals were bled by cardiac puncture 12 days later. Nonspecific antibodies were removed by adsorption with normal bovine serum and prolactin, as described by Hayashida and Li (1958).

Antiserum to bovine prolactin was prepared by

immunizing rabbits with a total dose of 3 mg/animal. An aqueous solution of the antigen, emulsified as before with Freunds' adjuvant, was injected as in the case of growth hormone, according to the following schedule: 0.5, 1.0 and 1.0 mg quantities at weekly intervals, followed by a booster dose of 0.5 mg after a 2 week rest period. Animals were bled by cardiac puncture after 12 days.

After addition of merthiolate to a final concentration of 1:10,000, the antisera were preserved frozen at -20°C.

With animals receiving prolactin, additional booster doses of 0.5 to 1.0 mg were given at monthly intervals and one week later animals were bled by cardiac puncture.

III. Results

A. Biosynthesis of prolactin and growth hormone in slices of bovine anterior pituitary tissue

1. Isolation procedure

Much work has been done on the isolation of pituitary hormones. The majority of methods described have been developed for the production of large quantities of purified hormones and start from kilogram amounts of pituitary tissue.

In adjusting the method of Ellis to a small scale procedure for the isolation of growth hormone, (GH), and prolactin, (LTH), it soon became apparent that greater losses might be anticipated when starting with a small amount of tissue. The extraction step with 0.1M $(NH_4)_2SO_4$, used in our original studies (Rao, et al., 1967) extracted GH as well as thyrotropic hormone, (TSH), and luteinizing hormone (LH). It was decided to check the losses of GH at this step. Table IV indicates that omission of the 0.1M $(NH_4)_2SO_4$ step resulted in 1.8 times greater yield in mg of GH purified to the Amberlite CG-50 stage. An approximately 1.4 times increase in the prolactin fraction was also observed.

Extraction of the $(NH_4)_2SO_4$ precipitate with pH 5.1 phosphate buffer containing 0.45M $(NH_4)_2SO_4$, and subsequent chromatography on Amberlite CG-50 column, is shown in Figure 1A. Nearly all of the labeling was recovered in the last peak eluted with 0.3 M phosphate buffer (pH 6.0). Rechromatography of the above radioactive component under the same conditions as

TABLE IV. MODIFIED PROCEDURE FOR ISOLATION OF GROWTH HORMONE FROM TISSUE SLICES[a]

Extraction Step	Growth Hormone CG-50		Prolactin DEAE-cellulose		Residual Protein
	cpm[b]	mg[b]	cpm	mg	cpm
Sample 1 and 2					
0.10 M $(NH_4)_2SO_4$	4,000	3.8			
0.25 M $(NH_4)_2SO_4$	7,100	4.5			
SUM:	11,100	8.3	12,300	7.3	60,800
Sample 3 and 4					
0.25 M $(NH_4)_2SO_4$ (no 0.1 M step)	12,400	8.2	17,400	10.3	68,400

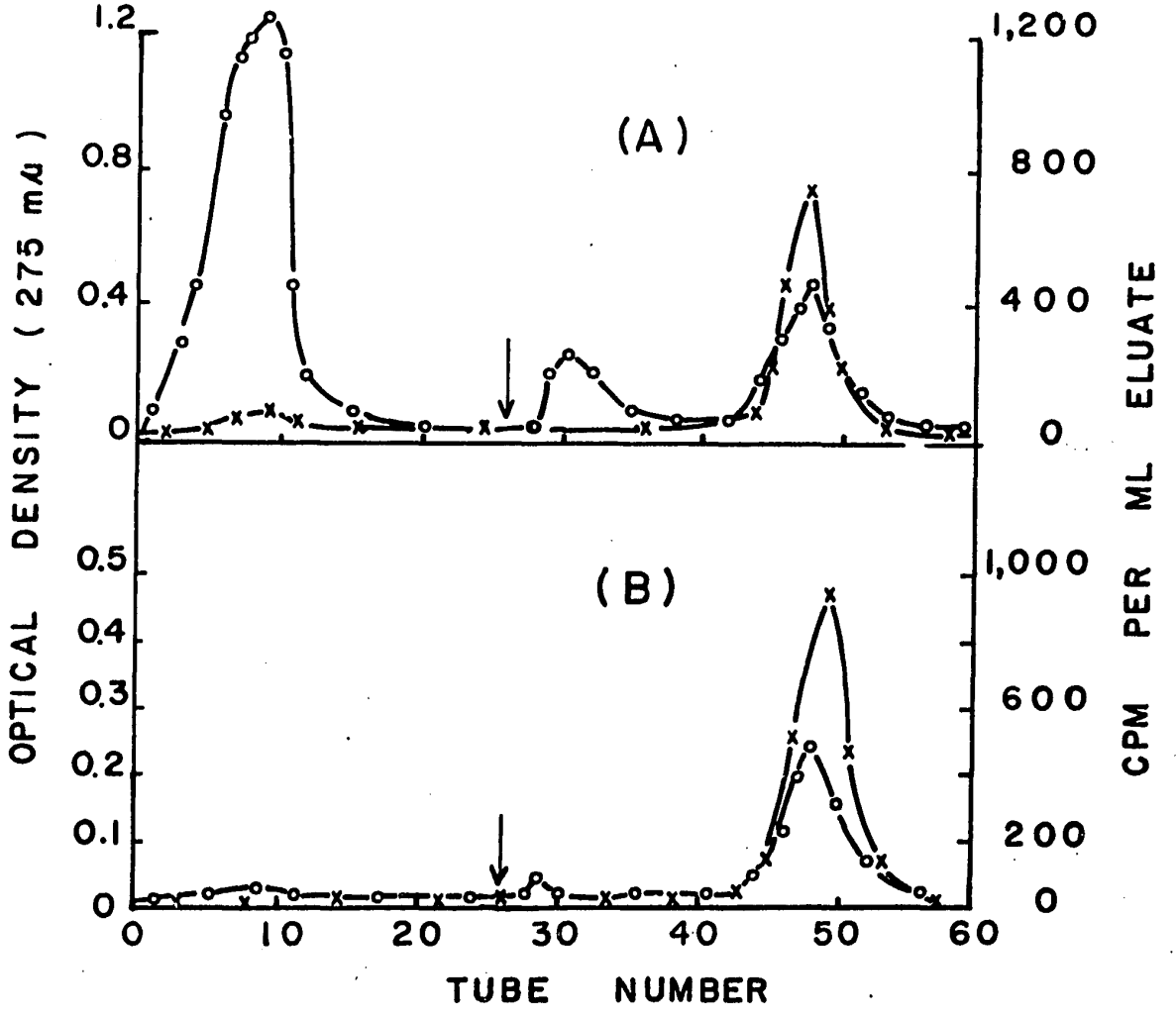
[a]Slices prepared from 12.3 g of anterior pituitary tissue were incubated for 4 hr at 37° with 50 μc ^{3}H-Phe in 50 ml of Krebs-Ringer solution. After stopping the reaction, the slices and media were homogenized, and the homogenate divided into four portions for the isolation of growth hormone and prolactin.

[b]The values are the averages of duplicate estimations, and are expressed per g of wet tissue.

FIGURE 1. CHROMATOGRAPHY ON AMBERLITE CG-50 COLUMN (1x7 cm) OF RADIOACTIVE PREPARATIONS OF GROWTH HORMONE ISOLATED FROM BOVINE ANTERIOR PITUITARY SLICES.

(A) Partially purified growth hormone, after precipitation of crude growth hormone fraction by half saturation with $(NH_4)_2SO_4$ (Table III).

(B) Rechromatography of pooled tubes 40-55 of (A), after dialysis and lyophilization. The arrows indicate a change from pH 5.1 to 0.3 M buffer of pH 6.0, circles, absorbance at 280 mμ, and X's radioactivity.


(A)
(B)
OPTICAL DENSITY (275 mμ)
CPM PER ML ELUATE
TUBE NUMBER
1.2
0.8
0.4
0
1,200
800
400
0
0.5
0.4
0.3
0.2
0.1
0
1,000
600
200
0
0
10
20
30
40
50
60

those used in the purification procedure resulted in a single peak (Fig 1B), possibly homogeneous in the sense that the optical density and radioactivity values were almost coincident. The NIH growth hormone standard showed identical chromatographic behavior under these conditions. When different amino acids (^{3}H-Leu, ^{3}H-Phe, ^{3}H-Thr, or ^{3}H-Pro) were used in the incubation mixture to label the product, virtually the same results were obtained, with very similar distributions of radioactivity.

The residue remaining after extraction of growth hormone was further extracted with 1% DOC (pH 8.4) to yield a fraction rich in prolactin. In the early studies with slices, the DOC extract was dialysed and lyophilized, and prolactin precipitated from an alkaline solution of the lyophilized powder by the isoelectric precipitation procedure of Cole and Li (1955). Estimations on duplicate samples varied greatly (22.8%).

Kwa, et al. (1965) described an isolation procedure for bovine prolactin suitable for small quantities of pituitary tissue, based on DEAE-cellulose column chromatography. It was decided to compare these two methods for the isolation of prolactin from pituitary slices.

As is indicated in Table V, the radioactivity recovered after isoelectric precipitation of prolactin at pH 5.6 is approximately equal to that obtained by stepwise elution of prolactin from a DEAE-cellulose column with 0.4 M NaCl.

TABLE V. FURTHER PURIFICATION OF PROLACTIN (1% DOC EXTRACT) OBTAINED FROM INCUBATION OF PITUITARY SLICES[a]

Sequence of steps in purification	cpm[b]	weight (mg)[b]
Isoelectric precipitation (Cole and Li, 1954)		
1. Crude prolactin, 1% DOC extract	136,000	-
2. Isoelectric precipitation at pH 5.6	55,600	33.4
3. Dialysis and filtration	88,700	24.0
4. Precipitation with 50% $(NH_4)_2SO_4$ and dialysis	71,800	20.2
Elution from DEAE-cellulose (Kwa, et al., 1965)		
1. Crude prolactin, 1% DOC extract	172,000	-
2. Stepwise elution from DEAE-cellulose with 0.4 M NaCl	56,300	23.6
3. Redialysis and filtration	66,400	18.9
4. Precipitation with 50% $(NH_4)_2SO_4$ and dialysis	60,700	15.0

[a]Slices prepared from 9 g of anterior pituitary tissue were incubated for 4 hr at 37° with 60 μc of ^{3}H-Phe in 48 ml of Krebs-Ringer solution. After stopping the reaction, the slices and media were homogenized together and the homogenate divided into four portions for the isolation of hormones.

[b]Values are the average of duplicate estimations and expressed per g of wet tissue.

However the quantities of material differed. On a weight basis, the isoelectric precipitation method resulted in 40% more material than the DEAE-cellulose method. After purification by precipitation with 50% $(NH_4)_2SO_4$, the weight of the isoelectric precipitation product was still 30% more than the DEAE-cellulose product, while the radioactivity was only 18% greater, indicating the presence of a probable non-protein contaminant in the isoelectric precipitation product.

Prolactin isolated from slice material by the DEAE-cellulose method showed a mean variation between duplicates of 3.2%. In subsequent studies this procedure was used for the separation of this hormone.

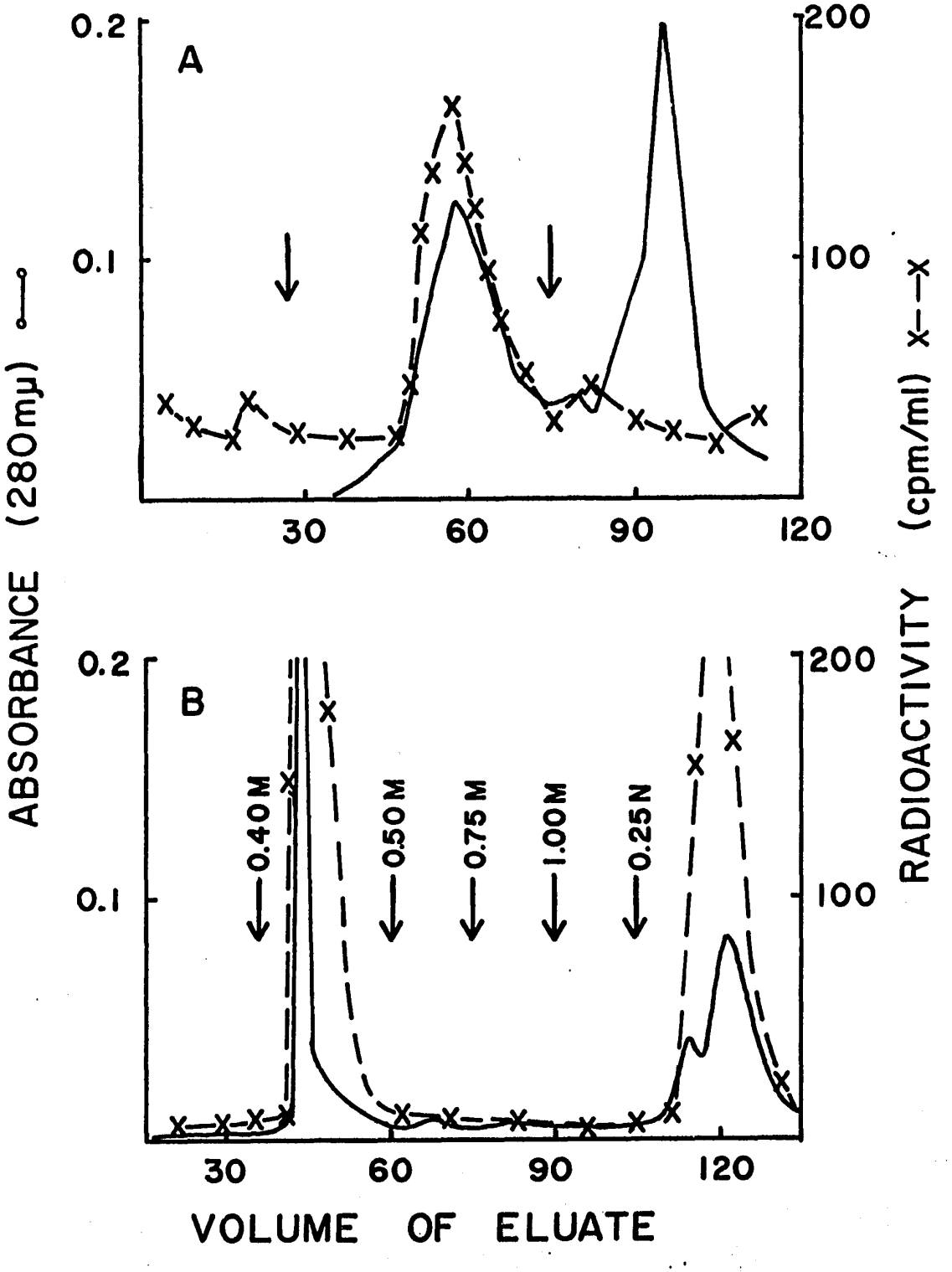
The behavior of labeled prolactin, when applied to DEAE-cellulose column and eluted with a NaCl gradient, is shown in Figure 2A. About 80% of the isotope was recovered in the first large ultraviolet absorbing peak (corresponding to prolactin). The second major peak absorbing in the ultraviolet, but not appreciably labeled, showed a 260/280 ratio of 2.0, suggesting the presence of nucleic acid.

For routine isolations, elution of the prolactin fraction in a single step with NaCl, appeared more desirable than a gradient elution requiring two changes of buffer. Figure 2B shows that elution of the prolactin fraction (first peak) from the DEAE-cellulose column was essentially complete after the addition of 0.4 M NaCl. The second large peak, eluted with 0.25 N NaOH, corresponds to an inactive fraction reported by

FIGURE 2. CHROMATOGRAPHY ON DEAE-CELLULOSE OF PROLACTIN ISOLATED FROM BOVINE ANTERIOR PITUITARY SLICES.

(A) Gradient elution pattern of DOC extract of pituitary slices on a column of DEAE-cellulose (1x8 cm). The initial eluant was 0.025 M borate buffer (pH 8.4). The first arrow indicates the beginning of the gradient to 0.4 M NaCl. The second arrow indicates a change of gradient to 1 M NaCl.

(B) Stepwise elution pattern of DOC extract of Residue III (Table III) on a column of DEAE-cellulose (1x8 cm). The initial eluant was 0.025 M borate buffer (pH 8.4). The arrows indicate the increase in NaCl concentration to 1.0 M. The arrow at 105 ml indicates a change to 0.25 N NaCl. Solid line, absorbance at 280 mμ, and broken line with X's, radioactivity.


A
B
0.2
0.1
200
100
30
60
90
120
0.40 M
0.50 M
0.75 M
1.00 M
0.25 N
ABSORBANCE (280mμ) o—o
RADIOACTIVITY (cpm/ml) x—x
VOLUME OF ELUATE

Sluyser and Li (1964). According to these authors this material represented only about 11% of the sample. A similar fraction eluted by 0.25 N NaOH, was obtained when prolactin was recovered from the column by gradient elution (data not included).

A comparison of stepwise versus gradient elution of prolactin from DEAE-cellulose column Table VI revealed the counts recovered in prolactin to be essentially the same by either method.

Table VII shows the yields of growth hormone and prolactin obtained from bovine anterior pituitary slices after purification to the Amberlite CG-50 and DEAE-cellulose stages, respectively. Additional gel filtration on Sephadex G-100 resulted in separation of a monomer peak in average yields of approximately 1.3 and 1.7 mg/gm wet tissue, for growth hormone and prolactin respectively.

In seeking to establish that the incubation and isolation procedure was fairly tissue specific for measuring the synthesis of the two hormones, a comparison was made of fractions isolated by the same procedure from posterior pituitary, hypothalamus, brain, heart, and liver slices (Table VIII). On the basis of g of tissue incubated, the anterior pituitary was approximately twice as active as the posterior pituitary and hypothalamus in incorporating radioactivity into residual protein, 11-12 times more label was isolated in the growth hormone fraction and 4-6 times more in the prolactin fraction.

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TABLE VI. COMPARISON OF STEPWISE VERSUS GRADIENT ELUTION OF PROLACTIN FROM DEAE-CELLULOSE COLUMNS

Method	Cpm applied[a]	Cpm recovered in prolactin component
Stepwise elution	50,000	13,360
Gradient elution	50,000	12,850

[a]The radioactive material was a crude prolactin fraction isolated from anterior pituitary slice incubations.

TABLE VII. YIELDS OF GROWTH HORMONE AND PROLACTIN FROM BOVINE ANTERIOR PITUITARY TISSUE[a]

Experiment Number	Growth Hormone		Prolactin	
	Isolation Step		Isolation Step	
	CG-50	G-100[b]	DEAE-cellulose	G-100
1	8.3	1.5	18.9	1.7[c]
2	8.2	1.2	8.5	2.1[b]
3	9.0	-	9.4	1.3[b]

[a]Values are expressed as mg/g wet bovine anterior pituitary tissue.

[b]Sephadex G-100 column equilibrated with 0.036 M Na acetate buffer, pH 4.0.

[c]Sephadex G-100 column equilibrated with 0.1 M $NaHCO_3$ buffer, pH 8.1.

TABLE VIII. COMPARISON OF ANTERIOR PITUITARY WITH SEVERAL OTHER BOVINE TISSUES, IN THE HORMONE BIOSYNTHETIC PROCEDURE

Source of Tissue Slices	Incorporation of ^{3}H-amino acids				
	Growth hormone CG-50		Prolactin DEAE-cellulose		Residual Protein
	cpm	%	cpm	%	cpm
Expt. 1[a]					
Anterior pituitary	24,400	2.96	53,750	6.53	823,000
Posterior pituitary	2,190	0.55	9,830	2.49	394,000
Hypothalamus	2,240	0.39	13,390	2.36	567,000
Expt. 2[b]					
Hypothalamus	2,260	0.25			906,500
Brain	550	0.69			79,500
Heart	190	0.48			40,550
Liver	830	1.42			58,150

[a]Approximately 1 g of each tissue was incubated for 4 hr at 37° with 10 μc of ^{3}H-L-Phe in 4 ml of Krebs-Ringer solution. Values are expressed as cpm/g wet tissue, and as percent of residual protein values.

[b]Five slices of each tissue were incubated for 4 hr at 37° with 10 μc of ^{3}H-DL-Phe in 4 ml of Krebs-Ringer solution. The radioactivity values are expressed as cpm/g dry tissue.

When the radioactivity recovered in the hormone fractions was expressed as a percentage of that in the residual protein, the anterior pituitary still had 3-8 times more label in these fractions than similar preparations isolated from other bovine tissues. Table VIII also indicates that 3.0% and 6.5% of the protein being synthesized by anterior pituitary tissue was growth hormone and prolactin, respectively, based on the "residual" protein values.

Since some labeled material was isolated by the hormone procedures from tissues other than anterior pituitary, the nature of this product was studied by gel filtration on Sephadex G-100. Figures 3 and 4 indicate that the radioactive material was isolated by the hormone procedure from posterior pituitary and hypothalamus tissues, was relatively insignificant and differed in molecular weight from the labeled protein of the anterior pituitary. The prolactin fraction isolated from hypothalamus appeared to be an exception (Fig 4C) but only accounts for 2% of the protein being synthesized compared to 6.5% for the anterior pituitary. Interestingly, the prolactin fraction isolated from posterior pituitary was composed of low molecular weight peptides.

2. Rates of hormone synthesis in bovine pituitary slices

Figure 5 reveals the time course for the incorporation of labeled amino acids into the hormone and protein fractions. In the case of general protein synthesis, the process proceeded at a linear rate for at least 6 hr. About 20% of

FIGURE 3. SEPHADEX G-100 FILTRATION OF FRACTIONS CORRESPONDING TO THE CG-50 PREPARATION OF GROWTH HORMONE, ISOLATED FROM VARIOUS BOVINE TISSUES.

The Sephadex column was 2.5x36 cm, and 0.036 M sodium acetate (pH 4.0) was the eluting buffer. Elution pattern of CG-50 fraction isolated from:

(A) bovine anterior pituitary slices;

(B) bovine posterior pituitary slices; and

(C) bovine hypothalamus.

Solid line, absorbance at 280 mμ, broken line with X's, radioactivity. Cross-hatched region corresponds to the elution volume of standard growth hormone.

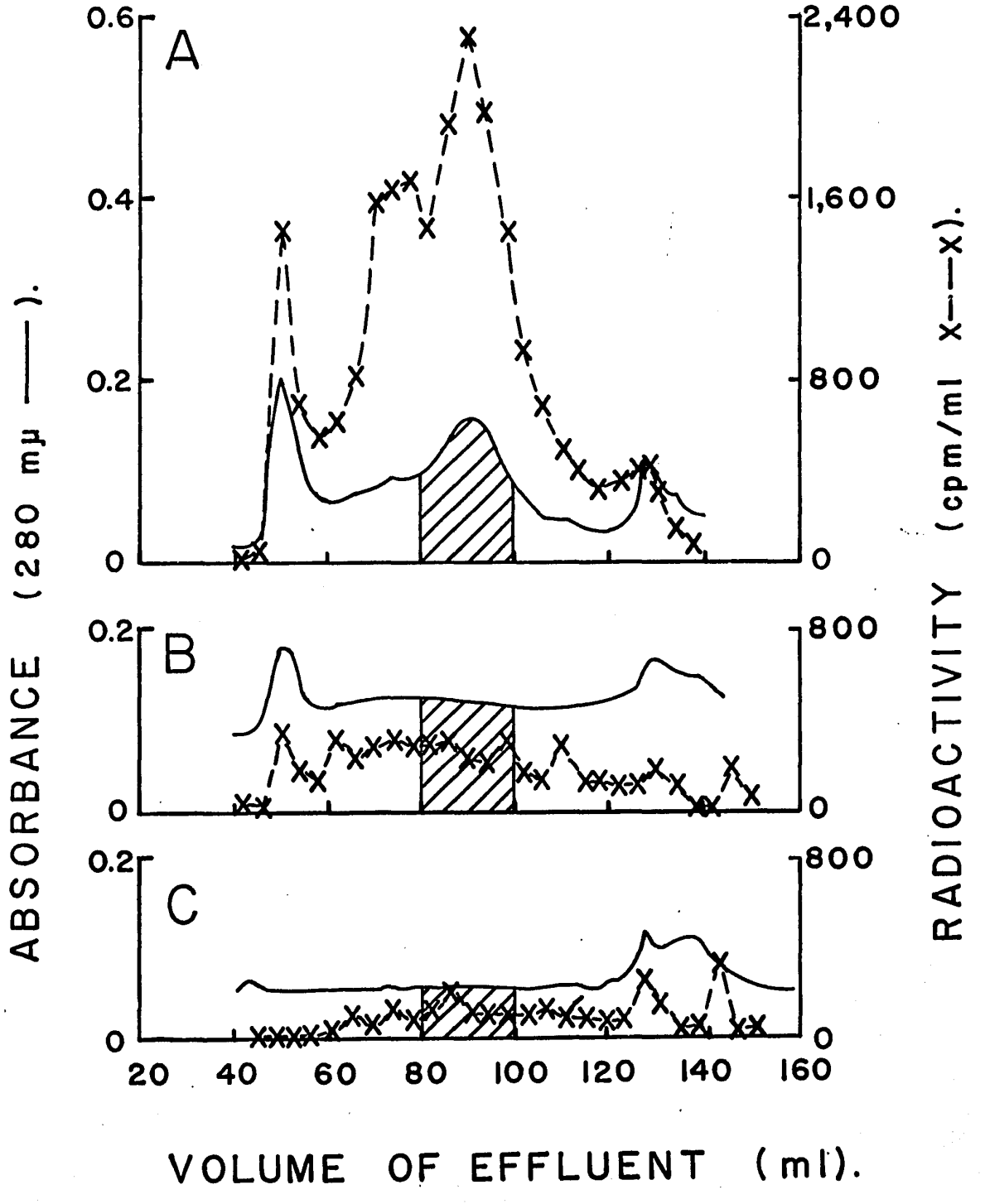

A
B
C
0.6
0.4
0.2
0
2,400
1,600
800
0
0.2
800
0
0.2
800
0
20
40
60
80
100
120
140
160
ABSORBANCE (280 mμ ——).
RADIOACTIVITY (cpm/ml x—x).
VOLUME OF EFFLUENT (ml).

FIGURE 4. SEPHADEX G-100 FILTRATION OF FRACTIONS FROM VARIOUS BOVINE TISSUES ISOLATED BY THE PROCEDURE DESCRIBED TO OBTAIN PROLACTIN.

The Sephadex column was 2.5x36 cm, and 0.036 M sodium acetate (pH 4.0) was the eluting buffer. Elution pattern of DOC extract from:

(A) bovine anterior pituitary slices;

(B) bovine posterior pituitary slices; and

(C) bovine hypothalamus.

Solid line, absorbance at 280 mμ, broken line with X's, radioactivity. Cross-hatched region corresponds to the elution volume of standard prolactin.

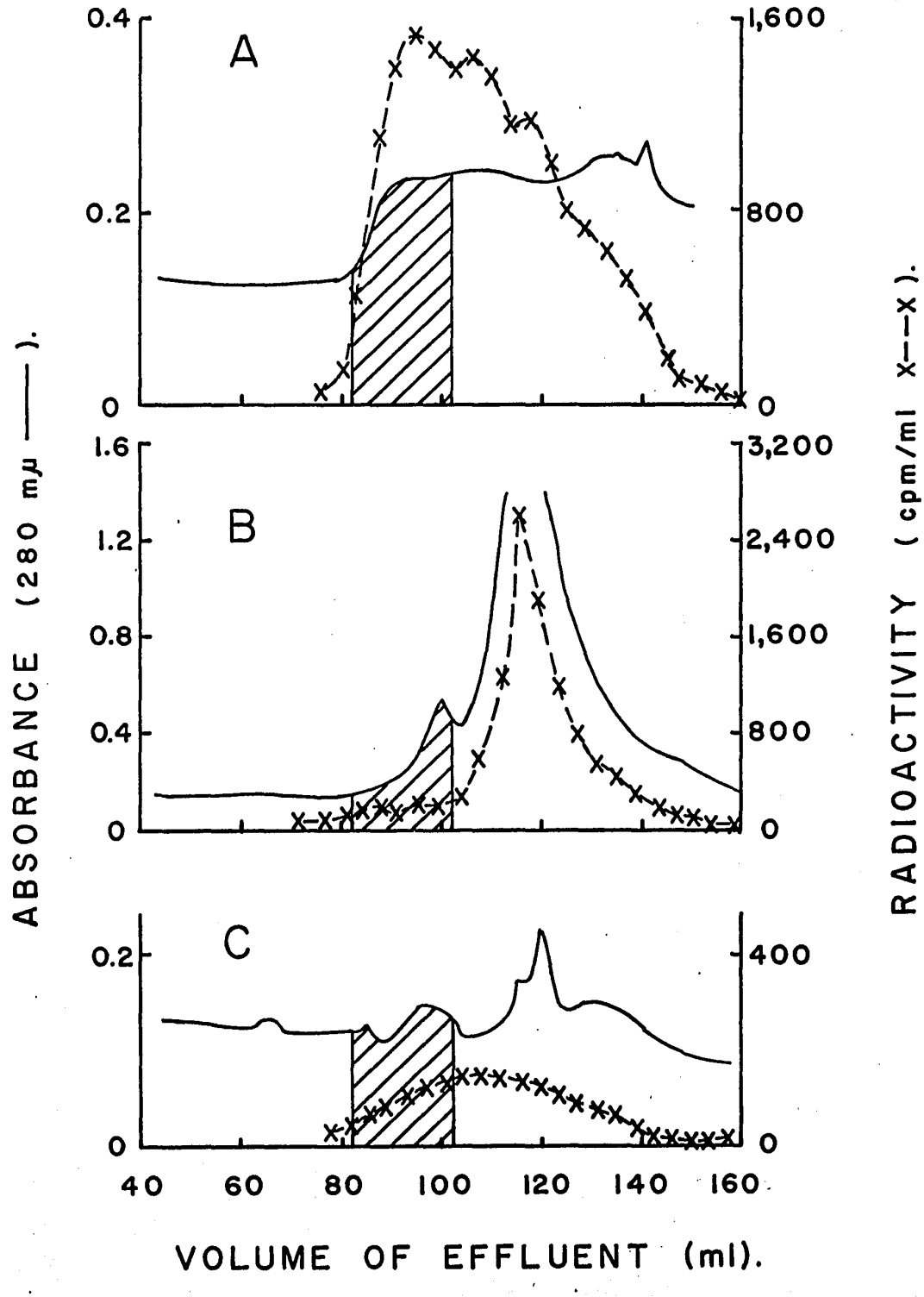
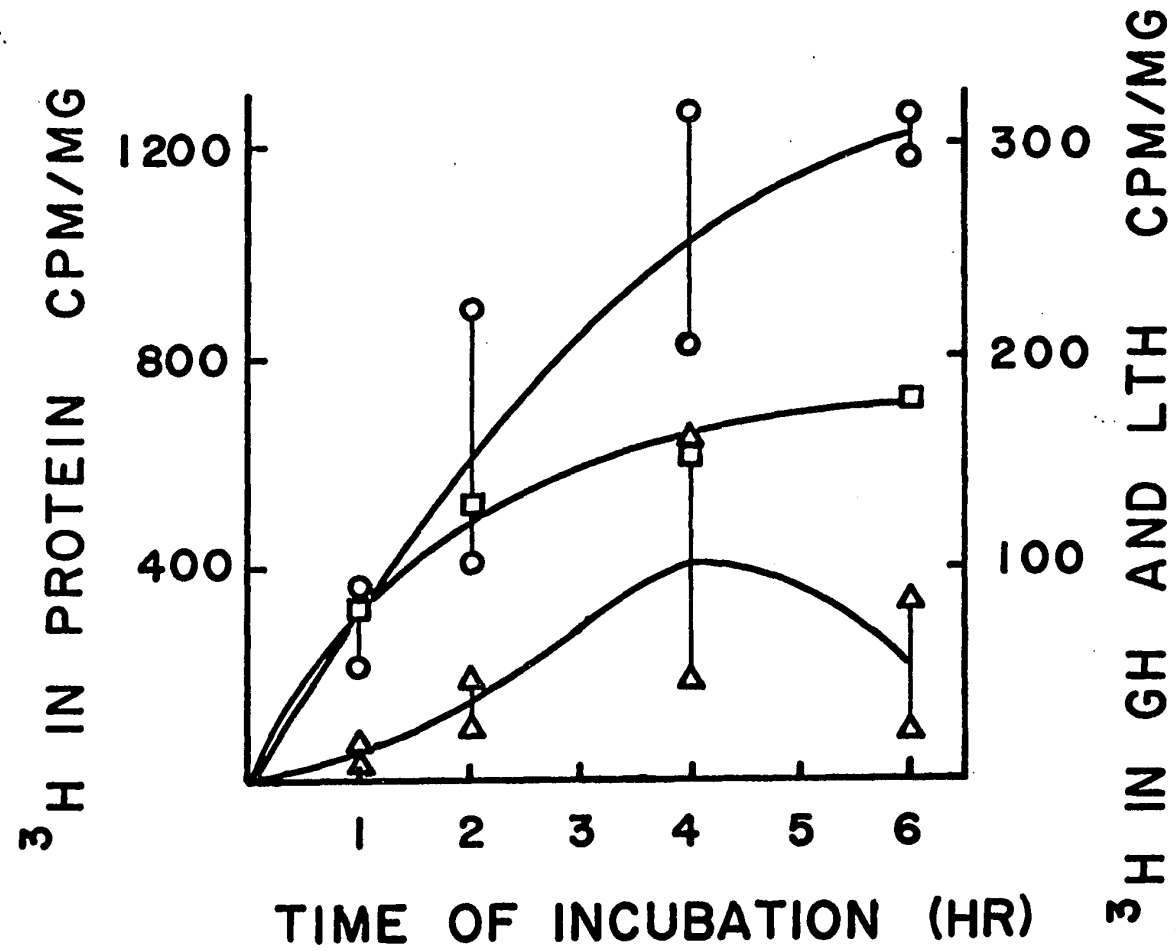

A
B
C
0.4
0.2
0
1,600
800
0
1.6
1.2
0.8
0.4
0
3,200
2,400
1,600
800
0
0.2
0
400
0
40
60
80
100
120
140
160
ABSORBANCE (280 mμ ——).
RADIOACTIVITY (cpm/ml X—X).
VOLUME OF EFFLUENT (ml).

FIGURE 5. RELATIVE REATES OF GROWTH HORMONE, PROLACTIN, AND GENERAL PROTEIN SYNTHESIS IN SLICES OF ANTERIOR PITUITARY TISSUE.

The growth hormone and residual protein curves represent average values of duplicate estimations obtained from two separate experiments carried out on different days. Circle, residual protein; triangle, growth hormone; and square, prolactin.


^{3}H IN PROTEIN CPM/MG
1200
800
400
300
200
100
1
2
3
4
5
6
TIME OF INCUBATION (HR)
^{3}H IN GH AND LTH CPM/MG

the total isotopic phenylalanine employed was utilized in this period of time. The rate of prolactin synthesis was rapid initially, but appeared to level off after about 4 hr. With growth hormone, the initial rate of ^{3}H-phenylalanine incorporation was relatively slow, and, after attaining maximum value at 4 hr, the extent of labeling thereafter decreased. This curious behavior was confirmed in repeated experiments. At 4 hr the radioactivities for each hormone corresponded to the utilization of approximately 3% of the original quantity of labeled amino acid initially present in the incubation procedure.

As a preliminary to the study of the effect of growth hormone releasing factor (GRF), on growth hormone synthesis and release (Section 4 to follow), the relative degrees of synthesis and release of growth hormone, prolactin, and total protein were measured after incubation of pituitary tissue for 2 and 4 hour periods. The results are presented in Table IX. After the shorter time, the relative proportion of protein released into the media (11%) was more than that in the case of either growth hormone (3%) or prolactin (5%). After 4 hr the extent of release of these three fractions appeared to be about equal (in the range of 2-5%).

When the radioactivity released in 2 hrs as growth hormone or prolactin was expressed as a percentage of the label released as total protein in the same period, more (16%) could be attributed as due to prolactin than to growth hormone (5%).

TABLE IX. RELATIVE RATES OF SYNTHESIS AND RELEASE OF GROWTH HORMONE, PROLACTIN, AND GENERAL PROTEIN IN SLICES OF ANTERIOR PITUITARY TISSUE

Procedure, with 1 g tissue slices in 5 ml medium	Incorporation of ^{3}H-amino acids					
	Growth Hormone CG-50		Prolactin DEAE-cellulose		Total Protein	
	cpm	%	cpm	%	cpm	%
Expt. 1[b]						
2 hr -Medium analysed	710	3	2,180	5	13,700	11
2 hr -Tissue analysed	22,000	97	38,000	95	107,000	89
4 hr -Medium analysed	2,250	4	2,370	2	16,100	5
4 hr -Tissue analysed	57,000	96	101,000	98	345,000	95
Expt. 2[c]						
4 hr -Medium analysed	3,340	2	4,580	2	21,000	1
4 hr -Tissue analysed	171,000	98	280,000	98	2,000,000	99

[a]Values are the average of duplicate determinations and expressed as cpm/g of slices and as a percent of the total label in medium and tissue for each hormone and protein estimation.

[b]10 μc of ^{3}H-Phe.

[c]10 μc each of ^{3}H-Phe, Pro, Leu, Val, and Lys.

This difference probably reflected the slower initial rate of synthesis of growth hormone, as compared to prolactin (Fig 5). At 4 hr the label in growth hormone represented 15% and prolactin 18% of the radioactivity in total protein, released in this time. The experiments indicated that hormones were constantly being released in the media without the stimulus of added GRF.

3. Characterization of radioactive product from slice incubations

In experiments designed to demonstrate the synthesis of a specific protein in slices or cell-free system, care must be taken to ensure that the radioactive preparation isolated is properly characterized, and that the associated radioactivity is not due to contamination. In order to establish the identity and purity of the preparations, labeled growth hormone and prolactin samples recovered at the Amberlite CG-50 and DEAE-cellulose stages, respectively, were subjected to a number of fairly rigorous techniques.

The gel filtration pattern of such growth hormone preparations on Sephadex G-100 is illustrated in Figure 6. With 0.036 M sodium acetate, pH 3.6 as buffer, a typical elution pattern showed three major peaks (Fig 6C), the fastest moving component (I) corresponding to the void volume of the column, and the slowest moving fraction to a standard preparation of NIH-growth hormone (Fig 6A). When the tubes from each of these peaks were pooled, dialyzed and relyophilized, the components reappeared in their original positions on the column (Fig 6D, E and F). Electrophoresis of

FIGURE 6. GEL FILTRATION OF GROWTH HORMONE PREPARATIONS FROM BOVINE PITUITARY SLICES ON SEPHADEX G-100 IN AN ACID BUFFER.

(2.5x36 cm, 0.036 M sodium acetate buffer, pH 4.0)

(A) 3.5 mg NIH-growth hormone standard.

(B) Rechromatography of tubes 70-95 of A, dialyzed and lyophilized.

(C) 26.0 mg of CG-50 growth hormone fraction isolated from bovine anterior pituitary slices incubated with ^{3}H-Phe.

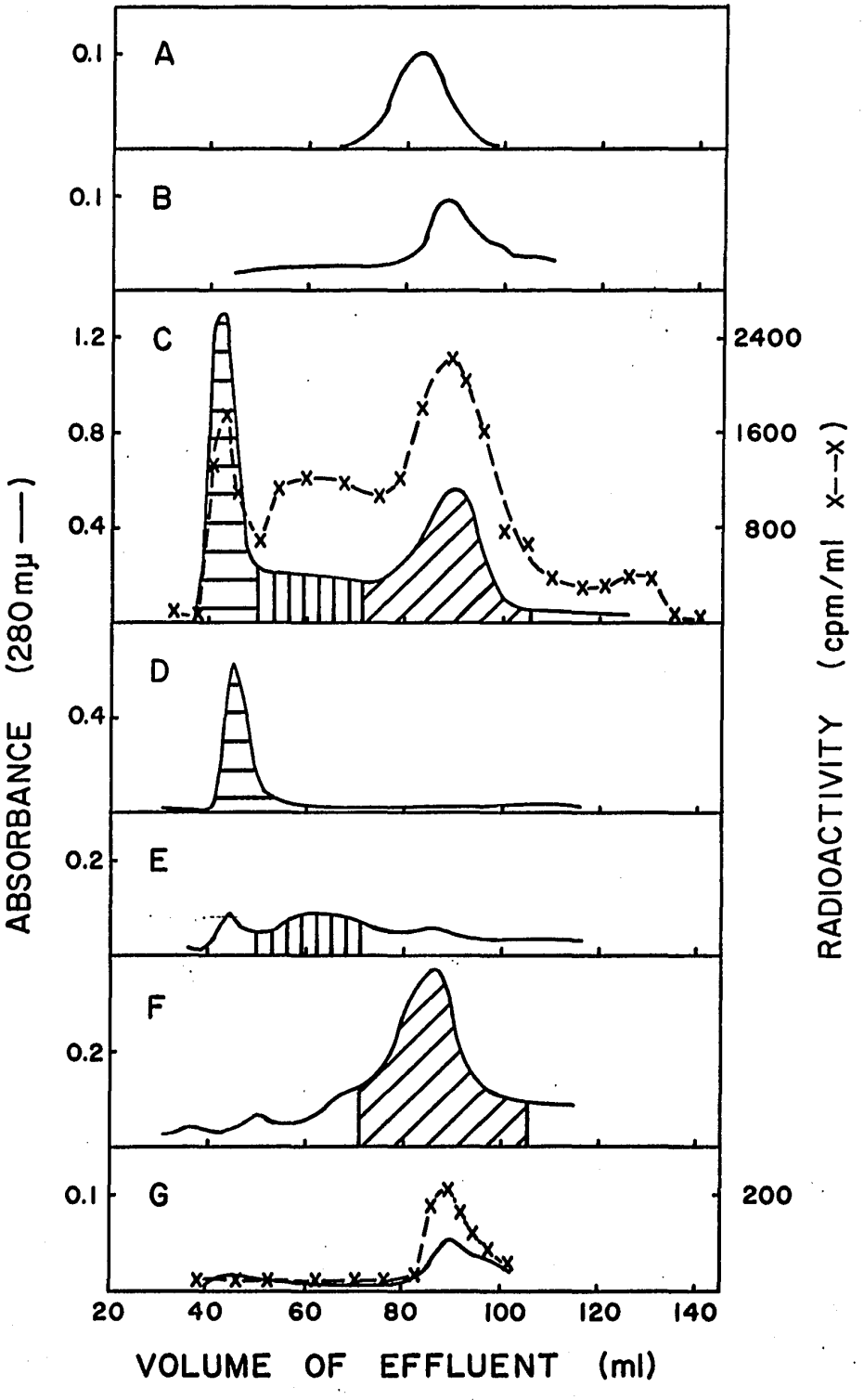
(D) Rechromatography of peak I, tubes 37-50, of C.

(E) Rechromatography of peak II, tubes 51-71, of C.

(F) Rechromatography of peak III, tubes 72-105, of C.

(G) CG-50 growth hormone fraction isolated from rat pituitaries plus 1 mg of NIH-growth hormone.

Solid line, absorbance at 280 mμ, broken line with X's, radioactivity.


A
B
C
D
E
F
G
0.1
0.1
1.2
0.8
0.4
0.4
0.2
0.2
0.1
2400
1600
800
200
ABSORBANCE (280 mμ ——)
RADIOACTIVITY (cpm/ml x--x)
20
40
60
80
100
120
140
VOLUME OF EFFLUENT (ml)

the three peaks on polyacrylamide gel (Fig 10B) indicated that growth hormone was the sole protein present. According to Dellacha, et al., 1968, the three fractions corresponded to a monomer (Peak III), dimer (Peak II) and aggregate form (Peak I) of growth hormone and accounted for 45%, 23% and 27% respectively of the total radioactive material applied.

Gel filtration of CG-50 purified radioactive growth hormone was conducted with two alkaline buffers: 0.15 M NH_4CO_3, pH 8.1 (Fig 7A and B) and 0.05 M borate-KCl, pH 8 (Fig 7C and D). As in the previous experiments, the presence of minor amounts of the aggregated material was revealed.

As part of the GRF studies, labeled growth hormone was isolated from rat pituitaries which had been incubated with ^3H-amino acids. The labeled material appeared at the same elution volume on Sephadex G-100 (Fig 6G), as standard bovine growth hormone, applied simultaneously. This supports the report by Ellis, et al. (1968) that the molecular weight of rodent growth hormone approximates that of bovine growth hormone.

Figure 8B shows the gel filtration pattern of DEAE-prolactin fraction on Sephadex G-100 with 0.036 M sodium acetate buffer, pH 3.6. Two components were present in a typical elution pattern; one corresponding to the void volume of the column and the second eluting at the same volume as a standard NIH prolactin preparation. Electrophoresis of material from each peak on polyacrylamide gel (Fig 8C) relieved patterns similar to the NIH-prolactin, with peak I showing two additional

FIGURE 7. SEPHADEX GEL FILTRATION OF GROWTH HORMONE PREPARATIONS FROM BOVINE PITUITARY SLICES IN ALKALINE BUFFERS.

(A) 1.9 mg of NIH-growth hormone. The Sephadex G-100 column (2.5x36 cm) was equilibrated with 0.15 M NH_4HCO_3 buffer, pH 8.1.

(B) 12.0 mg of CG-50 growth hormone isolated from bovine anterior pituitary slices incubated with ^{3}H-Phe. The Sephadex G-100 column was the one described in (A).

(C) 2.5 mg of NIH-bovine growth hormone. The Sephadex G-200 column (1.6x110 cm) was equilibrated with 0.05 M borate-0.1 M KCl, buffer, pH 8.6.

(D) CG-50 growth hormone isolated from bovine anterior pituitary slices incubated with ^{3}H-Leu. The Sephadex G-200 column was that described in (C).

Solid line, absorbance at 280 mμ, broken line with X's, radioactivity.

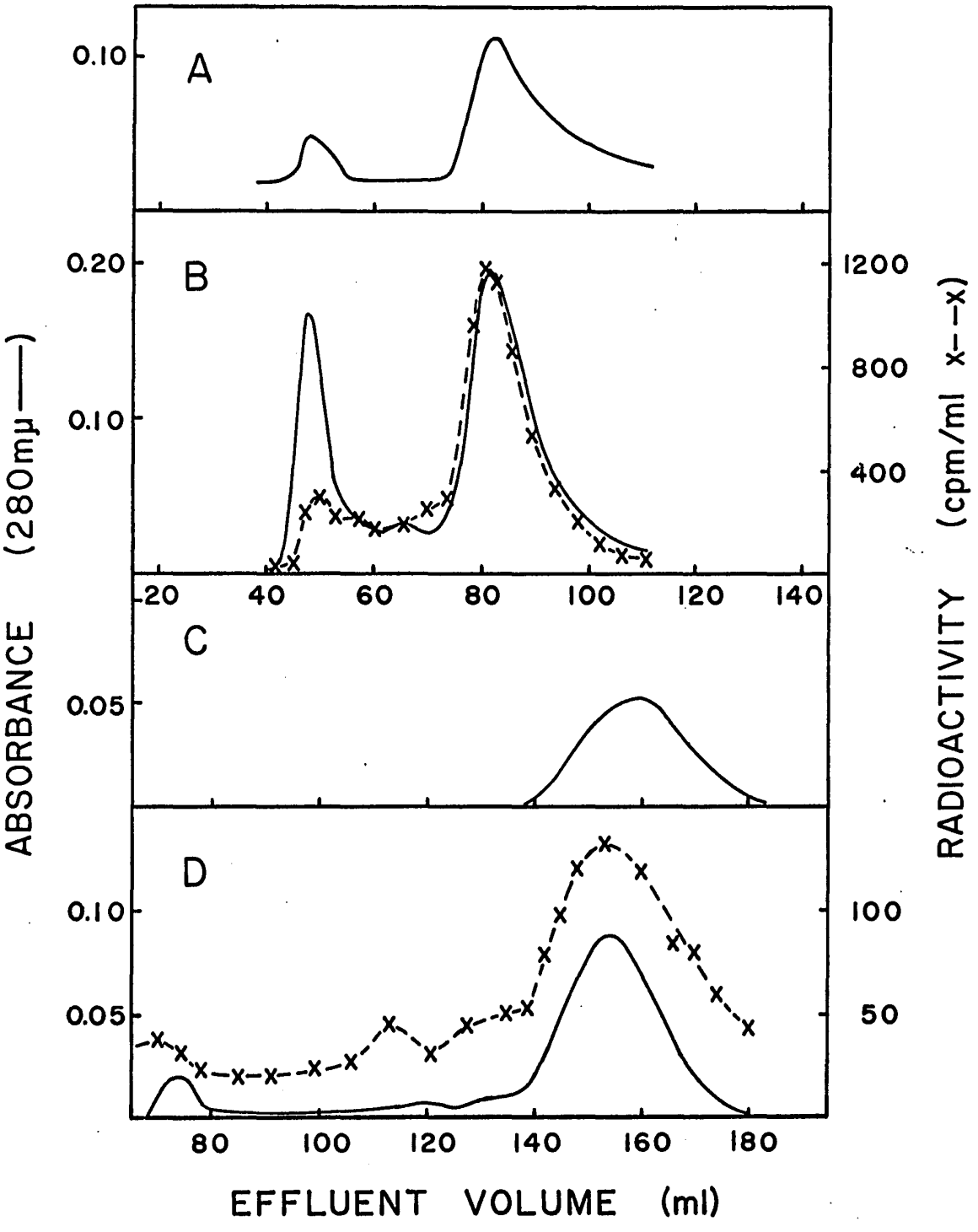

A
B
C
D
0.10
0.20
0.10
0.05
0.10
0.05
1200
800
400
100
50
20
40
60
80
100
120
140
80
100
120
140
160
180
ABSORBANCE (280mμ——)
RADIOACTIVITY (cpm/ml x--x)
EFFLUENT VOLUME (ml)

FIGURE 8. GEL FILTRATION OF PROLACTIN PREPARATIONS FROM BOVINE PITUITARY SLICES ON SEPHADEX G-100 IN AN ACID BUFFER.

(2.5 x 36 cm, 0.036 M sodium acetate buffer, pH 4.0)

(A) 2.5 mg of NIH-bovine prolactin.

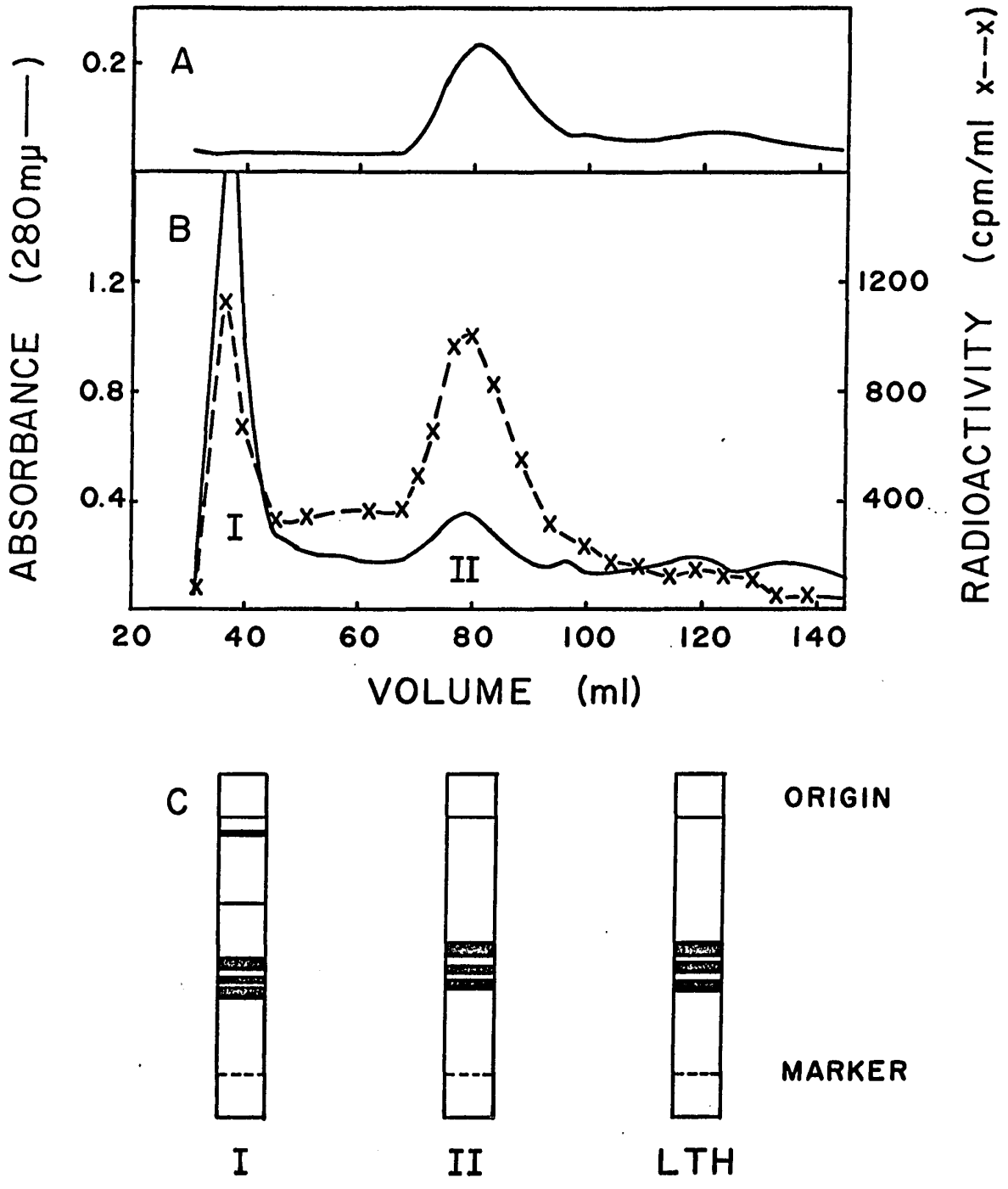
(B) 14.0 mg of DEAE-prolactin isolated from bovine anterior pituitary slices incubated with ^{3}H-Phe.

(C) Polyacrylamide gel electrophoresis at pH 8.9 of components separated in (B) above, after dialysis and lyophilization.

I, 300 µg of material from tubes 30-59 of (B).

II, 300 µg of material from tubes 60-110 of (B).

LTH, 100 µg of NIH-prolactin.


A
B
C
0.2
1.2
0.8
0.4
1200
800
400
I
II
20
40
60
80
100
120
140
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)
VOLUME (ml)
ORIGIN
MARKER
I
II
LTH

bands of minor components. Peak II, the monomer fraction usually accounted for approximately 70% of the applied radioactive material.

The gel filtration pattern of DEAE-prolactin fraction on Sephadex G-100 with an alkaline buffer, 0.05 M NH_4HCO_3, pH 8.1 is shown in Figure 9. Again two components were present corresponding to a monomer and aggregated form (Squire, et al., 1963). Upon gel electrophoresis (Fig 9C) only one band of the high molecular weight material (Peak I), the fastest moving band, seems to correspond to the midband of standard prolactin. The slightly slower migrating band of prolactin probably corresponds to the aggregated form altered somewhat by the alkaline buffer (Lewis and Cheever, 1965). Three bands of minor components were also detected. The pattern of the monomer fraction, Peak II, was similar to the NIH-standard.

The electrophoresis patterns of the labeled CG-50 growth hormone is shown in Figure 10A. The position of the major stained band was almost identical with that of the strongest band of a reference NIH sample, run simultaneously. At least two other bands corresponding to impurities were also noted. The distribution of radioactivity along the gel column shows that the label was concentrated to a marked degree in the growth hormone region, where major staining was observed. This zone corresponds to approximately 31% of the labeled material recovered. The considerable amount of radioactivity at the interface of the spacer gel corresponds to a band of

FIGURE 9. GEL FILTRATION OF PROLACTIN PREPARATIONS FROM BOVINE PITUITARY SLICES ON SEPHADEX G-100 IN AN ALKALINE BUFFER.

(2.0x55 cm, 0.05 M NH_4HCO_3 buffer, pH 8.1)

(A) 1.4 mg of NIH-bovine prolactin.

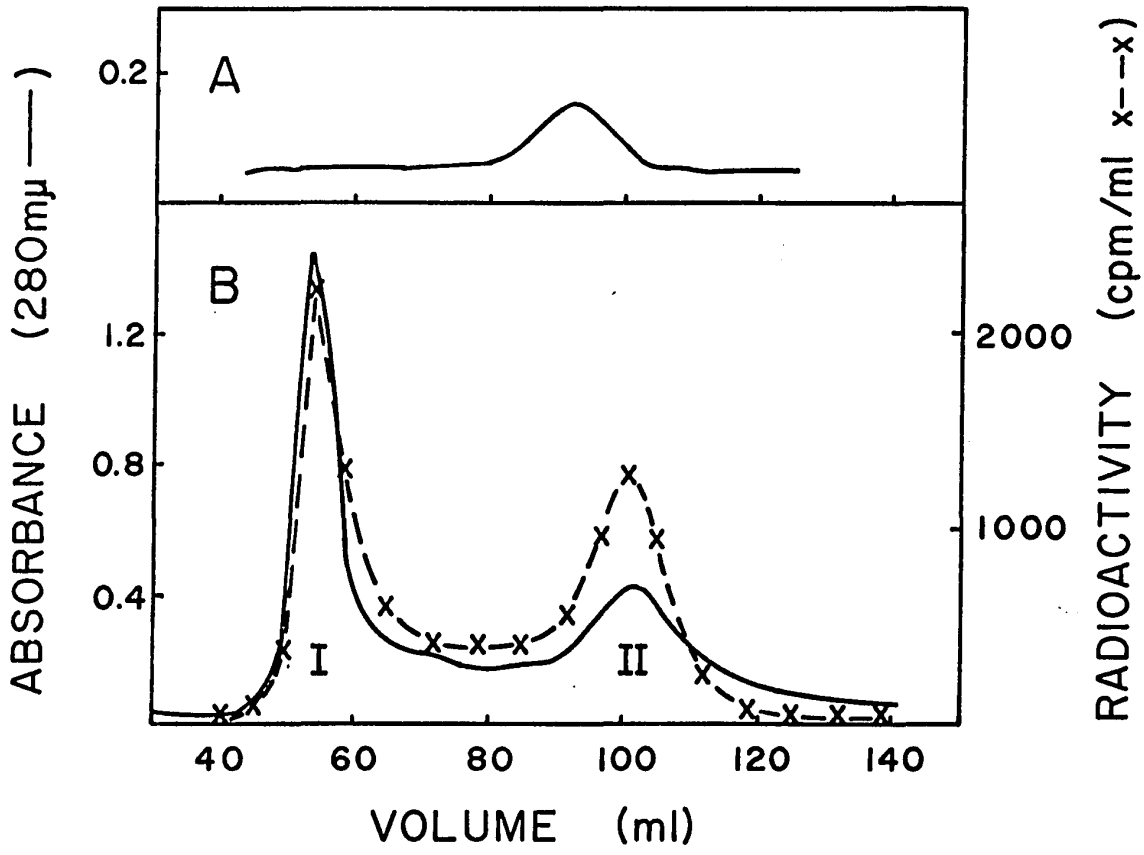
(B) 17.0 mg of DEAE-prolactin isolated from bovine anterior slices incubated with ^{3}H-Phe.

(C) Polyacrylamide gel electrophoresis at pH 8.9, of components separated in (B) above, after lyophilization.

I, 300 µg of material from tubes 45-86 of (B).

II, 300 µg of material from tubes 82-120 of (B).

LTH, 100 µg of NIH-prolactin.


A
B
0.2
1.2
0.8
0.4
2000
1000
I
II
40
60
80
100
120
140
VOLUME (ml)
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)

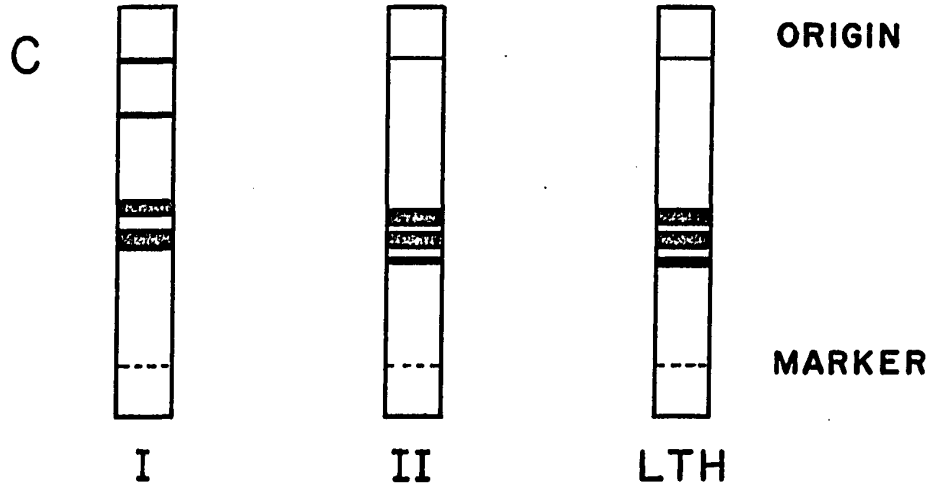

C
ORIGIN
MARKER
I
II
LTH

FIGURE 10. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 8.9 OF GROWTH HORMONE PREPARATIONS FROM BOVINE PITUITARY SLICES.

(A) GH, 100 μg of NIH-bovine growth hormone isolated from anterior pituitary slices incubated with ^{3}H-Phe. The cross-hatched area shows the distribution of radioactivity along the gel of a similar sample of ^{3}H-GH run concurrently.

(B) Components of growth hormone separated by gel filtration with 0.036 M sodium acetate buffer, pH 4.0 (Fig 6C).

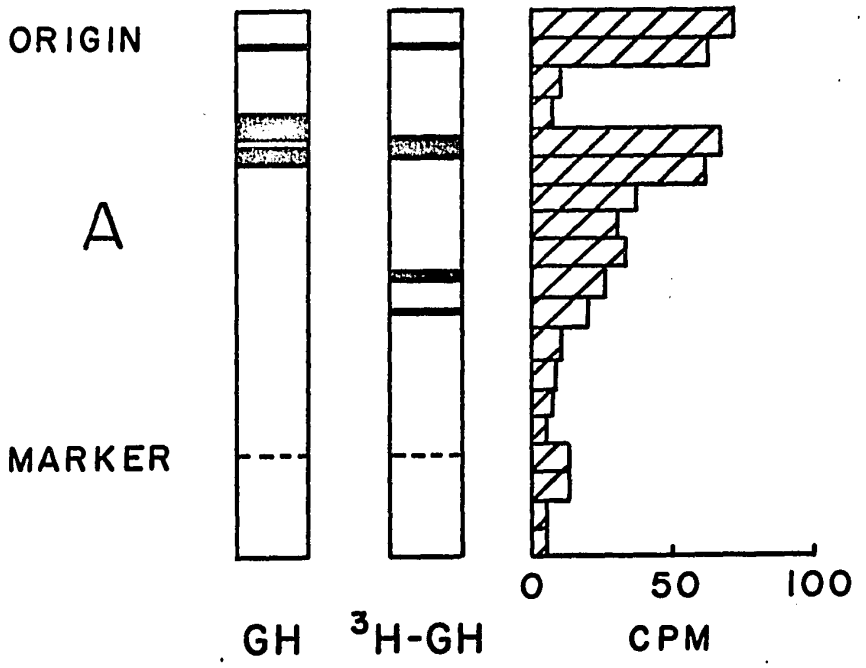
GH, 100 μg of NIH-growth.

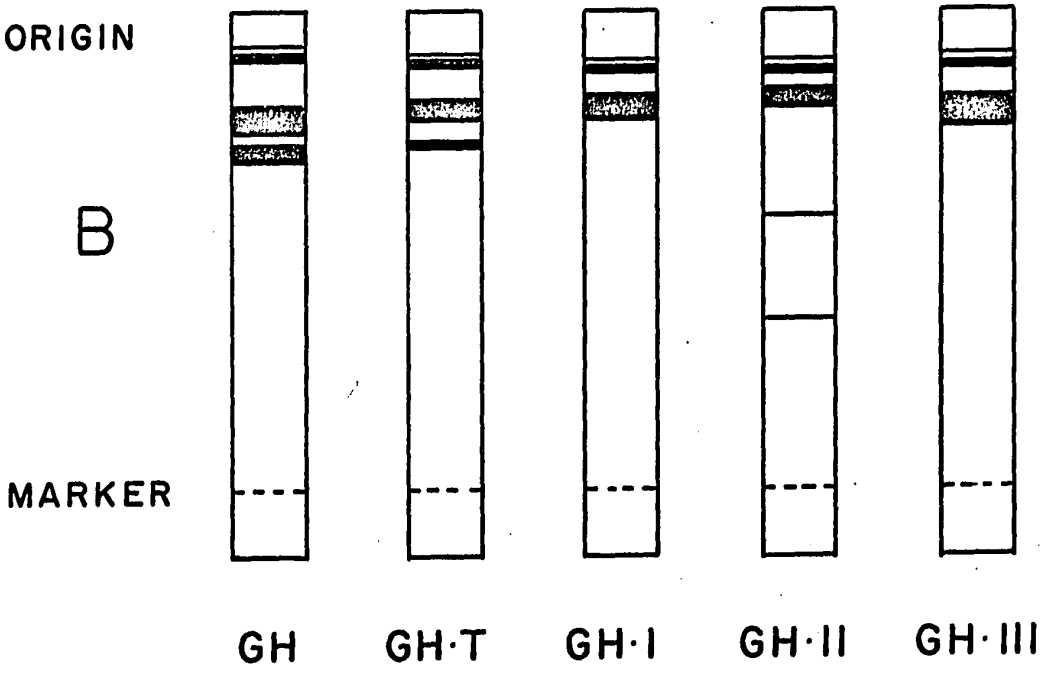
GH.T, 200 μg of CG-50 growth hormone.

GH.I, 200 μg of material corresponding to peak I (Fig 6D).

GH.II, 300 μg of material corresponding to peak II (Fig 6E).

GH.III, 300 μg of material corresponding to peak III (Fig 6F).


ORIGIN
A
MARKER
0
50
100
GH
^{3}H-GH
CPM


ORIGIN
B
MARKER
GH
GH·T
GH·I
GH·II
GH·III

high molecular weight material which did not enter the running gel, and accounts for 30% of the radioactivity recovered.

Prolactin isolated from slices and purified to the DEAE-cellulose stage exhibited three bands which migrated about 2/3 of the way down the gel towards the anode when analyzed by electrophoresis on polyacrylamide gels (Fig 11). The stained bands corresponded to those of a sample of NIH-prolactin run simultaneously. The radioactivity concentrated in the region corresponding to stained prolactin bands represented 35% of the total isotope.

The molecular weight of the labeled hormones was estimated by the gel filtration method of Andrews (Fig 12). A sample of tritiated growth hormone was eluted from a calibrated G-200 column at a volume of 124 ml (Fig 12B), corresponding to a molecular weight of 26,000, while the monomer peak of prolactin was eluted at 150 ml (Fig 12C), corresponding to 20,000 molecular weight. NIH standards filtered under identical conditions gave similar values. As was observed in previous experiments, the radioactivity peaks of the hormones coincided with those of ultraviolet absorbance.

A number of methods which rely on the endogenous hormones derived in the isolation procedure were also used to establish the identity of the radioactive material. One of these, immunoassay of the labeled growth hormone and prolactin preparations by the Ouchterlony gel diffusion method is shown in Figure 13. It may be seen that in both cases single

FIGURE 11. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 8.9 OF PROLACTIN PREPARATIONS FROM BOVINE PITUITARY SLICES.

(A) 100 μg of NIH-bovine prolactin.

(B) 1.0 mg of DEAE-cellulose prolactin isolated from anterior pituitary slices incubated with ^{3}H-Phe.

(C) Distribution of radioactivity along the gel in (B).

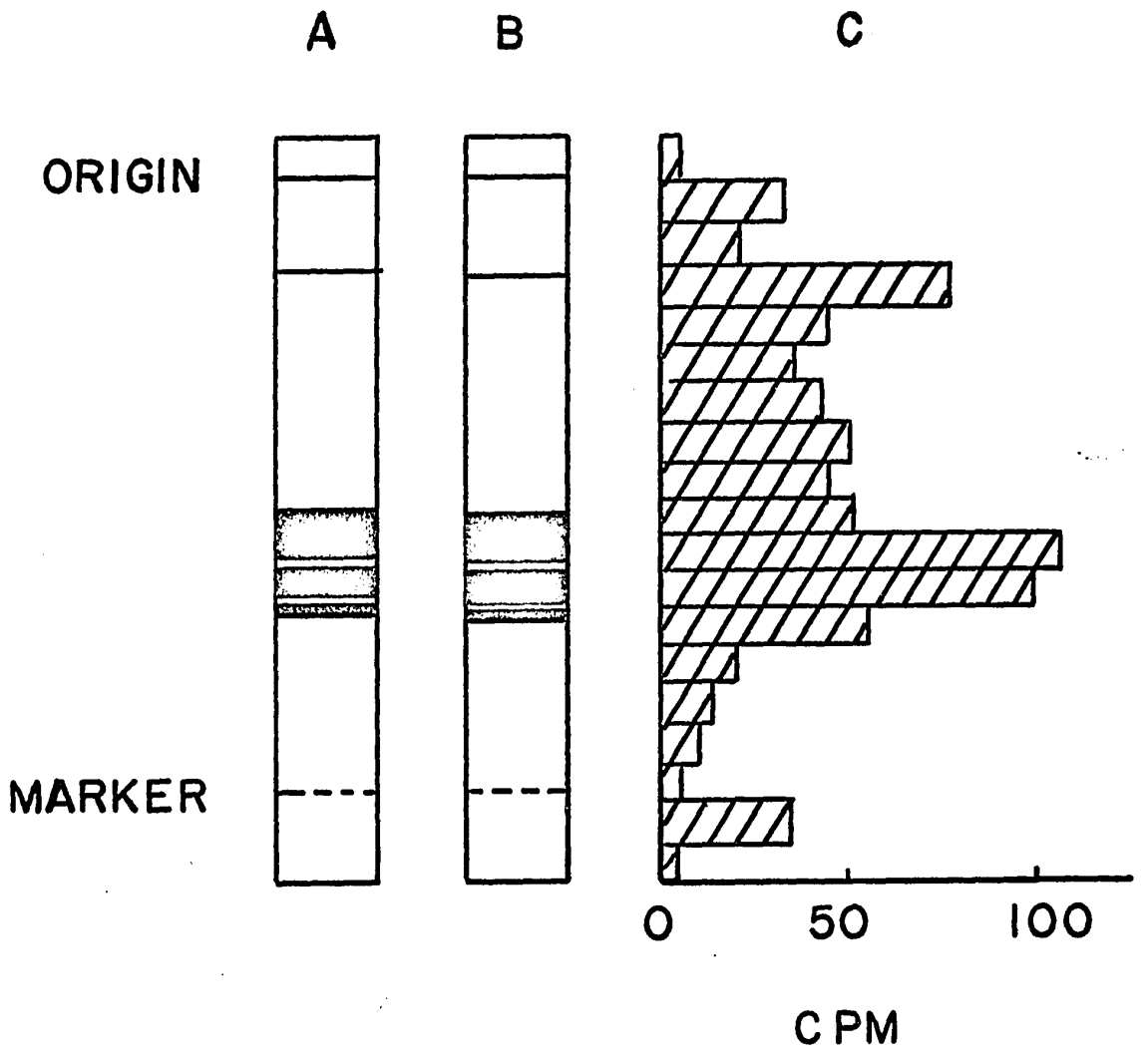

A
B
C
ORIGIN
MARKER
0
50
100
CPM

FIGURE 12. MOLECULAR SIZE OF THE HORMONES BY GEL FILTRATION ON SEPHADEX.

(A) Calibration curve for molecular weight estimation by Sephadex G-200 gel filtration. The column size was 1.6x110 cm, and the eluting buffer was 0.05 M Tris-0.1 M KCl (pH 7.5).

(B) Gel filtration on the above column of 5.0 mg of CG-50 growth hormone isolated from bovine adenohyphophyses.

(C) Gel filtration on the column described in (A) of 4.5 mg of DEAE-cellulose prolactin isolated from bovine adenohypophyses.

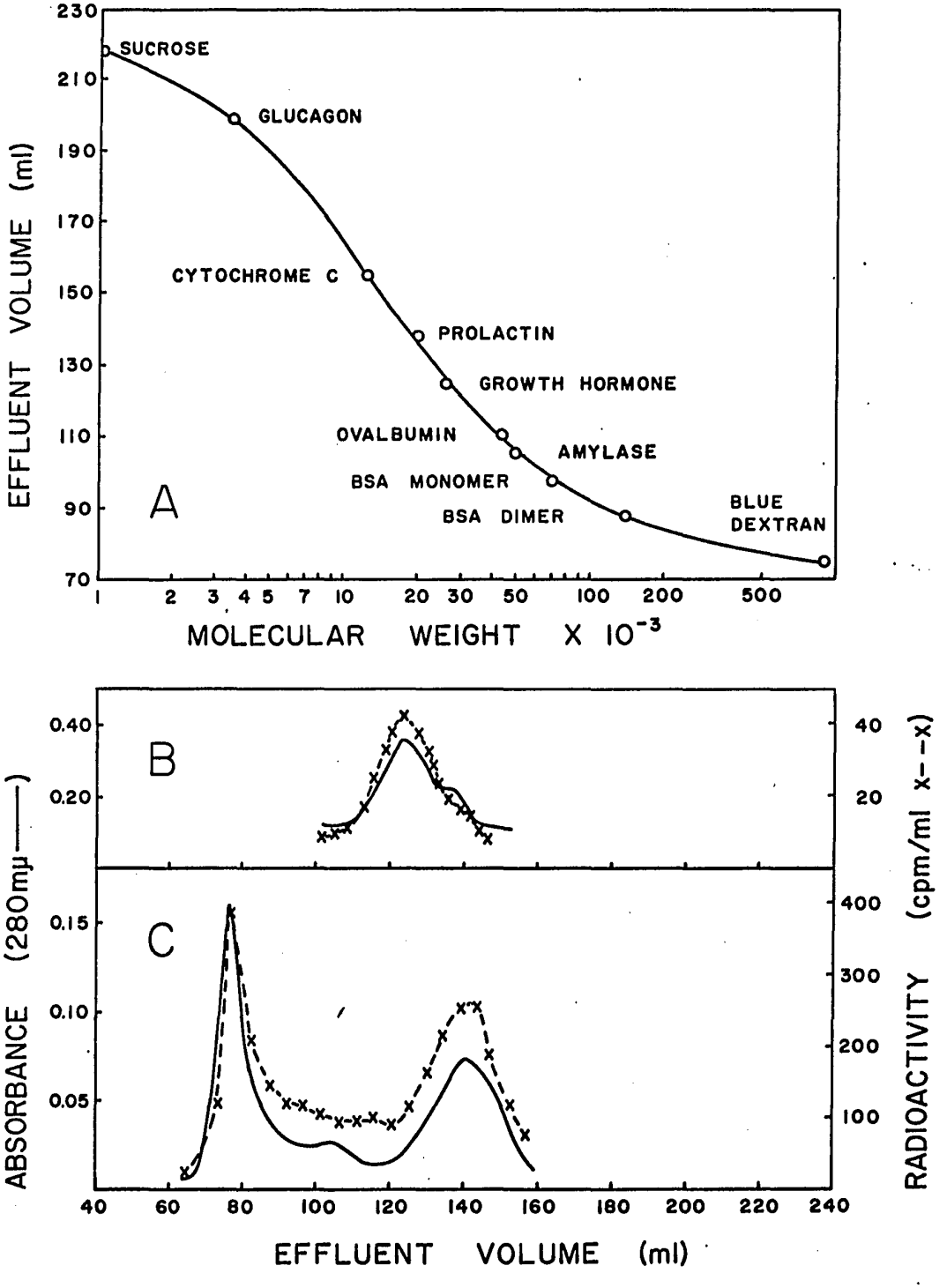
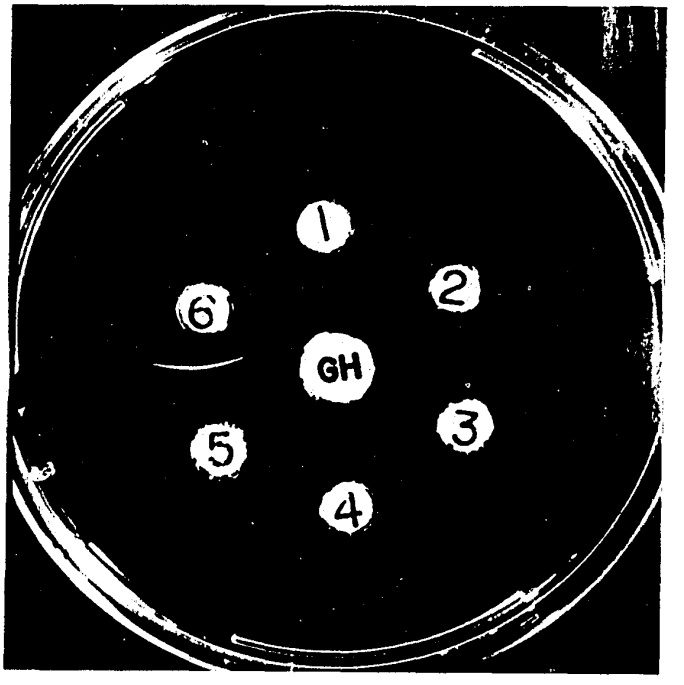
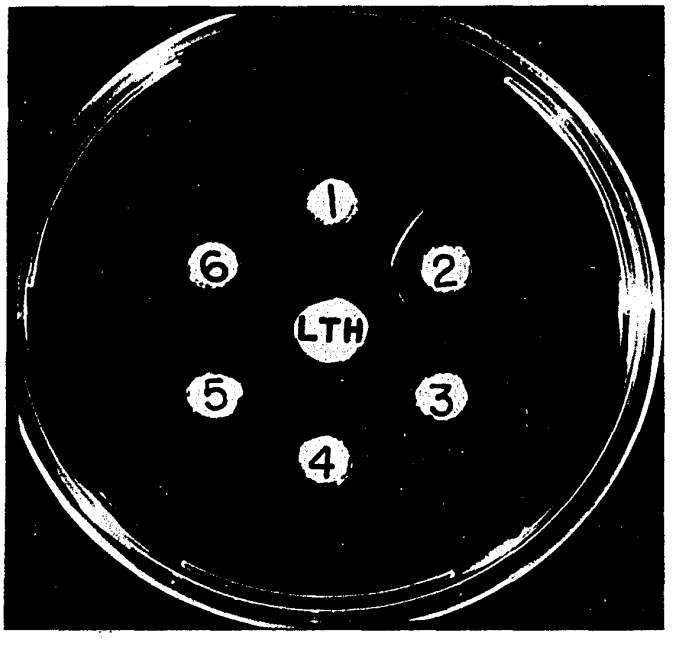

SUCROSE
GLUCAGON
CYTOCHROME C
PROLACTIN
GROWTH HORMONE
OVALBUMIN
AMYLASE
BSA MONOMER
BSA DIMER
BLUE DEXTRAN
A
EFFLUENT VOLUME (ml)
MOLECULAR WEIGHT X 10⁻³
B
C
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)
EFFLUENT VOLUME (ml)

FIGURE 13. OUCHTERLONY IMMUNODIFFUSION OF GROWTH HORMONE AND PROLACTIN ISOLATED FROM BOVINE ANTERIOR PITUITARY SLICES.

Upper figure: precipitin reactions between isolated CG-50 bovine growth hormone (center well-GH) and: 1) NIH-growth hormone, 2) anti-growth hormone-serum, 3) bovine serum, 4) anti-bovine albumin-serum, 5) NIH-prolactin, and 6) anti-prolactin-serum.

Lower figure: precipitin reactions between isolated DEAE-cellulose bovine prolactin (center well-LTH) and: 1) NIH-prolactin, 2) anti-prolactin-serum, 3) bovine serum, 4) anti-bovine albumin-serum, 5) NIH-growth hormone, and 6) anti-growth hormone-serum.


1
2
3
4
5
6
GH


1
2
3
4
5
6
LTH

precipitin lines confluent with those formed by NIH standards were obtained against homologous antisera. No cross-reaction developed with anti-bovine serum, even at a 1% concentration of the hormone solutions. This indicated the absence of significant contamination by serum components. Similarly, there were no cross-reactions of hormones with heterologous antisera.

Amino acid analysis of the labeled growth hormone and prolactin similarly reflected the endogenous hormones. Table X compares the composition of bovine growth hormone and prolactin isolated from tissue slice experiments with that obtained for a reference standard, and also with values reported in the literature. The number of residues present in both the GH and LTH samples were in fairly good agreement with those reported for bovine GH by Dellacha, et al. (1968) and for ovine prolactin by Lyons and Dixon (1966). Proteolytic activity in the pituitary may have removed some C- and N-terminal phenylalanine from growth hormone before the isolation was started accounting for the low values obtained.

To further establish the identity of the labeled hormones, end group analyses were also carried out (Table XI). The DNP method indicated phenylalanine and alanine as the amino acid residues at the N terminal position for bovine growth hormone, and threonone as N-terminal for prolactin. Enzymic digestion of growth hormone with DFP-treated carboxypeptidase released radioactive phenylalanine and a lesser amount of alanine. No other amino acids could be detected on paper chromatography.

TABLE X. AMINO ACID COMPOSITION OF GROWTH HORMONE AND PROLACTIN

Amino acid	Bovine growth hormone		Bovine prolactin		
	From tissue slice experiment	Dellacha et al (1967)	From tissue slice experiment	NIH standard	Ovine prolactin Lyons and Dixon (1966)
Alanine	14	14	11	12	10
Aspartic acid	17	15	23	23	23
Arginine	12	12	10	8	11
Glutamic acid	25	23	24	21	24
Glycine	12	10	14	12	11
Histidine	4	3	6	5	8
Isoleucine	7	7	9	8	10
Leucine	24	24	19	19	24
Lysine	14	12	10	9	10
Methionine	3	4	7	7	7
Phenylalanine	10	12	6	4	6
Proline	5	6	11	10	12
Serine	12	11	14	13	15
Threonine	11	11	11	8	9
Tyrosine	5	6	8	7	7
Valine	8	6	11	11	10

The number of residues of each amino acid was calculated on the basis of selected amino acids, stable on acid hydrolysis and present in both bovine growth hormone (molecular weight 22,000) and prolactin (23,000). The constituents were: ala, val, gly, lys, glu, arg, leu and ileu.

TABLE XI. N- AND C-TERMINAL ANALYSIS OF GROWTH HORMONE AND PROLACTIN

Hormone	N-terminal		C-terminal	
	Amino acid	Radioactivity cpm	Amino acid	Radioactivity cpm
Growth hormone[a]	Phenylalanine	50	Phenylalanine	205
	Alanine[b]			
Prolactin	Threonine[b]			

[a]1800 cpm of hormone (2 mg) isolated from incubation of anterior pituitary slices with ^{3}H-Phe and purified to the Amberlite CG-50 stage was used in each case.

[b]Nonlabeled; detected by the DNP and ninhydrin reactions.

The amount of label released from the N-terminus was considerably less than that liberated from the C-terminal end. With prolactin, no amino acid was liberated, even after digestion for 18 hrs.

4. Effect of growth hormone releasing factor (GRF), on the synthesis and release of growth hormone

Table XII shows that when porcine GRF was incubated in vitro with bovine anterior pituitary slices, a decrease occurred in both the synthesis and release of growth hormone, prolactin and general proteins. Although slightly less inhibition was noted in the sample receiving only 1 μg of GRF, a graded response to increasing amounts of GRF was not evident.

In order to obtain pituitaries from animals where pre-conditioning was feasible, rats were used. The effect of GRF on the release of growth hormone labeled in vitro, in pituitaries from normal rats is shown in Table XIII. Summation of the radioactivity present in the medium and tissue samples (i.e. total growth hormone) indicated that there was no significant difference in the total amount of growth hormone synthesized in the experimental and control group of pituitaries (2C and 2E). However with the experimental groups (1E and 2E) receiving the GRF, slightly more growth hormone was released into the media, compared with the control groups.

Miller, et al. (1967) reported that the exposure of rats to cold stress resulted in depletion of pituitary GH. Accordingly the effect of GRF on the release of labeled hormone

TABLE XII. EFFECT OF PORCINE GRF ON THE SYNTHESIS AND RELEASE OF GH, LTH AND PROTEIN IN BOVINE ANTERIOR PITUITARY SLICES[a]

GRF	Incorporation of ^{3}H-amino acids[b]					
µg	Growth Hormone		Prolactin		Residual Protein	
	Tissue	Medium	Tissue	Medium	Tissue	Medium
None	171	3.3	280	4.5	508	5.4
1	126	2.4	167	3.4	387	4.4
5	100	1.7	145	1.7	366	2.0
15	98	2.2	205	1.9	380	2.0
50	107	2.2	153	2.6	296	2.2

[a]Each g of slices was incubated for 4 hr in 5 ml of Krebs-Ringer containing 10 µc each of ^{3}H-Phe, Pro, Leu, Val and Lys. GRF was added to the indicated flasks at the beginning of the incubations.

[b]Values are expressed as cpm x 10^{-3}/ g wet tissue.

TABLE XIII. IN VITRO EFFECT OF GRF ON THE RELEASE OF PRE-LABELED GROWTH HORMONE, FROM PITUITARIES OF NORMAL RATS[a]

Expt. No.[b]	GRF µg	Inhibitor	Incorporation of ^{3}H-amino acids					
			Growth Hormone cpm x 10^{-2}/mg			Protein cpm x 10^{-2}/mg		
			Tissue	Medium	Total	Tissue	Medium	Total
1C[b]	-	-	lost	5.6	-	-	35	-
1E	1.0	-	22.1	8.5	30.6	690	27	717
2C	-	-	37.4	7.7	45.1	724	21	745
2E	4.0	-	29.8	11.2	41.0	658	62	720
3C	-	Puromycin 4 x 10^{-4}M	8.1	3.5	11.6	404	56	460
3E	4.0	Puromycin 4 x 10^{-4}M	11.8	8.7	20.5	506	18	524
4C	-	Actinomycin D 2 µg	17.7	5.5	23.2	582	58	640
4E	4.0	Actinomycin D 2 µg	20.7	4.9	25.6	624	100	724

[a]Preincubation: 2 hr with 23.5 µc each of ^{3}H-Phe, Pro and Leu.
Subsequent incubation: 5 hr with fresh media plus GRF.

[b]C - control groups; E - experimental groups; each flask contained eight pituitary halves.

was measured, using pituitaries from rats which had been stressed by exposure to 4° for approximately 1 hr. The pituitaries from these animals were labeled *in vitro* by preincubation for 2 hr with tritiated amino acids. As may be seen in Table XIV, the amount of labeled hormone released into the medium during this preincubation period was approximately equal for each duplicate pool of pituitary halves (i.e., 1C versus 1E, 2C versus 2E). The addition of GRF to the experimental group resulted in an increased release of growth hormone into the media in Experiment 1, and a decrease in Experiment 2. In both cases little difference was noted in the total amount of protein released into the media during the 6 hr incubation period. In Experiment 3, Table XIV, the addition of puromycin (3E), did not depress this release, but seemed to cause some inhibition of further incorporation of the adhering labeled amino acids, not removed with the preincubation medium during the change of medium. This effect was also noted in Experiment 3 of Table XIII, when normal rat pituitaries were used.

The effect of GRF on both the synthesis and release of growth hormone was measured by incubating pituitaries from cold stressed rats in media containing the tritiated amino acids and GRF from the beginning of the experiment. Two different samples of porcine GRF, differing in specific activity when bioassayed, were used. The GRF sample used in experiments reported in Tables XII-XV, was fully active *in vivo*

TABLE XIV. IN VITRO EFFECT OF GRF ON THE RELEASE OF PRE-LABELED GROWTH HORMONE FROM PITUITARIES OF COLD STRESSED RATS[a]

Expt. No.[c]	GRF µg	Inhibitor	Incorporation of ^{3}H-amino acids							
			Growth Hormone cpm x 10^{-2}/mg				Protein cpm x 10^{-2}/mg			
			Pre-Media[b]	Tissue	Medium	Total	Pre-Media	Tissue	Medium	Total
1C	-	-	7.5	12.6	5.7	18.3	43	839	91	930
1E	1.0	-	9.4	12.4	11.0	23.4	60	917	83	1000
2C	-	-	2.9	14.0	5.9	19.9	50	617	81	698
2E	4.0	-	4.5	11.3	3.8	15.1	29	692	83	775
3C	-	Puro 4 x 10^{-4}M	3.7	9.2	2.9	12.1	77	540	34	574
3E	4.0	Puro 4 x 10^{-4}M	4.1	7.7	4.5	12.2	50	540	38	578
4C	-	Act D 2 µg	2.2	8.9	6.2	15.1	28	577	83	660
4E	4.0	Act D 2 µg	4.1	13.5	2.6	16.1	46	576	109	685

[a]Preincubation: 2 hr with 23.5 µc each of ^{3}H-Phe, Pro and Leu.
Subsequent incubation: 6 hr in fresh media plus GRF.

[b]Preincubation Media - removed after 2 hr incubation with ^{3}H-amino acids, before addition of GRF.

[c]C - control group; E - experimental group; each flask contained eight pituitary halves.

TABLE XV. EFFECT OF GRF ON BOTH THE SYNTHESIS AND RELEASE OF ^{3}H-LABELED GROWTH HORMONE, BY PITUITARIES OF COLD STRESSED RATS. I.[a]

Expt. No.[b]	GRF µg	Inhibitor	Incorporation of ^{3}H-amino acids					
			Growth Hormone cpm x 10^{-2}/mg			Protein cpm x 10^{-2}/mg		
			Tissue	Medium	Total	Tissue	Medium	Total
1C	-	-	51	30	81	1600	128	1728
1E	1.0	-	58	19	77	1885	111	1996
2C	-	-	87	22	109	1715	216	1931
2E	4.0	-	79	39	118	1860	144	2004
3C	-	Puromycin 4 x 10^{-4}M	0.9	1.5	2.4	25	13	38
3E	4.0	Puromycin 4 x 10^{-4}M	0.8	0.6	1.4	29	6	35
4C	-	Actinomycin D 2 µg	42	52	94	1324	494	1818
4E	4.0	Actinomycin D 2 µg	96	15	112	955	297	1252

[a]Incubation: 8 hr with media containing 23.5 µc each of ^{3}H-Phe, Pro and Leu, plus GRF from the beginning.

[b]C - control groups; E - experimental groups; each flask contained eight pituitary halves.

in rats when assayed by Dr. Schally at doses of 1-2 μg, while the sample of GRF used in experiments reported in Table XVI was prepared from a different batch of porcine hypothalmi and was active at 10-100 ng. As is indicated in Tables XV and XVI, no difference was noted in the total amount of growth hormone or of general protein synthesis during the incubation periods with either sample of GRF used.

Of the nine experimental groups in Tables XIII-XVI receiving GRF but no antibiotic, an increase of growth hormone release into the media was noted in 5 cases (55%). Of the total 16 experimental groups receiving GRF, 8 groups (50%) showed an increased release of growth hormone into the media. With puromycin, an increase in growth hormone release occured in 3 cases (75%), while with actinomycin D a decrease occured in 2 cases (65%). The presence of puromycin from the beginning of the incubation caused a 98% inhibition of labeled amino acids incorporation into growth hormone and general protein, while with actinomycin D a 33% inhibition of general protein synthesis resulted (Table XV).

B. Biosynthesis of growth hormone and prolactin in a bovine pituitary cell-free system

1. Isolation procedure

The studies with slices had indicated that a reasonable yield of hormone could be obtained by modifying a large scale isolation procedure to the requirements of a tissue slice incubation (Table VII). Little was known of the effect of

TABLE XVI. EFFECT OF GRF ON BOTH THE SYNTHESIS AND RELEASE OF ^{3}H-LABELED, GROWTH HORMONE BY PITUITARIES OF COLD STRESSED RATS. II.[a]

Expt. No.[b]	GRF µg	Inhibitor	Incorporation of ^{3}H-amino acids					
			Growth Hormone cpm x 10^{-2}/mg			Protein cpm x 10^{-2}/mg		
			Tissue	Medium	Total	Tissue	Medium	Total
1C	-	-	84	67	151	2190	202	2392
1E	10	-	85	81	166	2311	158	2469
2C	-	-	91	45	136	2345	74	2419
2E	100	-	93	34	127	2383	93	2476
3C*	-	-	80	18	98	1750	58	1808
3E*	100	-	66	11	77	1532	42	1574
4C	-	Puromycin 4 x 10^{-4}M	1.1	0.8	1.9	36	15	51
4E	100	Puromycin 4 x 10^{-4}M	1.8	0.9	2.7	27	9	36

[a]Incubation: 3* and 7 hr with media containing 23.5 µc each of ^{3}H- Phe, Pro and Leu, plus GRF from the beginning.

[b]C - control groups; E - experimental groups; each flask contained eight pituitary halves.

applying this procedure to the subcellular components, present in a cell-free incubation system.

When a small amount of radioactive material is being isolated it is usually advisable to add unlabeled material to the mixture to act as a carrier during the isolation procedure. The recovery of counts from a pituitary cell-free system after adding various carrier to the incubation mixture prior to isolation of the hormones is shown in Table XVII. The addition of 10 mg of anterior pituitary powder gave the best recovery of counts in both hormone fractions and was used routinely in subsequent isolations. The presence of BSA alone or in combination with NIH-growth hormone and NIH-prolactin favored the recovery of growth hormone, but at the expense of prolactin. When only prolactin was isolated from the incubation mixture, the recovery was almost three times as much as when growth hormone was also isolated. However the greatest recovery of counts in the prolactin region was achieved when anterior pituitary powder was present.

Radioimmunoassays were carried out on aliquots of the initial incubation system with a known quantity of standard NIH-prolactin present, and on the final prolactin fraction, carried through the isolation procedure to the DEAE-cellulose stage. These determinations indicated the 43% of the prolactin detectable in the initial incubation mixture was recovered at the DEAE-cellulose step.

TABLE XVII. EFFECT OF CARRIER ON THE ISOLATION OF GROWTH HORMONE AND PROLACTIN FRACTIONS FROM CELL-FREE ANTERIOR PITUITARY SYSTEMS[a]

Carrier	Incorporation of ^{3}H-amino acids[b]		
	Growth Hormone CG-50	Prolactin DEAE-cellulose	Residual Protein
Control (no carrier)	1.87	1.11	7.6
10 mg BSA	2.22	1.00	10.6
10 mg anterior pituitary powder	3.08	3.38	23.4
0.5 mg NIH-LTH + 0.5 mg NIH-GH	2.37	1.79	15.3
10 mg BSA + 0.5 mg NIH-GH + 0.5 mg NIH-LTH	4.24	1.35	11.9
10 mg anterior pituitary powder + 0.5 mg NIH-GH + 0.5 mg NIH-LTH	2.36	3.43	24.2
10 mg BSA (prolactin only isolated)	-	2.96	13.8

[a]Pituitary ribosomes, pH 5 enzyme, cofactors, energy sources, and 10 μc each of ^{3}H-Leu and ^{3}H-Phe, per ml incubation mixture.

[b]% of counts in total protein recovered in the growth hormone, prolactin, and residual protein fractions.

Figure 14 shows the chromatography on Amberlite CG-50 of the growth hormone fraction isolated from the cell-free system. Compared with the same preparation from slices (Fig 1), there was large proportion of radioactivity present which was not absorbed onto the CG-50 resin. When the incubation mixture was treated with 20 μg RNAse after the incubation period, but prior to isolation of growth hormone (Fig 14B), the amount of label in this first fraction decreased, implying that the radioactive material was bound to RNA. The quantity of labeled substance in the growth hormone eluate (pH 6.0-phosphate buffer) was slightly less after the RNAse treatment.

The profile of crude prolactin from the cell-free system on DEAE-cellulose is given in Figure 15. Comparison with Figure 2 for slices indicates the similarity of the patterns in the two cases. Prolactin was isolated from the same RNAse-treated incubation tube that was used in the above-mentioned growth hormone experiment (Fig 15B). The second large peak absorbing in the ultraviolet, but not appreciably labeled, disappeared after treatment with RNAse, again suggesting the presence of nucleic acid. The number of counts in the prolactin region was approximately the same as that in the untreated preparation.

Table XVIII presents the variations observed between duplicate samples in isolating growth hormone and prolactin fractions from cell-free incubation mixtures. Similar variations were noted for both estimations; that is, 7.2% for

FIGURE 14. CHROMATOGRAPHY ON AMBERLITE CG-50 OF GROWTH HORMONE ISOLATED FROM A PITUITARY RIBOSOMAL SYSTEM.

(A) Partially purified growth hormone, after precipitation of crude growth hormone fraction by 50% saturation with $(NH_4)_2SO_4$ (Table III).

(B) Similar preparation as (A), but isolated from a cell-free incubation mixture treated with RNAse after the incubation period, but prior to isolation.

The arrows indicate a change from pH 5.1 to 0.3 M buffer of pH 6.0. Solid line, absorbance at 280 mμ, and broken line with X's, radioactivity.

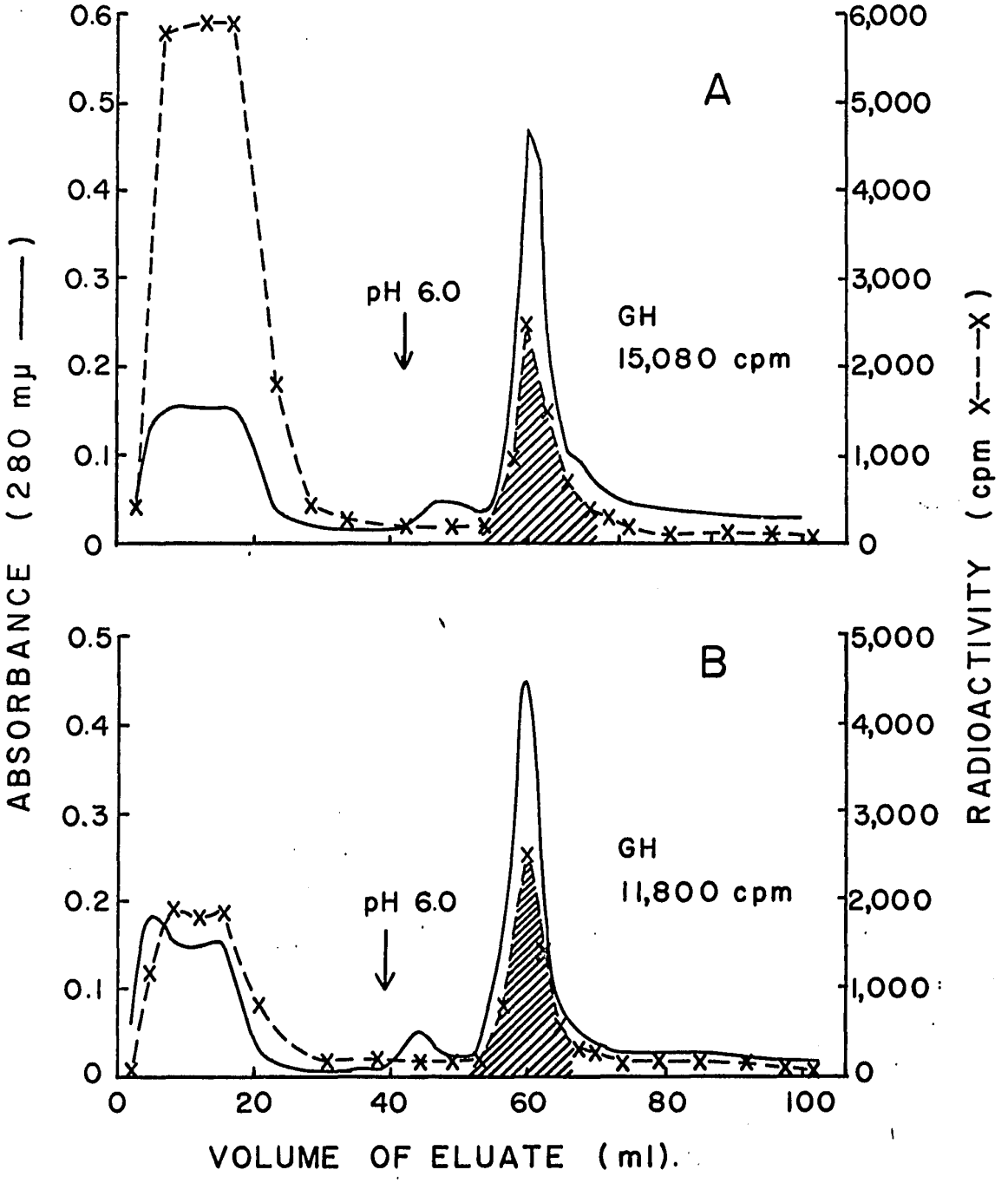

A
B
pH 6.0
pH 6.0
GH
15,080 cpm
GH
11,800 cpm
ABSORBANCE (280 mμ ——)
RADIOACTIVITY (cpm X—X)
VOLUME OF ELUATE (ml).
0.6
0.5
0.4
0.3
0.2
0.1
0
6,000
5,000
4,000
3,000
2,000
1,000
0
0
20
40
60
80
100

FIGURE 15. CHROMATOGRAPHY ON DEAE-CELLULOSE OF A PROLACTIN FRACTION ISOLATED FROM A PITUITARY RIBOSOMAL SYSTEM.

(A) Gradient elution pattern of DOC extract of a pituitary cell-free incubation mixture. The initial eluent was 0.025 M borate buffer (pH 8.4). The first arrow (40 ml) indicates the beginning of the linear gradient to 0.4 M NaCl. The second arrow (75 ml) indicates a change of gradient to 1 M NaCl.

(B) Gradient elution pattern of DOC extract, isolated from a pituitary cell-free incubation mixture treated with RNAse after the incubation period, but prior to isolation (same sample as 15(B)). Chromatographic conditions were as described in (A).

Solid line, abosrbance at 280 mμ, broken line with X's, radioactivity.

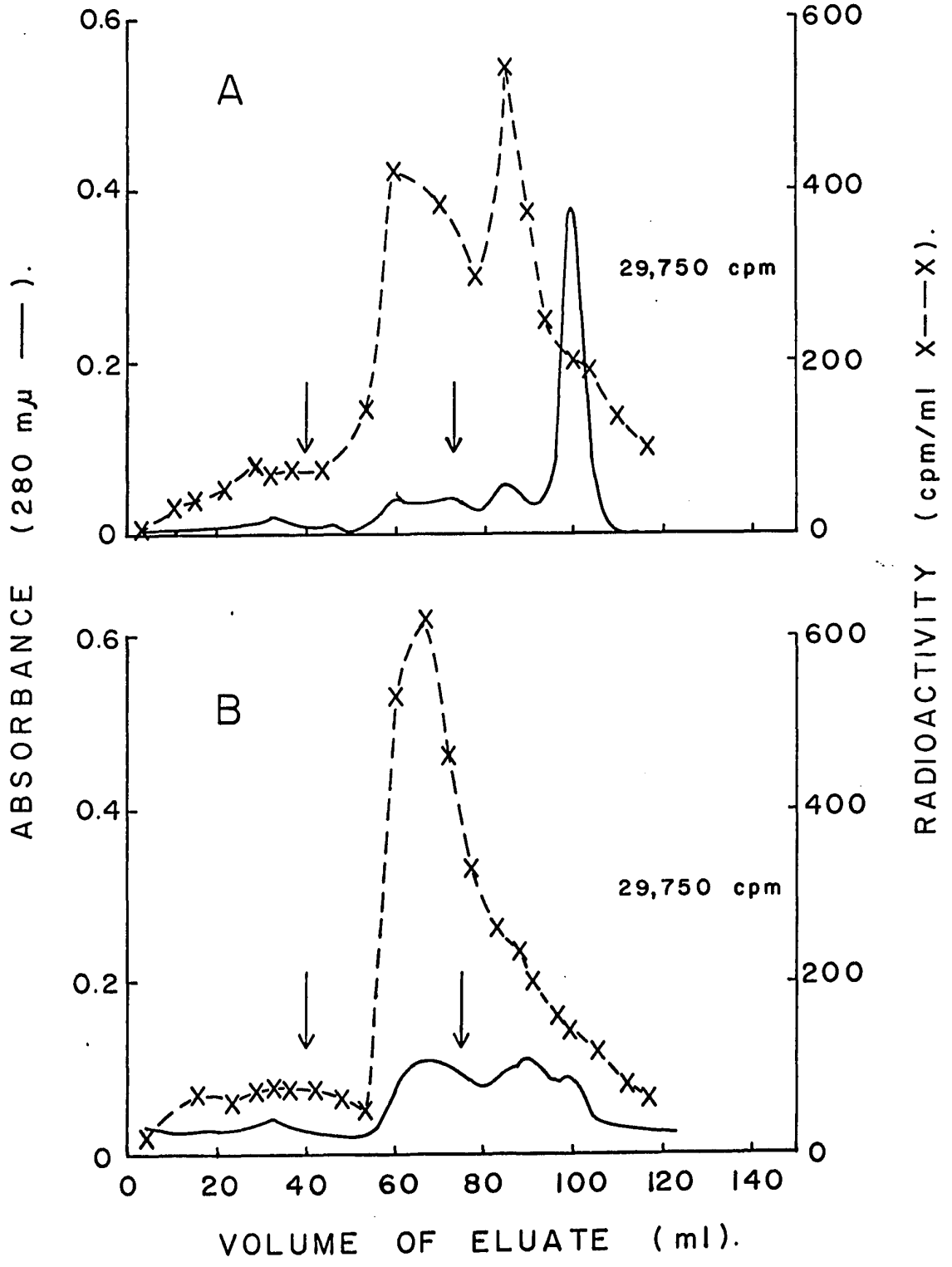

A
B
29,750 cpm
29,750 cpm
0.6
0.4
0.2
0
600
400
200
0
0 20 40 60 80 100 120 140
ABSORBANCE (280 mμ ——).
RADIOACTIVITY (cpm/ml X—X).
VOLUME OF ELUATE (ml).

TABLE XVIII. VARIATION BETWEEN DUPLICATE ESTIMATIONS OF PRODUCTS FROM CELL-FREE INCUBATIONS

Estimation	Variation between duplicate samples[a]		
	Mean %	% Range	No. of duplicate samples
Total protein	6.6	0.2 - 15.4	17
Residual protein	7.6	0.6 - 26.4	18
Growth hormone - CG-50	7.2	0.5 - 23.8	22
Prolactin - DEAE-cellulose	7.8	0 - 25.2	22

[a]Each estimation was made on duplicate samples from similar incubation mixtures. The total number of counts was determined for each sample, and the average between these two values calculated. The percentage by which the determined values varied from the average value was then calculated for each set of duplicates.

growth hormone and 7.8% for prolactin. In most cases the "residual protein" accounted for only 30% of the total protein.

Characterization studies to be reported in the following section have shown that a large portion of the radioactive material present in the CG-50 growth hormone fraction is indistinguishable from authentic growth hormone. However similar characterization studies with the DEAE-cellulose prolactin are most questionable. To date, the nature of the labeled fraction associated with the hormone has not yet been determined. In results to be reported in the following sections, radioactive material, separated by DEAE-cellulose chromatography, will be referred to as the prolactin-fraction since endogenous prolactin was isolated in this fraction.

2. Study of the components of the incubation mixture

a. Importance of energy sources and amino acid mixture

It may be seen (Table XIX) that omission of ATP resulted in marked decrease in hormone and protein biosynthesis. The omission of PEP, and PK resulted in only a slight to moderate reduction, indicating that these ATP generators are not particularly effective. Similar results were obtained when PK, PEP, and ATP were all omitted. Although the omission of GTP resulted in a 32% fall in protein biosynthesis, the addition of GTP did not seem to influence growth hormone or prolactin biosynthesis markedly. The presence of the pH 5 supernatant factor in this experiment may have influenced the results, since all the

TABLE XIX. IMPORTANCE OF ENERGY SOURCES AND AMINO ACID MIXTURE FOR BIOSYNTHESIS

Constituent omitted from standard incubation mixture[a]	Incorporation of ^{3}H-amino acids					
	Growth Hormone CG-50		Prolactin fraction DEAE-cellulose		Total Protein	
	cpm[b]	%[c]	cpm	%	cpm	%
Control (none)	8,700	100	22,200	100	222,580	100
ATP	1,702	20	2,610	12	18,820	8
PEP, PK	6,760	73	22,800	103	163,000	73
PEP, PK, ATP	1,690	20	2,720	12	23,000	10
GTP	7,740	89	18,800	85	151,800	68
Amino acid mixture	11,000	127	23,400	105	188,000	85

[a]The standard system contained 0.63 mg ribosomal RNA, 5.0 mg pH 5 enzyme-protein, 3.8 mg supernatant factor protein, and 10 μc each of ^{3}H-Phe in a total volume of 1 ml. The tubes were incubated for 45 min. at 37°.

[b]Values are expressed as cpm/ml incubation tube.

[c]Values are expressed as percent of the control.

GTP may not have been removed during the short dialysis period (2 hr) used in the preparation of this component. The removal of the amino acid mixture from the incubation mixture had no effect on the incorporation of labeled amino acids into prolactin, and even led to an increased incorporation into growth hormone. This observation may be related to the size of the amino acid pool, since it was found that maximum incorporation was reached in the presence of 30 μc of the ^{3}H-amino acids.

b. Role of the subcellular constituents

For ease of comparison, Experiment 1 of Table XX, containing ribosomes and pH 5 enzyme in the standard incubation media, was taken as the standard. The incorporation of labeled amino acids into both hormones was highest in this system. Addition of the pH 5 supernatant factor had little or no effect on the incorporation of isotope into growth hormone, but caused an approximately 20% decrease into the prolactin fraction and a 10 to 20% increase in the incorporation into total protein. When unfractionated pH 5 supernatant was included (Experiment 3), growth hormone and protein biosynthesis was reduced by more than 50%, and the incorporation into prolactin fraction by more than 70%. Substitution of the original 105,000 x g supernatant for the pH 5 enzyme (Experiment 4) caused an approximately 80% reduction in synthesis of all three fractions. However, if this supernatant was passed through

TABLE XX. IMPORTANCE OF PITUITARY SUBCELLULAR COMPONENTS FOR GH, LTH, AND PROTEIN BIOSYNTHESIS

Expt. No.	Ribosomes	pH 5 enzyme	pH 5 supt. factor[a]	pH 5 supt.[b]	Whole supt.[c]	Cell sap[d]	^{3}H-amino acid incorporation (% of control) Growth Hormone %	S.D.	Prolactin %	S.D.	Total Protein %	S.D.
1 control	+	+	-	-	-	-	100		100		100	
2	+	+	+	-	-	-	91	± 10	77	± 9	110	± 14
3	+	+	-	+	-	-	42		26		45	
4	+	-	-	-	+	-	16		19		14	
5	+	-	-	-	-	+	40		53		49	
6	+	-	-	-	-	-	9	± 8	19	± 10	10	± 4
7	-	-	-	-	-	+	10		9		13	
8	-	+	-	-	-	-	15	± 4	11	± 1	8	± 1
9	-	-	+	-	-	-	6		10		7	
10	+	-	+	-	-	-	40	± 17	99	± 21	52	± 23

[a] pH 5 supernatant factor - precipitated from pH 5 supernatant by 65% $(NH_4)_2SO_4$, 3.8 mg protein.

[b] pH 5 supernatant - 5.8 mg protein.

[c] Whole supernatant - 105,000 g supernatant before G-25 filtration, 5.6 mg protein.

[d] Cell sap - 105,000 g supernatant after G-25 filtration, 6.4 mg protein.

a column of Sephadex G-25 prior to addition to the incubation mixture (Experiment 5), the amount of incorporation was increased in all cases, but still reached only 50% of the control value. These experiments, in agreement with the observations of others (Munro, et al. 1964, Hussa, 1968) suggest that a inhibitory factor, present in the 105,000 g supernatant (Experiment 4), can be largely removed by gel filtration (Experiment 5). The remaining inhibitor was still present in the pH 5 supernatant (Experiment 3), but was not appreciably precipitated by 65% $(NH_4)_2SO_4$. Ribosomes alone had low activity (Experiment 6). In the absence of ribosomes neither the whole supernatant (Experiment 7) nor its component fractions, pH 5 enzyme (Experiment 8) or pH 5 supernatant factor (Experiment 9) exhibited significant biosynthetic activity. However, the addition of the pH 5 supernatant factor to ribosomes resulted in a sizable incorporation of ^3H-amino acids into the hormone fractions. In the case of prolactin, this effect was equivalent to that in the standard system. Apparently the pH 5 supernatant factor contained components (transferases, synthetases, etc.) similar to those present in the pH 5 enzyme.

c. Effect of inhibitors

Two types of evidence supporting the view that the hormones have synthetic mechanisms similar to those for general proteins, are presented in Table XXI. The incorporation of labeled amino acids was inhibited in each

TABLE XXI. INHIBITION OF GROWTH HORMONE, PROLACTIN, AND PROTEIN BIOSYNTHESIS BY PUROMYCIN AND RNAse

Inhibitor	Growth Hormone CG-50		Prolactin DEAE-cellulose		Total Protein	
	cpm[a]	%[b]	cpm	%	cpm	%
Control[c] (none)	9,100	100	9,520	100	203,000	100
Puromycin (100 µg)	2,220	24	1,310	14	29,380	15
RNAse (20 µg)	2,420	27	860	9	50,670	25

[a]Values are expressed as cpm/ml incubation tube.

[b]Values are expressed as a percent of the control.

[c]Standard incubation mixture containing 0.53 mg ribosomal RNA, 5.0 mg pH 5 enzyme-protein, and 10 µc each of ^{3}H-Leu and ^{3}H-Phe per ml. Incubation time was 40 min. at 37°.

case by puromycin. Similarly, synthesis was invariably depressed by the addition of RNAse.

3. Optimum conditions for biosynthesis

Although studies in this laboratory had established the optimal conditions for biosynthesis of ACTH and protein in a cell-free system from bovine anterior pituitary tissue (Adiga, et al., 1966), it was considered desirable and pertinent to conduct similar experiments for prolactin and growth hormone synthesis.

a. Ribosome concentration

The relationship between the concentration of ribosomes and the biosynthetic activity is presented in Figure 16. The incorporation of labeled Leu and Phe into protein and prolactin continued to rise even at a level of 3 mg of r-RNA/ml of incubation mixture. The protein result is in contrast to that of Adiga, et al. (1966), who observed that incorporation of labeled proline into protein approached a maximum value at a level corresponding to about 2 mg of r-RNA. Our results may reflect the shorter incubation time (30 min, in contrast to 60 to 90 min used by Adiga, et al.) and also the different labeled amino acids used. Leu and Phe are usually more abundant in proteins than is proline. A two fold increase in the concentration of ribosomes did not result in a doubling of the incorporation of isotope into the hormones on protein. The quantity of isotope present in growth hormone

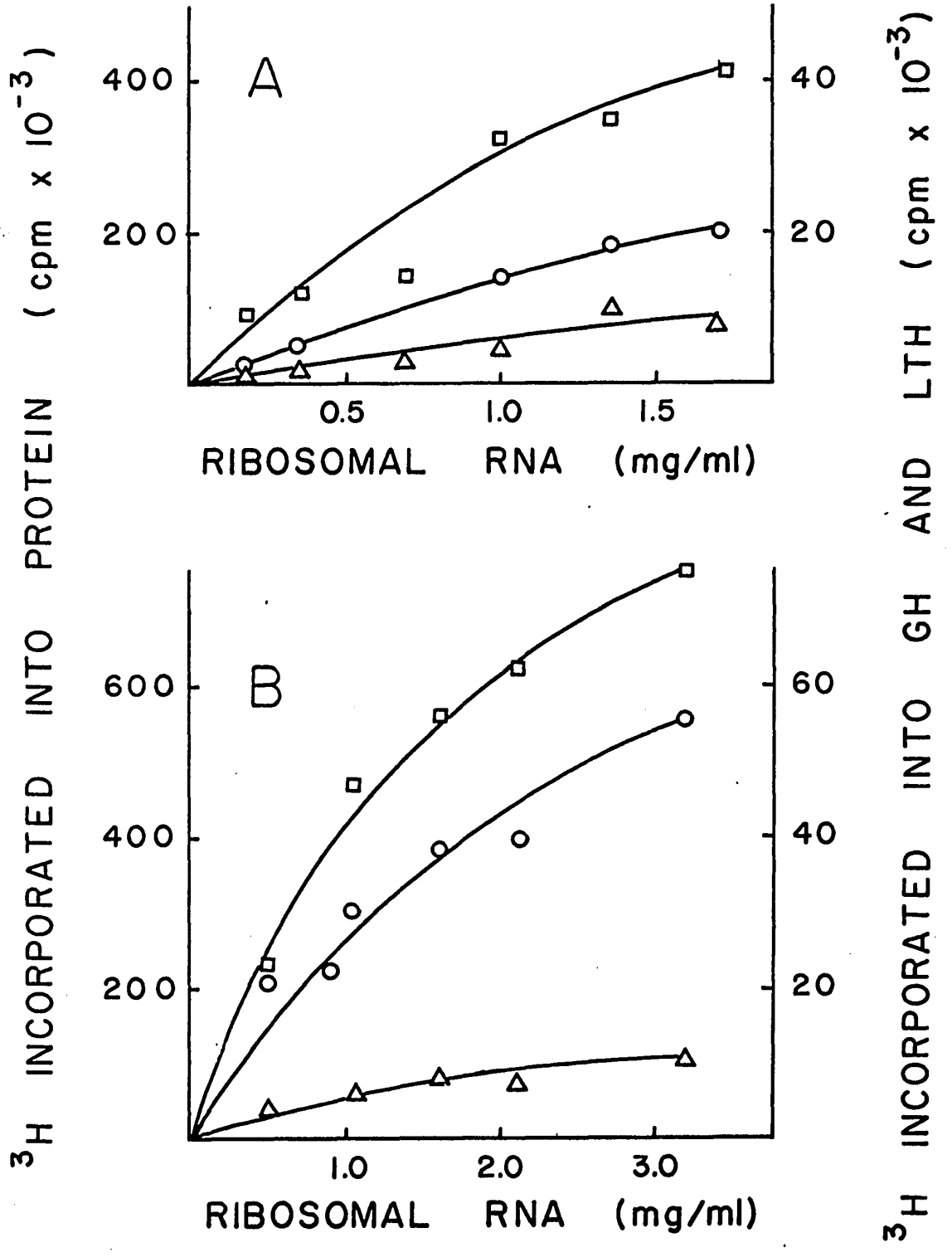
FIGURE 16. RELATIONSHIP OF RIBOSOMAL CONCENTRATION TO THE EXTENT OF INCORPORATION OF LABELED AMINO ACIDS INTO GROWTH HORMONE, PROLACTIN AND PROTEIN FRACTIONS IN A PITUITARY CELL-FREE SYSTEM.

Two incubation times were studied:

(A) 60 minutes with 5.0 mg of pH 5 enzyme protein and 10 μc each of ^{3}H-Phe and ^{3}H-Leu/ml of standard incubation mixture.

(B) 30 minutes with 3.2 mg of pH 5 enzyme protein and 10 μc each of ^{3}H-Phe and ^{3}H-Leu/ml of standard incubation mixture.

Triangle, growth hormone; square, prolactin fraction, isolated to the CG-50 and DEAE-cellulose stage, respectively; circle, total protein.


A
400
200
40
20
0.5
1.0
1.5
RIBOSOMAL RNA (mg/ml)
^{3}H INCORPORATED INTO PROTEIN (cpm x 10^{-3})
^{3}H INCORPORATED INTO GH AND LTH (cpm x 10^{-3})
B
600
400
200
60
40
20
1.0
2.0
3.0
RIBOSOMAL RNA (mg/ml)

represented 2% of that in the total protein when 0.5 to 1.5 mg of RNA was present and dropped only slightly to 1.8% at higher ribosome concentration (2-3 mg).

b. Optimum Mg^{++} level

The sensitivity of the biosynthetic process to Mg^{++} concentration is seen in Figure 17A. The curves for the synthesis of the two hormones and protein appear rather similar, all with optimal Mg^{++} concentration in the vicinity of 8 to 10 μ moles/ml. Adiga, et al. (1966) reported an optimal of 10 to 12 μ moles/ml, for both ACTH and protein. A comparison was made of the optimal Mg^{++} concentrations for pituitary and liver ribosomal systems (Fig 17B). The requirements for Mg^{++} were found to be slightly higher for pituitary system (8 to 10 μ moles/ml) than for a liver system (6 to 8 μ moles/ml), prepared under identical conditions.

c. pH 5 enzyme

The effect of varying the concentration of pH 5 enzyme fraction is shown in Figure 18. This variation was studied at two levels of ribosomal RNA. Incorporation of labeled Phe and Leu into growth hormone and prolactin was found to be optimal at 4 to 5 mg and 2 to 3 mg of pH 5 protein/ml, respectively. The rate of biosynthesis of total protein was most rapid at 5 mg of pH 5 protein/ml (data not included). The peaks were somewhat sharper for the prolactin fraction as compared to the growth hormone and "residual protein"

FIGURE 17. INFLUENCE OF MAGNESIUM CONCENTRATION ON THE INCORPORATION OF LABELED AMINO ACIDS IN A RIBOSOMAL SYSTEM.

(A) Optimal Mg^{++} concentration for the biosynthesis of growth hormone, prolactin and protein in a pituitary ribosomal system. The tubes contained 0.84 mg r-RNA, 5.8 mg pH 5 enzyme protein and 10 μc each of ^{3}H-Phe and ^{3}H-Leu/ml incubation mixture. They were incubated for 1 hr at 37°C.

Triangle, growth hormone; square, prolactin fraction; circle, total protein.

(B) Comparison of the optimal Mg^{++} concentration for pituitary and liver ribosomal systems. The liver system contained 0.94 mg r-RNA, 6.8 mg pH 5 enzyme protein/ml incubation mixture and the pituitary system was described in (A). Incubations were conducted on one quarter the standard scale, that is, 0.25 ml final incubation mixture.

Open circle, total pituitary protein; closed circle, total liver protein.

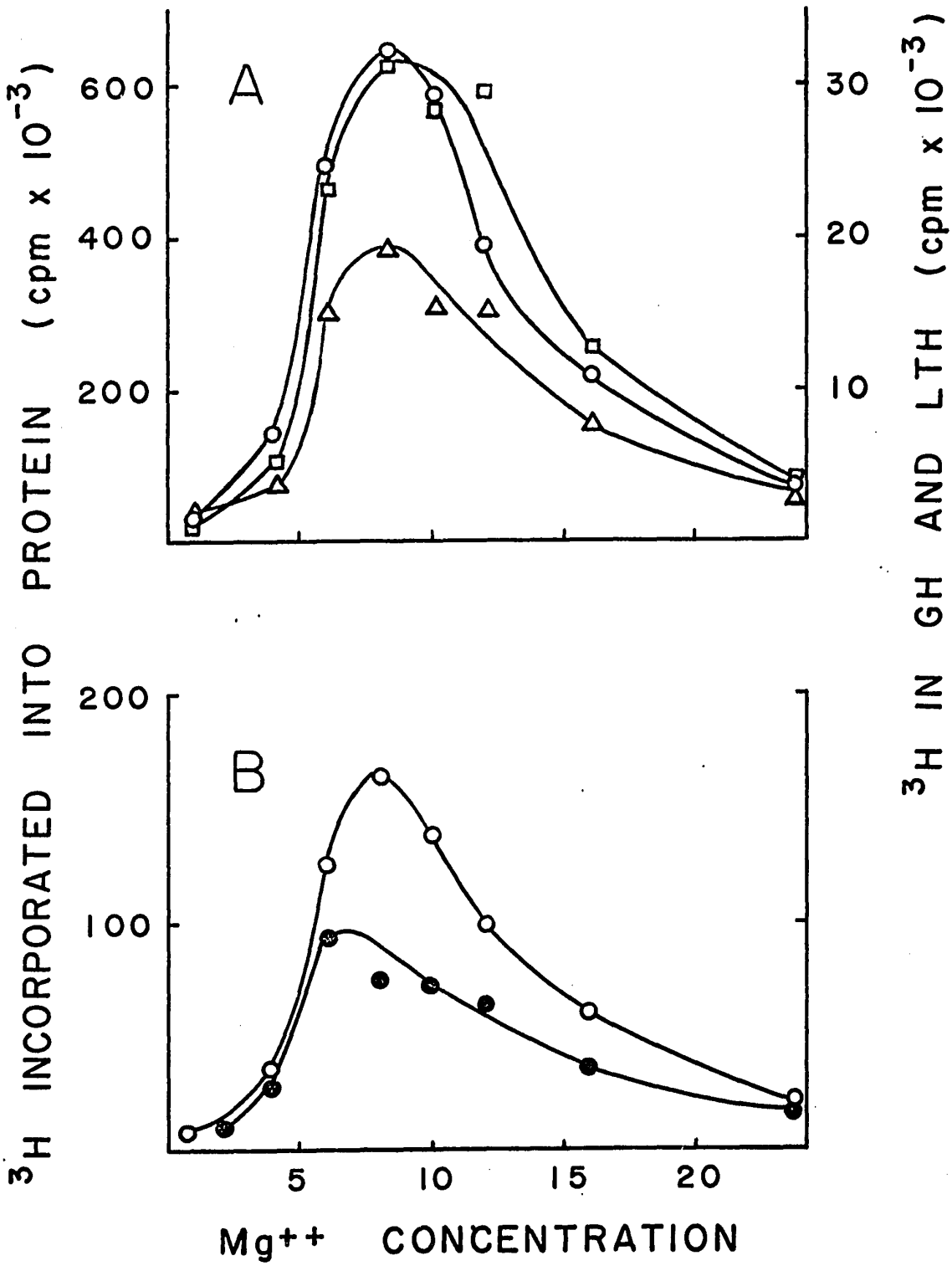

A
B
600
400
200
30
20
10
200
100
5
10
15
20
^{3}H INCORPORATED INTO PROTEIN (cpm x 10^{-3})
^{3}H IN GH AND LTH (cpm x 10^{-3})
Mg++ CONCENTRATION

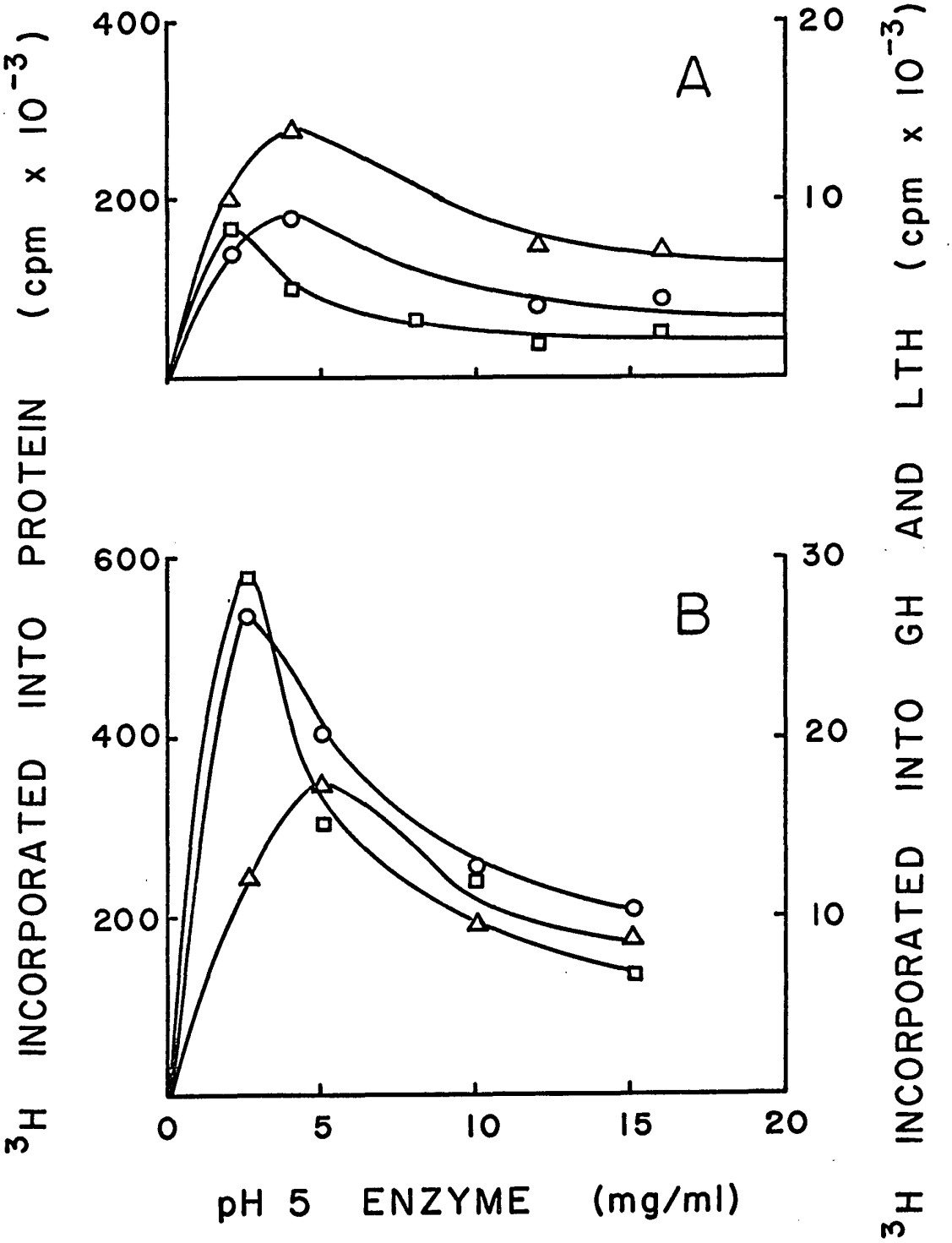
FIGURE 18. EFFECT OF VARYING QUANTITIES OF pH 5 ENZYME ON THE BIOSYNTHESIS OF GROWTH HORMONE, PROLACTIN AND PROTEIN IN A PITUITARY CELL-FREE SYSTEM.

Two concentrations of ribosomal-RNA were studied. Incubations were for 1 hr at 37°C with:

(A) 0.2 mg r-RNA and 10 μc each of ^{3}H-Leu, ^{3}H-Phe and ^{3}H-Pro/ml incubation mixture, and

(B) 0.86 mg r-RNA and 10 μc each of ^{3}H-Leu, and ^{3}H-Phe/ml incubation mixture.

Triangle, growth hormone; square, prolactin fraction; circle, "residual protein."


400
200
20
10
A
600
400
200
30
20
10
B
0
5
10
15
20
pH 5 ENZYME (mg/ml)
^{3}H INCORPORATED INTO PROTEIN (cpm x 10^{-3})
^{3}H INCORPORATED INTO GH AND LTH (cpm x 10^{-3})

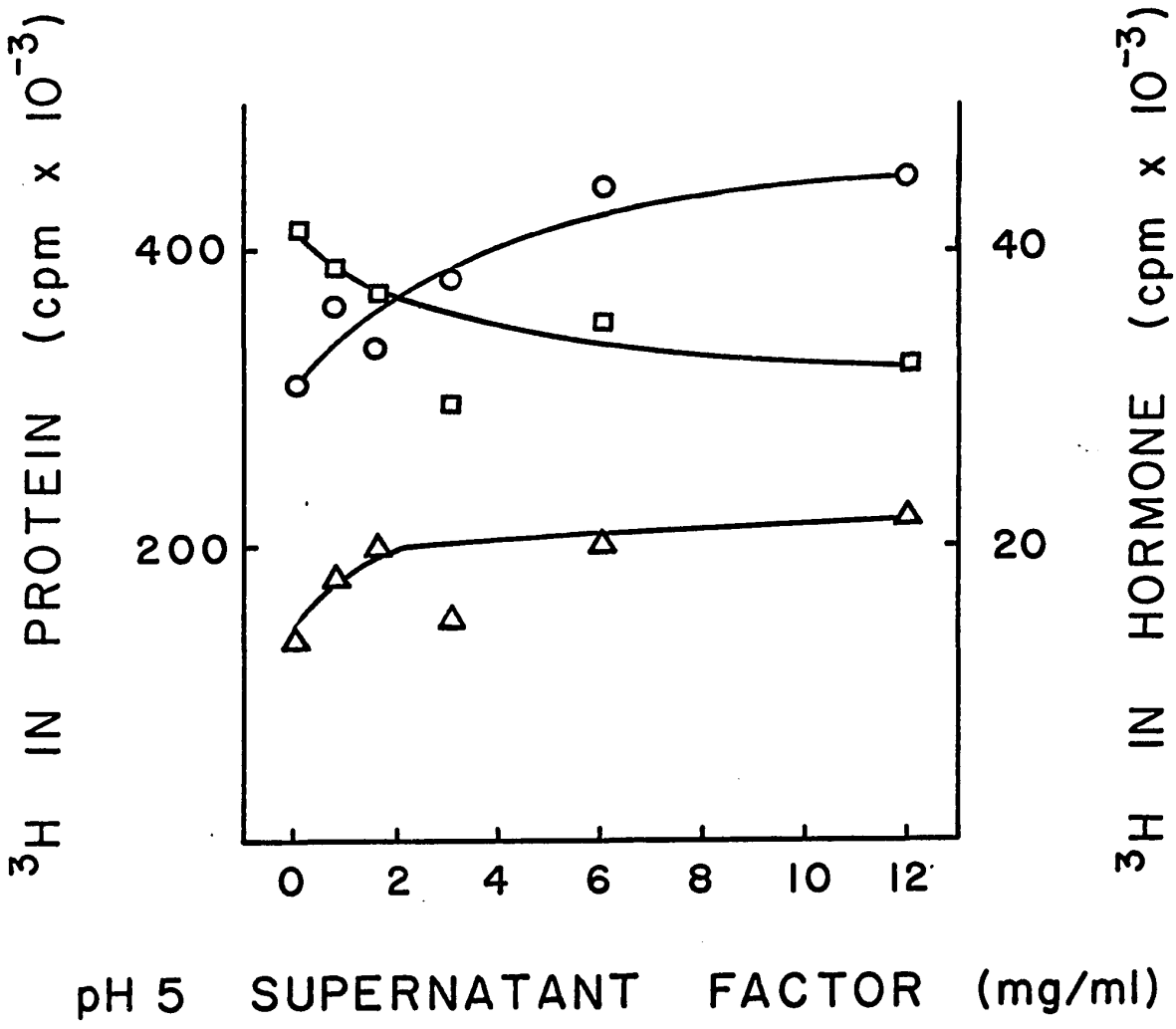
estimations. The fall in activity at higher levels probably reflected the inhibitory effect of some supernatant phase, which may have adhered to the (unwashed) pH 5 protein. Adiga, et al. (1966) noted an optimal effect of 1 mg of protein/ml for ACTH, and 2 to 3 mg/ml for protein synthesis. Gospodarowicz and Laporte (1968) did not determine an optimal level of supernatant protein in their pituitary polysomal-200,000 g_{max} supernatant system but demonstrated stimulation of ^{3}H-amino acid incorporation in the presence of 4 mg supernatant protein/ml. The difference in amino acid content of ACTH and GH may account for this difference in pH 5 enzyme requirements, since the level of certain transferases and s-RNA may be limiting. However, since LTH and GH contain all amino acid residues, the difference in requirements between these proteins are very surprising. This may reflect a difference in regulatory mechanisms or that the protein in the prolactin fraction is not the same molecular weight as that in the growth hormone fraction.

d. pH 5 supernatant factor

The effect of the addition of the pH 5 supernatant factor to a pituitary ribosomal-pH 5 enzyme incubation mixture is shown in Figure 19. Incorporation into growth hormone was stimulated 1.5 times by the addition of 2 mg of pH 5 supernatant protein/ml and then leveled off. On the other hand, incorporation into prolactin showed the reverse

FIGURE 19. EFFECT OF VARYING QUANTITIES OF pH 5 SUPERNATANT FACTOR ON THE EXTENT OF GROWTH HORMONE, PROLACTIN AND PROTEIN BIOSYNTHESIS IN A PITUITARY CELL-FREE SYSTEM.

The tubes contained 0.45 mg r-RNA, 4.5 mg pH 5 enzyme protein and 20 μc ^{3}H-Phe/ml incubation mixture. They were incubated for 1 hr at 37°C. Triangle, growth hormone; square, prolactin fraction; circle, total protein.


^{3}H IN PROTEIN (cpm x 10^{-3})
400
200
^{3}H IN HORMONE (cpm x 10^{-3})
40
20
0
2
4
6
8
10
12
pH 5 SUPERNATANT FACTOR (mg/ml)

affect, and decreased about 10%. The greatest response to this factor was in the labeling of total protein. Here a stimulation of synthesis took place until approximately 6 mg protein/ml had been added. The results presented in Table XX, Experiment 2, had indicated that the pH 5 supernatant factor could cause a reduction, as well as a stimulation of incorporation of isotopic amino acids into growth hormone. Stimulation occurred only when the concentration of pH 5 enzyme was less than 5 mg/ml. This is explicable if the pH 5 supernatant factor is contributing essentially the same components necessary for the synthesis of growth hormone as those present in the pH 5 enzyme. Addition of pH 5 supernatant factor to a suboptimum level of pH 5 enzyme increases the concentration of these components to an optimum level. In this case a stimulation is observed. When the pH 5 supernatant factor is added to an already optimum level of pH 5 enzyme (i.e. 5 mg/ml) a reduction is noted.

e. Time of incubation

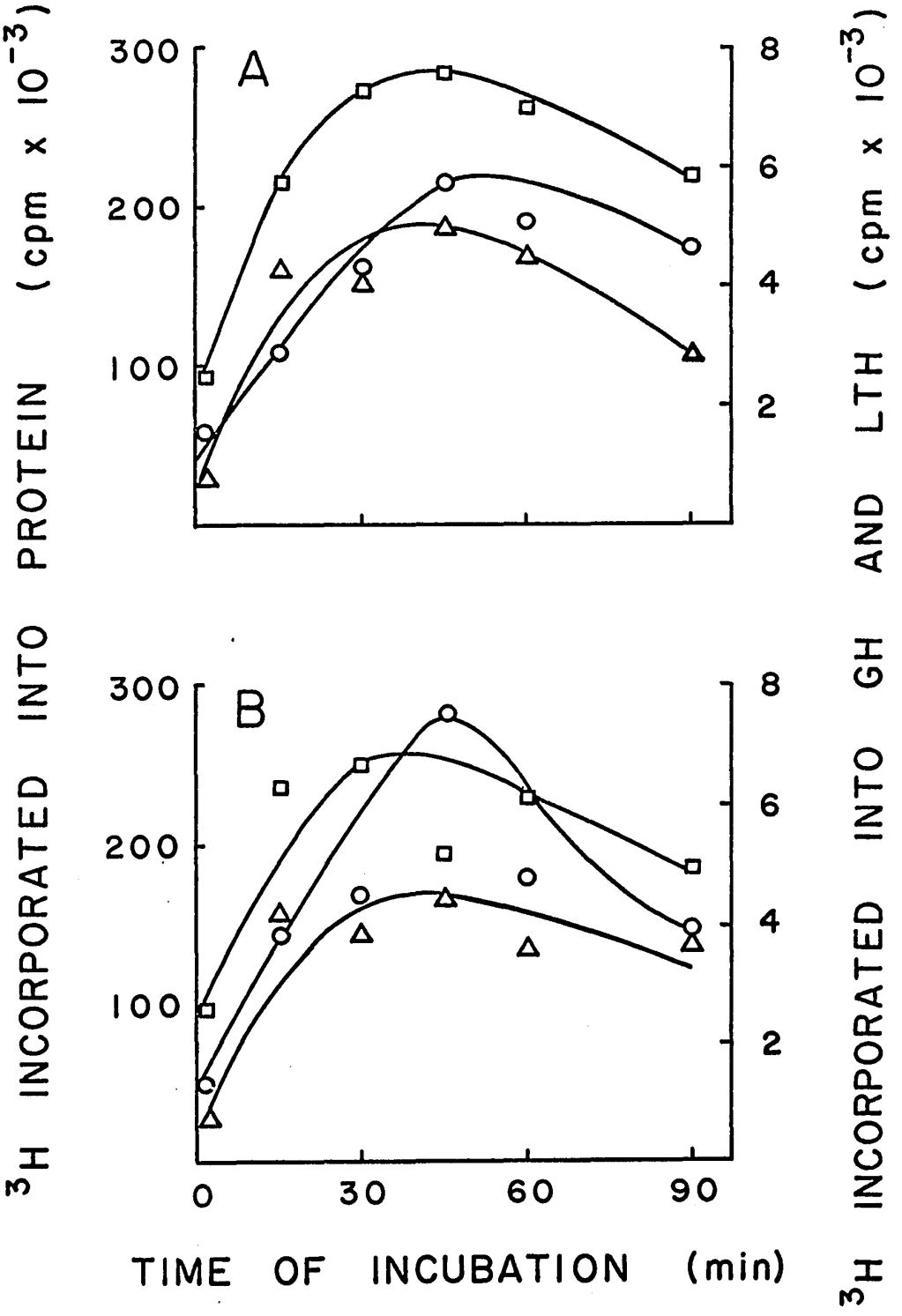
Although a 1 hr incubation was used for much of the earlier work with ribosomal systems, subsequent experiments with an optimal concentration of pH 5 enzyme indicated that a shorter reaction time was preferable. In Figure 20A it may be seen that the incorporation of isotopic amino acids into growth hormone and prolactin was initially rapid, reaching a maximum at 30 to 45 min, and then decreasing over

FIGURE 20. TIME COURSE FOR GROWTH HORMONE, PROLACTIN AND PROTEIN BIOSYNTHESIS IN A PITUITARY CELL-FREE INCUBATION MIXTURE.

(A) The tubes contained 0.22 mg r-RNA, 4.9 mg pH 5 enzyme protein and 10 μc each of ^{3}H-Leu, ^{3}H-Phe and ^{3}H-Pro/ml standard incubation mixture.

(B) Experimental conditions were as described in (A), each tube contained in addition 3.1 mg of pH 5 supernatant factor.

Triangle, growth hormone; square, prolactin fraction; circle, total protein.


A
B
300
200
100
8
6
4
2
0
30
60
90
^{3}H INCORPORATED INTO PROTEIN (cpm x 10^{-3})
^{3}H INCORPORATED INTO GH AND LTH (cpm x 10^{-3})
TIME OF INCUBATION (min)

longer time interval. In the case of protein, the rate of labeling reached a maximum slightly later (45 to 60 min), and thereafter declined. When the pH 5 supernatant factor was added to this system (Fig 20B) there was essentially no change in the rate or optimum time for growth hormone and prolactin synthesis. The rate of decrease in growth hormone synthesis after 45 min appeared to decline more slowly, suggesting that factors in the pH 5 supernatant factor were reducing the breakdown of growth hormone. As was anticipated from other experiments, the presence of the pH 5 supernatant factor stimulated the rate of incorporation into protein. The maximum was still at 45 min, while the subsequent decline was more abrupt. Adiga, et al. (1966) reported that the rate of incorporation of ^{3}H-proline into protein in their comparable system did not depart greatly from linearity over a 1.5 hr period of incubation. The present results, and those obtained in other experiments (data not included) indicated that the rate of incorporation of ^{3}H-Phe and ^{3}H-Leu into protein declined after a 60 min incubation period. With the pituitary polysomal system of Gospodarowicz and Laporte (1968) incorporation of ^{3}H-proline into proteins reached a maximum level after 45 minutes in the presence of 4 mg supernatant protein. With lower levels of supernatant protein, 0.5, 1.0 and 1.35 mg the rate of incorporation was slower, still showing an increase after 90 min. No decline was noted.

4. Biosynthetic efficiency of the pituitary ribosomal system

In Table XIX, the values of 8,700 cpm for growth hormone and 22,200 cpm for prolactin represent the utilization of approximately 0.1 and 0.3% respectively, of the total radioactivity employed. The 222,580 cpm recovered in the protein fraction correspond to the utilization of 2.8% of the labeled amino acids for protein synthesis. These efficiencies were found to vary somewhat between experiments but, as indicated in Table XXII-A are typical of the range of values found. If the label in the hormone fractions is expressed as a percent of the radioactivity recovered as total protein (Table XXII-B), growth hormone and prolactin accounted for 3.5 and 7.0% respectively, of the total protein synthesized. Not surprisingly these values are lower than those obtained for slices after a 4 hour incubation period when growth hormone and prolactin account for 10 and 18% respectively of the total protein synthesized (Table IX). The ability of the pituitary ribosomal preparation to incorporate amino acids into total protein was compared with similar systems prepared from bovine liver, brain and kidney tissues (data not included). Based on the RNA content, thepituitary system was approximately 1.7 and 3.5 times as active as the liver and kidney, respectively. However, ribosomes prepared from the brain were 1.6 times as active as those from the pituitary.

5. Biosynthetic activity of pituitary polysomes

a. Polysome-enriched ribosome preparation

TABLE XXII. EFFICIENCY OF INCORPORATION OF ^{3}H-AMINO ACIDS IN A PITUITARY CELL-FREE INCUBATION SYSTEM[a]

A. Percent of total counts in incubation mixture recovered in each fraction[b]

Estimation	Mean	Range	No. of samples
Total protein	3.60	2.0 - 6.0	11
Growth hormone	0.11	0.04 - 0.17	11
Prolactin	0.24	0.06 - 0.37	11

B. Percent of counts in total protein recovered in hormone fractions[c]

Estimation	Mean	Range	No. of samples
Growth hormone (CG-50)	3.5	1.6 - 7.1	9
Prolactin (DEAE-cellulose)	7.0	2.4 - 13.2	9

[a]Conditions were optimal for pH 5 enzyme (5 mg/ml) and Mg^{++} concentration, but the quantity of ribosomes varied.

[b]The number of counts recovered in each fraction was expressed as a percentage of the total number of counts originally present in the incubation mixture.

[c]The number of counts recovered in each hormone fraction was expressed as a percentage of the number of counts recovered in total protein estimations carried out on similar samples.

Many reports have implicated polysomes, orderly aggregates of ribosomes linked together by a strand of m-RNA, as the active structures for protein synthesis. Sedimentation of a DOC-treated microsome fraction through a denser sucrose solution, 2 M instead of the usual 1 M, removed some of the lighter monosomes and resulted in a polysome-enriched fraction. Comparison of this preparation with the usual mixed ribosomes (Table XXIII) showed that the polysomes had approximately 2.5 times the amino acid incorporating ability of the ribosomes for hormone and general protein synthesis. However, the yield in terms of RNA, of polysome-enriched ribosomes was only 20% that of the mixed ribosome fraction, and for this reason the mixed ribosome preparation was used routinely.

b. Polysome profile on sucrose density gradients

Resolution of the polysome preparation by this method is shown in Figure 21-A. Monosomes were the predominant component and represented (on the basis of r-RNA content) about 35-40% of the material resolved on the gradient, and 25-30% of the total material applied. Seven heavier entities (ranging up to octasomes) were detected on the gradient profile as UV-absorbing peaks (numbered 2-8). The small peak (s) preceding the monosome region may correspond to the native 55s peak observed in schlieren patterns by Adgia and Hussa (1968a). It is possible that the polydisperse region at the bottom of the gradient

TABLE XXIII. COMPARISON OF THE BIOSYNTHETIC ACTIVITY OF PITUITARY POLYSOME-ENRICHED RIBOSOMES AND MIXED RIBOSOMES

Preparation	Incorporation of ^{3}H-amino acids (cpm)[a]		
	Growth Hormone CG-50	Prolactin DEAE-cellulose	Total Protein
Polysome-enriched (2M)[b]	3,550 ± 130	7,800 ± 300	103,500 ± 7,800
Mixed ribosomes (1M)[c]	1,380 ± 120	3,560 ± 300	35,400

[a]Values are the average of duplicate estimations and expressed per 100 µg RNA.

[b]Prepared by sedimenting the DOC treated microsomes through a discontinuous sucrose gradient (2.5 ml of 0.5 M sucrose in Medium M, layered over 3.0 ml of 2.0 M sucrose in Medium M).

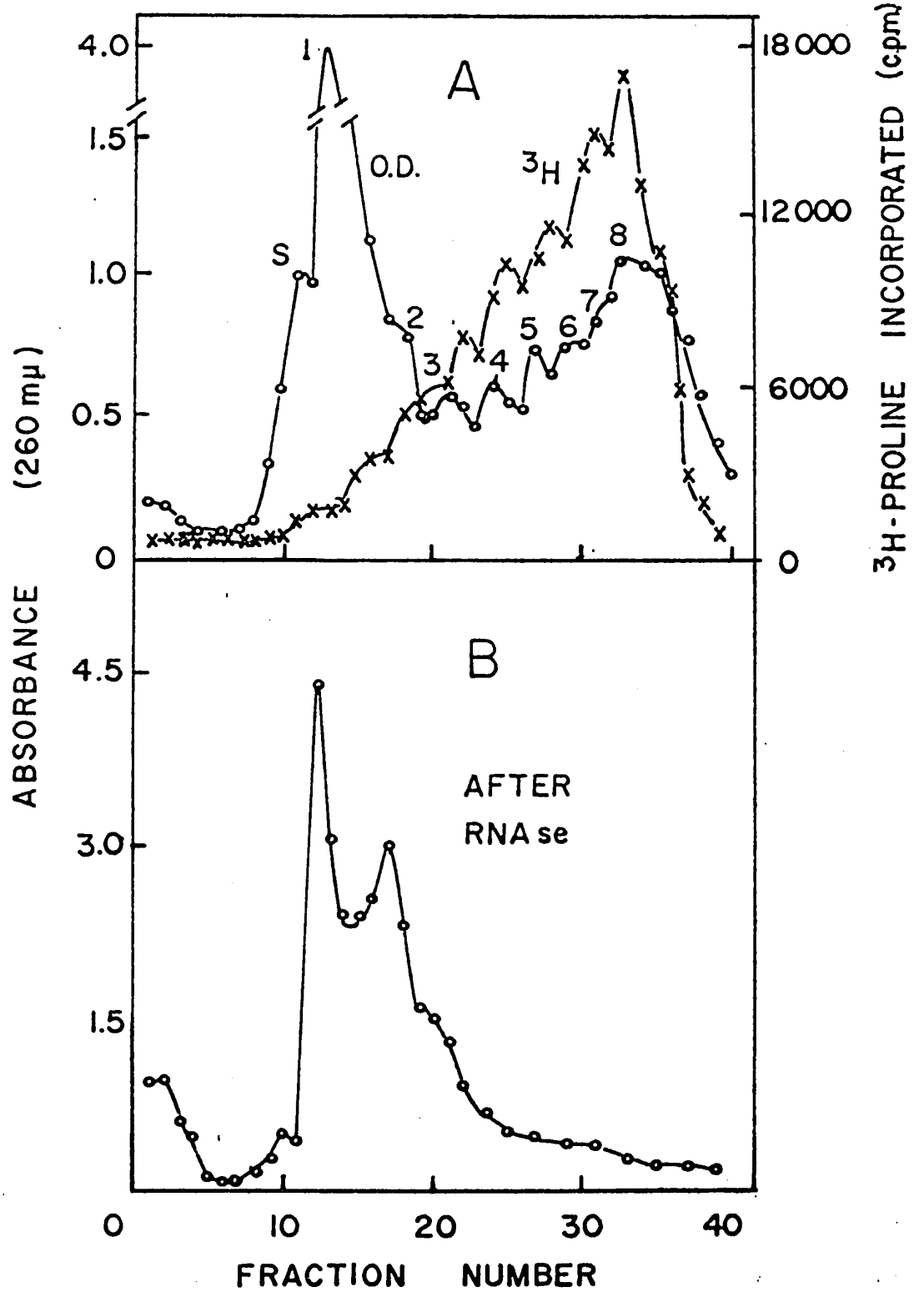
[c]Prepared by sedimenting the DOC treated microsomes through 5.5 ml of 1M sucrose in Medium M.

FIGURE 21. RESOLUTION ON SUCROSE DENSITY GRADIENT OF PITUITARY POLYSOMES, BEFORE AND AFTER RNAse TREATMENT.

(A) Biosynthetic activity of individual fractions.

(B) Sucrose gradient pattern after preliminary treatment of polysome preparation (0.7 mg r-RNA) with 1 μg of crystalline pancreatic RNAse for 5 minutes at 0°C.

Centrifugation was for 270 minutes at 25,000 rpm on linear 15-30% sucrose gradients in Medium M minus β-mercaptoethanol. The SW 25.1 swinging bucket rotor was used (Adiga, et al., 1968b).


A
B
O.D.
^{3}H
S
1
2
3
4
5
6
7
8
4.0
1.5
1.0
0.5
0
18000
12000
6000
0
ABSORBANCE (260 mμ)
^{3}H-PROLINE INCORPORATED (cpm)
AFTER
RNAse
4.5
3.0
1.5
0
10
20
30
40
FRACTION NUMBER

represented aggregation due to neutralization of ribosomal charges by the high ionic environment (Peterman, 1964, 1967).

In order to test the biosynthetic ability of the isolated polysomes, fractions taken along the entire sucrose gradient were individually incubated for one hr in the complete amino acid-incorporating system (containing ^{3}H-proline), and subsequently the radioactivity associated with the isolated protein preparations was measured. Figure 21A shows a progressive increase in the labeling of protein with increasing aggregate size up to peak 8, followed by a decline at the heaviest region. Very little, if any, isotope appeared in the monosome peak.

When specific incorporative activity (cmp in protein/mg r-RNA) was compared with polysome size, a similar pattern was obtained, except that the labeling reached a plateau at peak 7 (data not included).

c. Effect of RNAse on polysome stability

To rule out the possibility that the heavier ribosomal species fractionated on the gradient were contaminated with random aggregates of monosomes, the effect of a minute quantity of RNAse on the polysome profile was tested. Figure 21B shows that an almost complete breakdown of heavier aggregates occurred, with a concomitant increase in monomers, dimers, and some trimers. In addition, an increase in UV-absorbing material was found at the top of the gradient, equivalent to about 2-3% of the total input.

This component probably reflected nucleotides released by the RNAse action. The small amounts of dimers and trimers remaining after RNAse action is in accord with the results of Manner, et al. (1967), and may reflect the presence of RNAse-insensitive types of bonds (Peterman, 1967).

d. Polysome size and hormone synthesis

The current hypothesis is that the maximum aggregate size of a functional polysome should be compatible with that of the synthesized protein (Rich, et al., 1963). It was of considerable interest, therefore, to measure the incorporation of labeled amino acids into the two hormones (of molecular weight 21,000-26,000) under the influence of polysome aggregates of different sizes. For this purpose, the corresponding fractions from three separate sucrose gradients were pooled, in order to provide adequate material. The data in Table XXIV, Experiment 1, show that the radioactivity recovered in growth hormone, prolactin and residual protein fraction rose progressively with increasing size of the polysome cluster, reaching a maximum in the case of the hormones with polysomes containing six to seven ribosomal units. Thereafter the quantities of isotope declined. In experiment 2, Table XXIV (employing radioactive leucine, proline and phenylalanine simultaneously), in which labeling of total protein was measured, highest specific incorporative activity (cpm in protein/mg RNA) was again found in hexamers to heptamers, followed by a decline in synthesis above this region.

TABLE XXIV. HORMONE AND PROTEIN SYNTHESIS WITH POLYSOME FRACTIONS OF VARYING SIZE

Tube No.[b]	Polysome aggregate number	Incorporation of ^{3}H-amino acids[a]			
		Expt. 1[c]			Expt. 2[d]
		Growth Hormone cpm/A_{260}	Prolactin fraction cpm/A_{260}	Residual Protein cpm/A_{260}	Total Protein cpm/A_{260}
9-11	55 s sub-unit	133	177	3,540	42,000
12-14	1	116	211	2,480	21,000
15-17	1-2	525	1,070	12,200	77,000
18-20	2	1,020	2,620	29,600	190,000
21-23	3	2,180	3,610	37,500	280,000
24-26	4	2,420	5,170	42,200	370,000
27-29	5	3,260	6,430	45,900	300,000
30-32	6-7	3,350	7,200	43,800	410,000
33-35	7-9	1,930	3,800	27,000	340,000
36-36	Polydisperse region (above 9)	1,420	2,720	16,700	290,000
38-41	Bottom of gradient	540	1,920	10,800	200,000

[a]Values are expressed as cpm/absorbance unit at 260 mμ.

[b]The corresponding fractions from three separate gradients were pooled in order to provide adequate quantities of polysomes.

[c]Tubes were incubated for 1 hr with 5 μc ^{3}Leu per ml incubation mixture.

[d]^{3}H-Pro, ^{3}H-Leu, and ^{3}H-Phe (5 μc of each amino acid) were used per ml of incubation mixture.

The rather similar results obtained for general protein synthesis suggested that the average molecular weight of the mixed proteins synthesized in the pituitary cell-free system was likewise in the approximate range of 20,000 to 30,000. Chromatography of the total soluble proteins from the incubated cell-free system on a Sephadex G-100 column (Fig 22) did indeed show that the over 50% of the newly synthesized proteins were in this molecular weight range.

6. Characterization of radioactive products isolated from bovine pituitary cell free incubation mixtures

a. Growth hormone

i. Gel filtration

As reported in an earlier section, growth hormone labeled in tissue slice experiments displayed two to three components when subjected to gel filtration on Sephadex G-100 columns. Comparable preparations from the cell-free system, purified to the Amberlite CG-50 stage, were similarly analyzed by gel filtration. Figure 23E shows the profile of the growth hormone on Sephadex G-100 with 0.026 M sodium acetate, pH 3.6, as buffer. In contrast to the slice-labeled material, there was one major peak corresponding to the standard preparation of NIH-growth hormone (Fig 23D) and a second slower moving fraction corresponding to smaller peptides and free amino acids. No dimer and aggregate fractions were detected.

FIGURE 22. SEPHADEX GEL FILTRATION IN AN ACID AND AN ALKALINE BUFFER OF THE TOTAL SOLUBLE PROTEINS FROM A BOVINE PITUITARY RIBOSOMAL SYSTEM.

After incubation the reaction was stopped by diluting with Medium M, and centrifuged at 105,000 g for 2 hr. The supernatant containing the soluble proteins was removed and dialyzed against water to remove free ^{3}H-amino acids. The solution was divided into two portions and subjected to gel filtration on Sephadex G-100 columns (2.0 x 55 cm) equilibrated with:

(A) 0.1 M glycine-HCl buffer, pH 4.0, and

(B) 0.05 M NH_4HCO_3 buffer, pH 8.1.

Solid line, absorbance at 280 mμ, broken line with X's, radioactivity. The cross-hatched area corresponds to the region of elution of 20,000-30,000 molecular weight material.

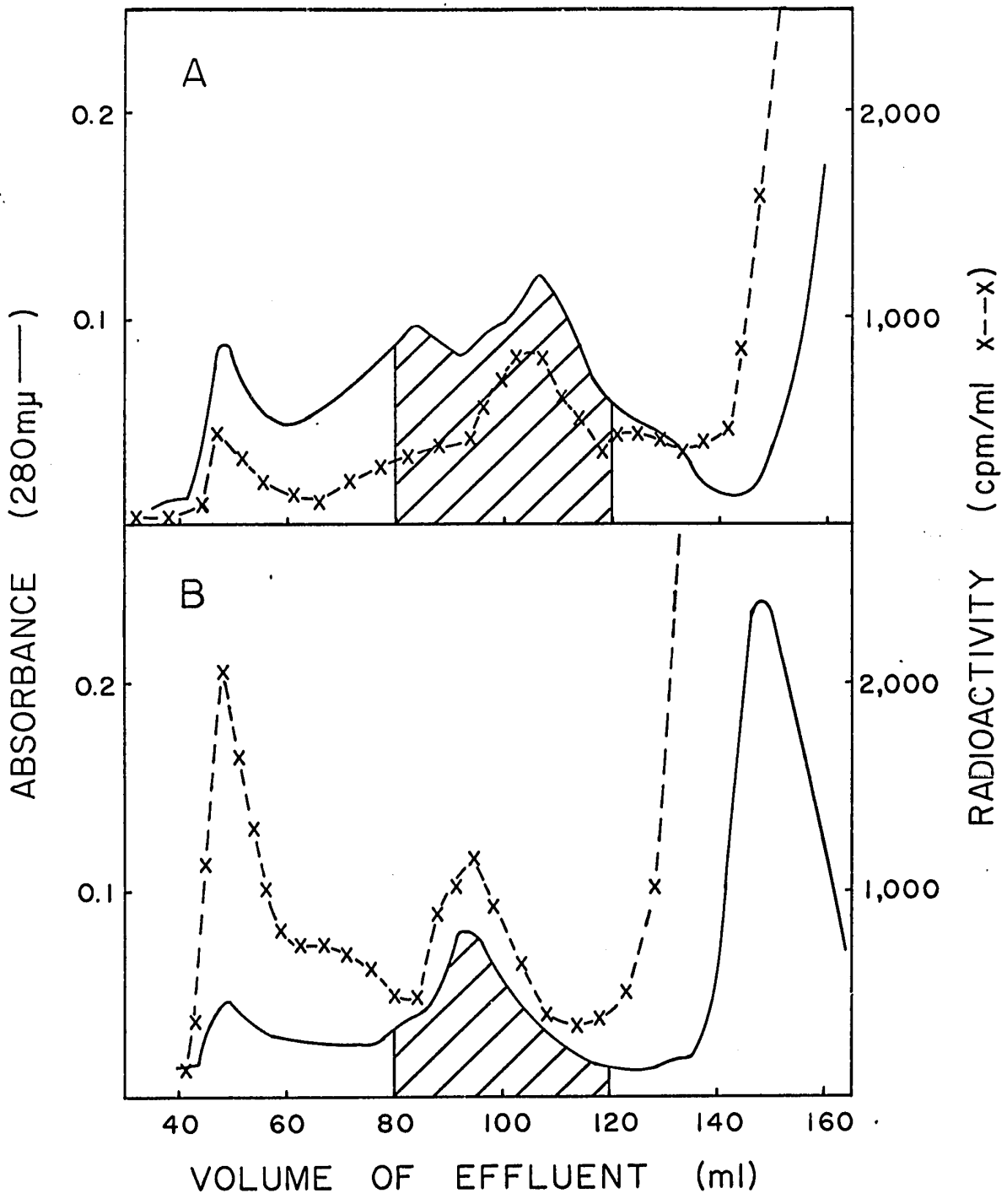

A
B
0.2
0.1
0.2
0.1
2,000
1,000
2,000
1,000
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)
40
60
80
100
120
140
160
VOLUME OF EFFLUENT (ml)

FIGURE 23. GEL FILTRATION OF GROWTH HORMONE PREPARATIONS FROM BOVINE PITUITARY RIBOSOMAL SYSTEM ON SEPHADEX G-100, USING DIFFERENT BUFFERS.

(A) 3.5 mg of standard growth hormone (Calbiochem). The Sephadex column (2.0 x 55 cm) was equilibrated with 0.1 M glycine-HCl buffer, pH 3.6.

(B) Growth hormone fraction isolated to the CG-50 stage, from cell-free system, plus 3.0 mg of Calbiochem, applied to the same column as (A).

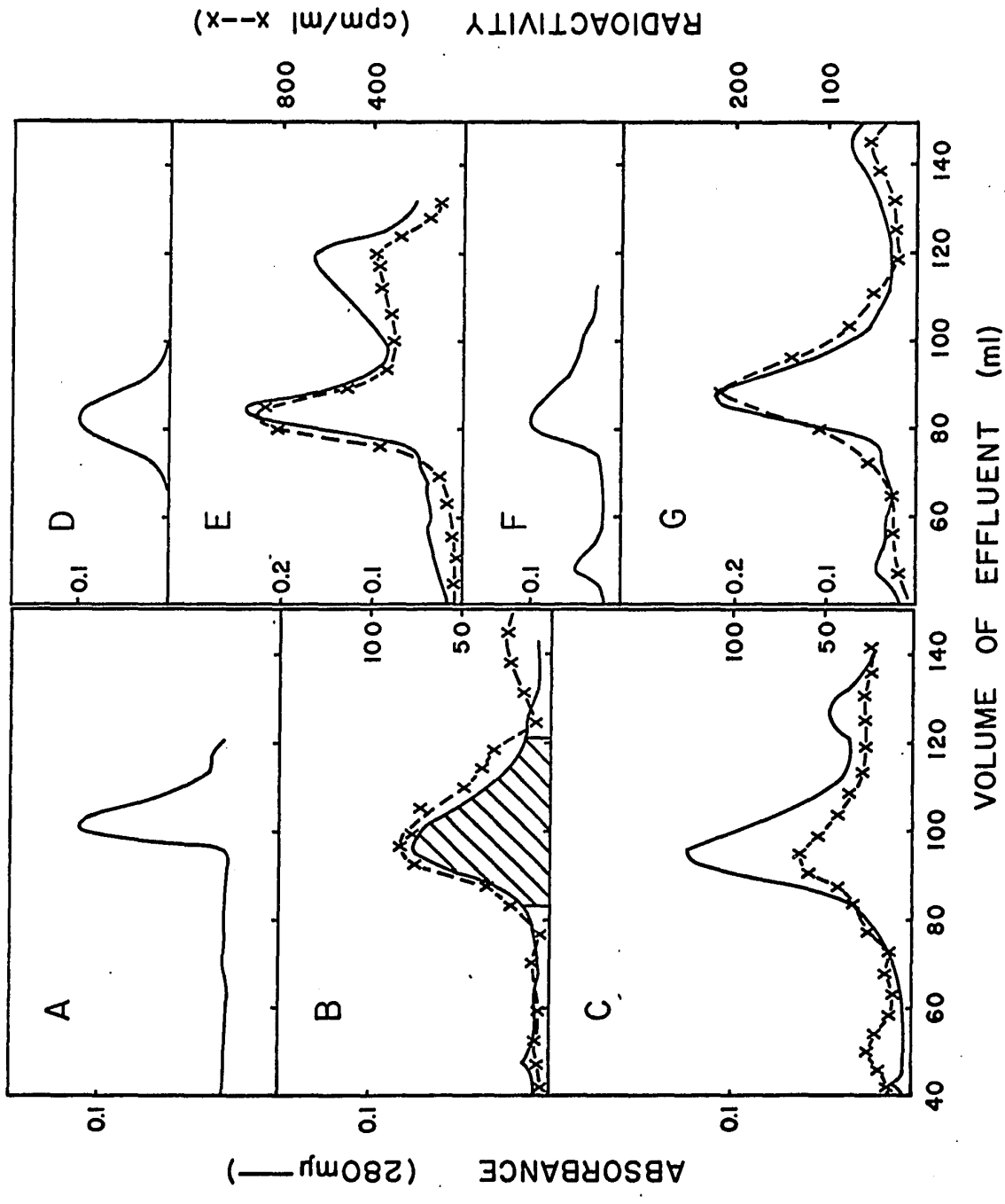
(C) Tubes 80-120 of (B) (cross-hatched area), were pooled, 2.0 mg of Calbiochem growth hormone added and precipitated by the addition of $(NH_4)_2SO_4$ to 50% saturation. The precipitate was dissolved in 0.1 M glycine buffer and applied to the column described in (A).

(D) 3.5 mg of NIH-growth hormone. The Sephadex G-100 column (2.5 x 36 cm) was equilibrated with 0.036 M sodium acetate buffer, pH 4.0.

(E) Labeled growth hormone from cell-free system plus 1 mg of NIH-growth hormone were applied to the same column as (D).

(F) 2.0 mg of NIH-growth hormone. The Sephadex G-100 column (2.0 x 54 cm) was equilibrated with 0.05 N NH_4HCO_3, pH 8.1.

(G) Labeled growth hormone from ribosomal system applied to the same column as (F).


A
B
C
D
E
F
G
0.1
0.2
100
50
800
400
200
40
60
80
100
120
140
VOLUME OF EFFLUENT (ml)
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)

Gel filtration with 0.1 M glycine-HCl buffer, pH 3.6 (Fig 23B) similarly revealed only one large peak of radioactive material, corresponding to the NIH-standard (Fig 23A). This peak was pooled, and reprecipitated with the aid of additional growth hormone carrier, by 50% $(NH_4)_2SO_4$. Upon re-filtration the labeled material reappeared in the same position on the column (Fig 23C). Gel filtration of the radioactive hormone in an alkaline buffer, 0.05 M NH_4HCO_3, pH 8.1 (Fig 23G), again indicated a main component, corresponding to standard growth hormone (Fig 23F). This procedure separated peptides and free amino acids from protein component.

ii. Polyacrylamide gel electrophoresis

Upon gel electrophoresis, growth hormone from the Amberlite CG-50 stage, exhibited two bands similar in position to those of standard growth hormone (Fig 24). Three regions of radioactivity were present. One corresponding to the origin of application represented aggregated material which did not enter the spacer gel. The fastest moving material which migrated with the buffer front corresponded to free amino acids. The radioactivity in the growth hormone region represents approximately 35% of the applied isotope.

iii. Density gradient centrifugation

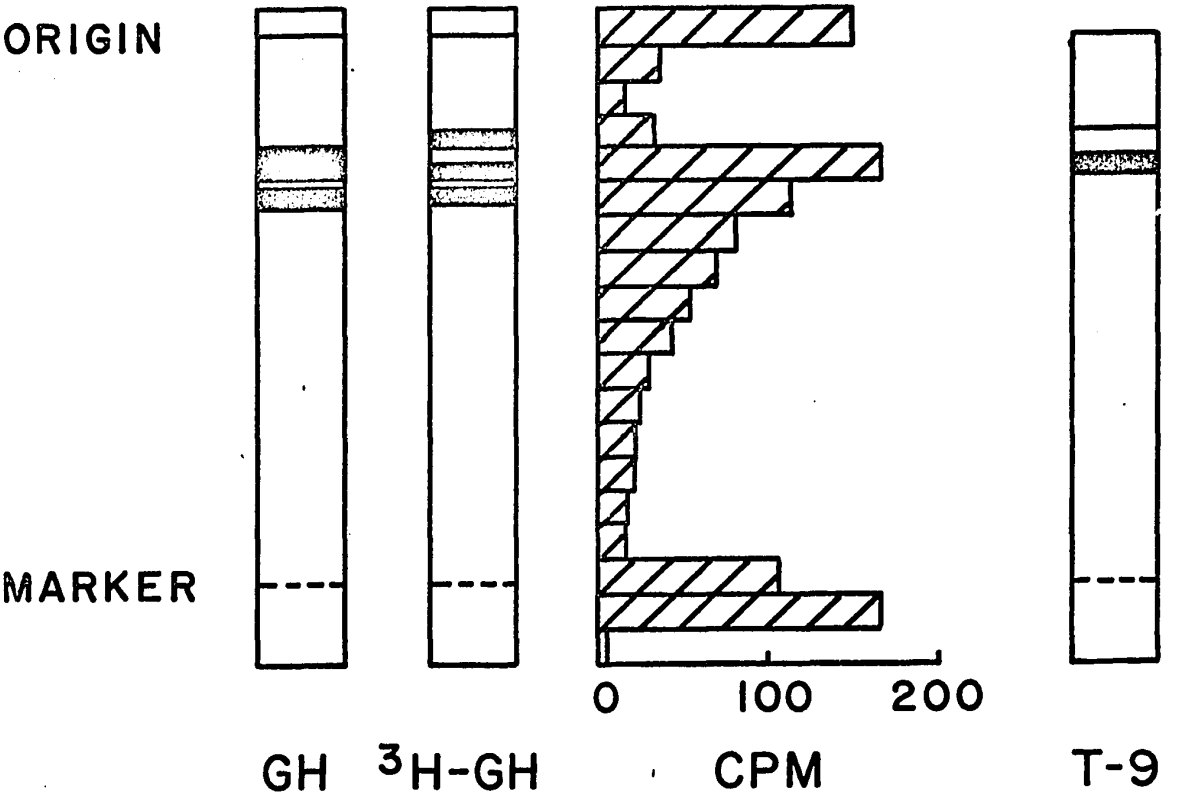
The growth hormone preparation fractionated on

FIGURE 24. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 8.9 OF GROWTH HORMONE PREPARATIONS FROM BOVINE PITUITARY CELL-FREE SYSTEM.

GH, 100 μg of NIH-growth hormone.

^{3}H-GH, CG-50 growth hormone fraction isolated from bovine pituitary ribosomal system. The cross-hatched area shows the distribution of radioactivity along the gel of a smilar preparation run concurrently.

T-9, material from tube 9 of Figure 25(B).


ORIGIN
MARKER
0
100
200
GH
3H-GH
CPM
T-9

5-20% sucrose gradient is shown in Figure 25B. After centrifugation at 40,000 rpm for 36 hr, standard growth hormone had moved approximately 1/3 of the distance along the gradient, corresponding to value of 2.3 S. A sample of free tritiated ^{3}H-Phe applied with the growth hormone standard did not enter the gradient and showed no binding to the standard (Fig 25A). It may be seen in Figure 25B that the labeled preparation moved to the same position on the gradient as did the standard. This radioactive component accounted for 34% of the applied material. Gel filtration of the material from tubes 8-9 of this gradient exhibited one stained band in the same position as in the case of growth hormone standard (Fig 24F-9).

iv. Antibody binding

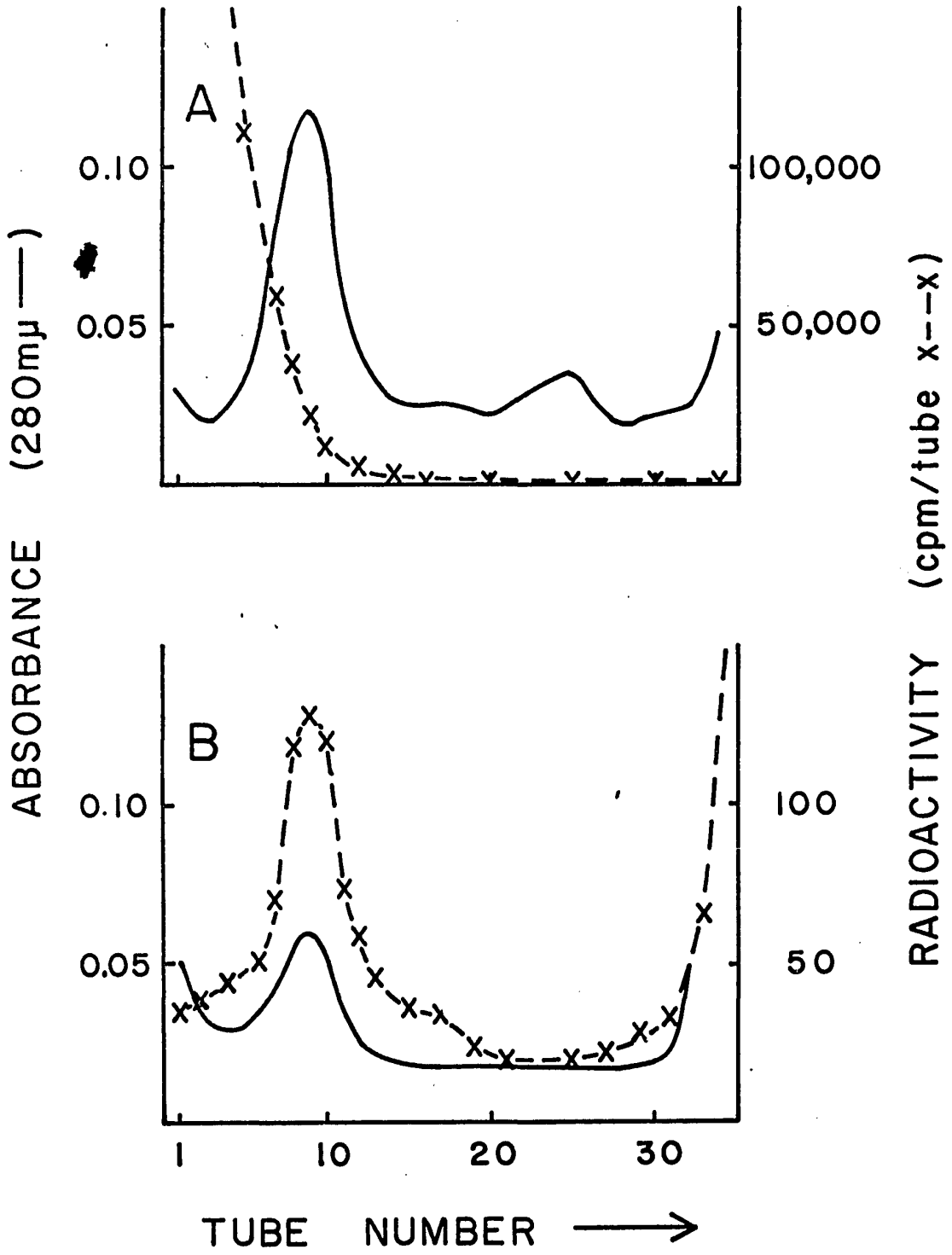
Short of sequence analysis of newly synthesized protein, the best means of identification is immunochemical, since this will recognize even rather small fragments of a molecule. In order to obtain highly labeled material for this study, the growth hormone was isolated without the addition of carrier from an incubation mixture containing 40 μc each of ^{3}H-Leu, Pro, Phe, Lys and Glu. The material was further purified by gel filtration on Sephadex G-100 and only the region corresponding to 20,000 - 25,000 Mol. Wt. was used.

FIGURE 25. DENSITY GRADIENT CENTRIFUGATION OF GROWTH HORMONE PREPARATIONS.

(A) 1 mg NIH-growth hormone plus 1 μc ^{3}H-Phe.

(B) CG-50 growth hormone isolated from the bovine pituitary ribosomal system.

Gradients, 5-20% sucrose, were prepared in 0.1 M glycine-HCl buffer, pH 3.6. Centrifugation was carried out for 36 hr at 40,000 rpm in the SW 41 rotor.


A
B
0.10
0.05
100,000
50,000
100
50
1
10
20
30
TUBE NUMBER
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/tube x--x)

Rechromatography of an aliquot of this material (Fig 26B) after incubation for 4 days at 4°C, revealed the presence of monomer material which eluted at a position corresponding to NIH- growth hormone (Fig 26A) and a small quantity of degraded material (16-18% of total label). After incubation for 4 days at 4°C bound and free material was separated by gel filtration on Sephadex G-100. When the labeled material was incubated with 0.4 ml of rabbit anti-bovine growth hormone-serum (Fig 26C), 16% of the material was bound to the globulin fraction. This binding could be inhibited by NIH-bovine growth hormone, added at the beginning of the incubation period (Fig 26G). Incubation with 0.8 ml of antiserum (Fig 26D) resulted in 35% of bound label. With 1.6 ml of antiserum, a precipitate formed but 40% of the soluble material was bound to the globulin (Fig 26E). In both cases the binding was inhibited by the addition of NIH-growth hormone (Fig 26H and J). There was no formation of globulin complexes with a rabbit anti-bovine albumin-serum (Fig 26F) indicating that the binding was specific.

b. Prolactin fraction

i. Gel filtration

Gel filtration of the labeled prolactin preparation on Sephadex is presented in Figure 27. In

FIGURE 26. ANTIBODY BINDING OF GROWTH HORMONE PREPARATIONS FROM PITUITARY CELL-FREE SYSTEM.

The bound and free hormone were separated by gel filtration on Sephadex G-100 (2.0 x 42 cm) in 0.05 M NH_4HCO_3 buffer, pH 8.1. Solutions (B-J) were incubated for 4 days at 4°C prior to filtration.

(A) 2.5 mg standard growth hormone (Calbiochem).

(B) Refiltration of an aliquot of the 80-100 ml region of highly labeled growth hormone, filtered under similar conditions as Figure 23G.

(C) Aliquot of growth hormone equivalent to (B), plus 0.4 ml of rabbit anti-bovine growth hormone-serum.

(D) Aliquot of growth hormone, equivalent to (B), plus 0.8 ml of anti-bovine growth-serum.

(E) The soluble portion of a reaction mixture similar to (D), but containing 1.6 ml of anti-bovine growth hormone-serum.

(F) Aliquot of growth hormone, equivalent to (B), plus 0.4 ml of rabbit anti-BSA-serum.

(G) Similar to (C), plus 8.0 mg NIH-growth hormone.

(H) Similar to (D), plus 8.0 mg NIH-growth hormone.

(J) Similar to (E), plus 8.0 mg NIH-growth hormone.

Solid lines, absorbance at 280 mμ, broken line with X's, radioactivity.

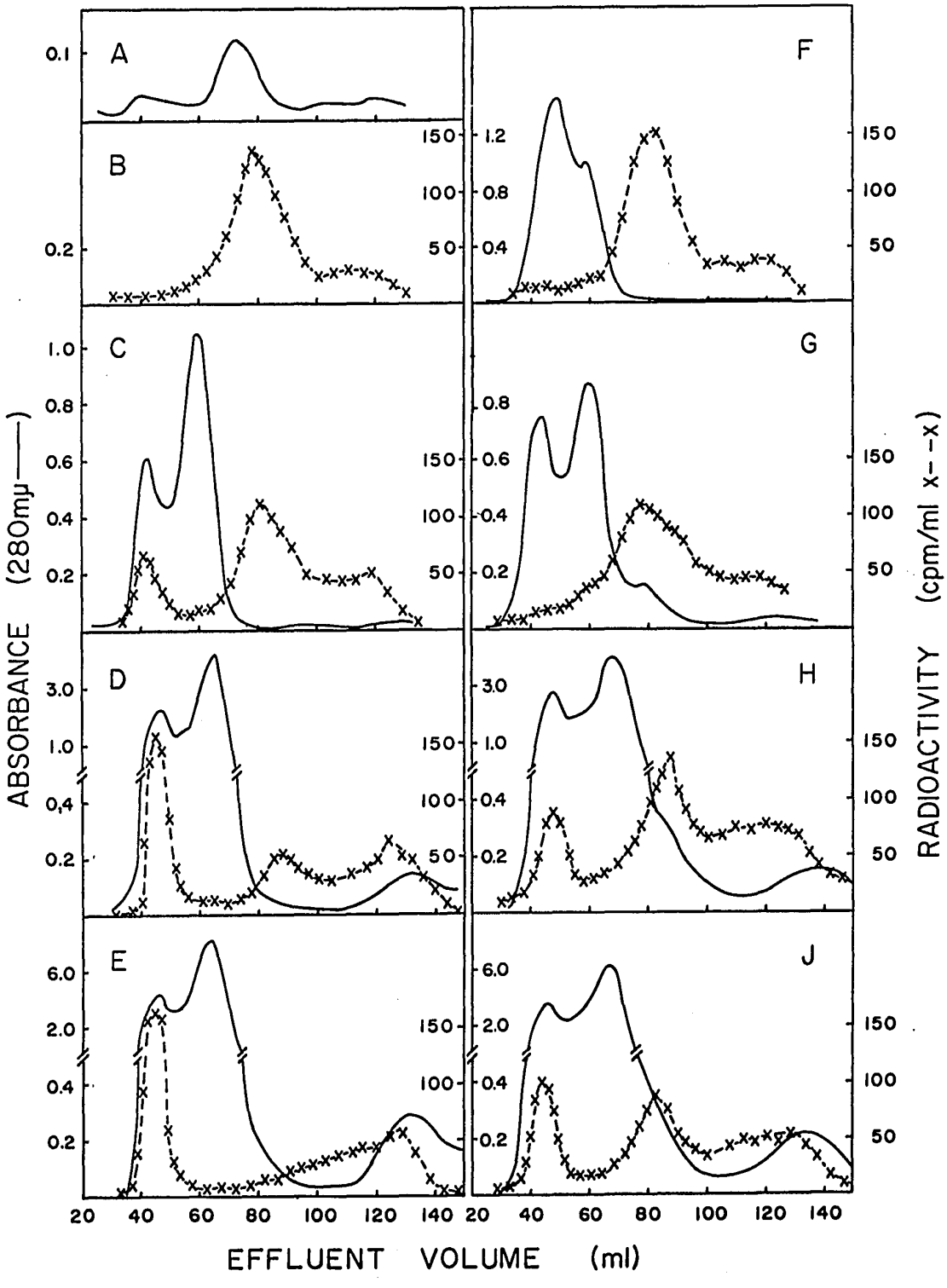

A
B
C
D
E
F
G
H
J
ABSORBANCE (280mμ——)
RADIOACTIVITY (cpm/ml x- -x)
EFFLUENT VOLUME (ml)
20 40 60 80 100 120 140

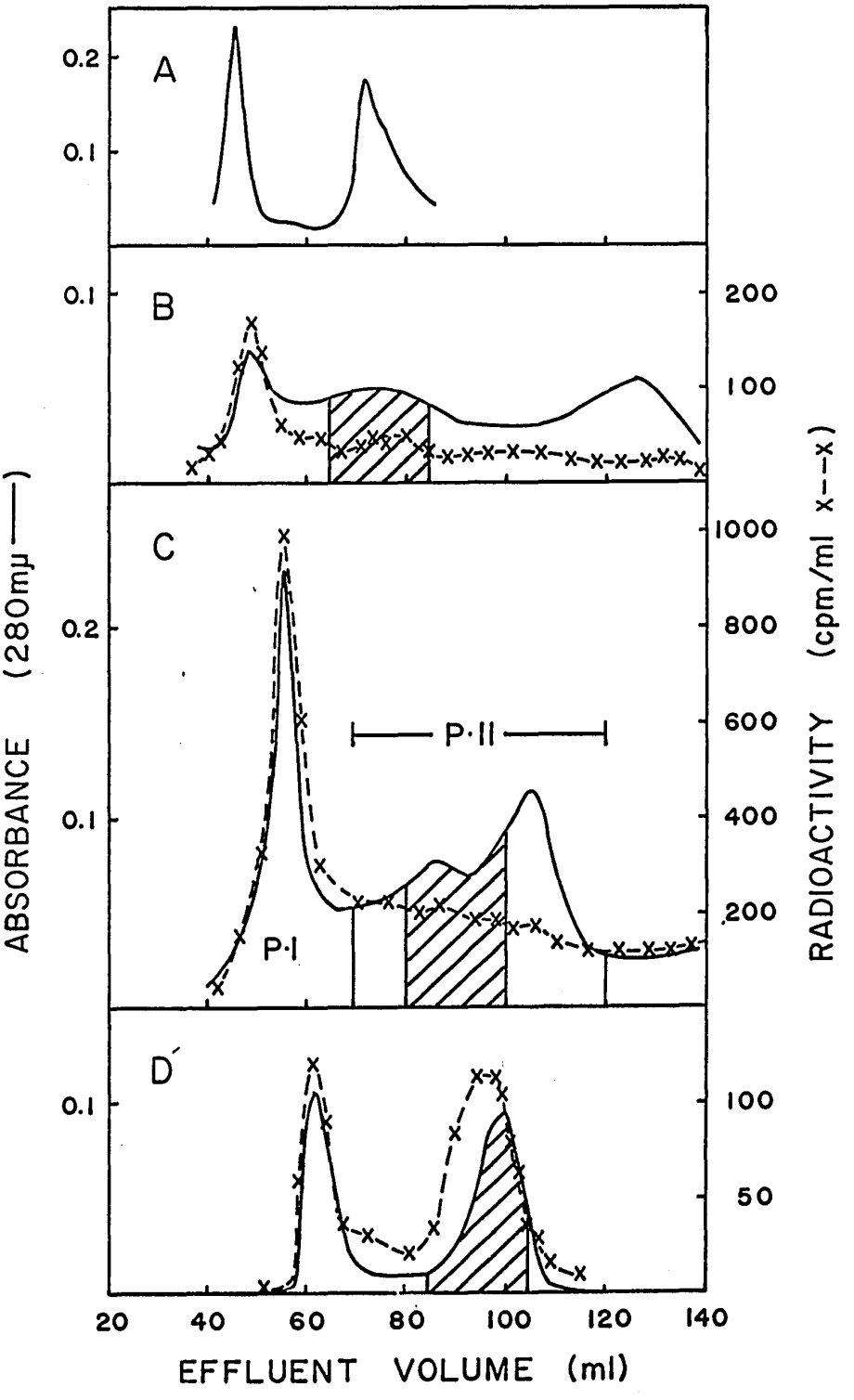
FIGURE 27. GEL FILTRATION ON SEPHADEX OF PROLACTIN FRACTION ISOLATED FROM PITUITARY CELL-FREE SYSTEM.

(A) 3.5 mg NIH-prolactin plus Blue dextran on a Sephadex G-75 column (2.5 x 36 cm) equilibrated with 0.05 M borate-0.1 M KCl buffer, pH 8.6. The first peak at 45 ml corresponds to Blue dextran, the second, at 72 ml to prolactin.

(B) DEAE-prolactin fraction from pituitary ribosomal system. The column used was described in (A).

(C) DEAE-prolactin fraction from pituitary ribosomal system plus 1.5 mg NIH-prolactin. The Sephadex G-100 column (2.0 x 55 cm) was equilibrated with 0.05 M NH_4HCO_3 buffer, pH 8.1.

(D) DEAE-prolactin fraction isolated from pituitary ribosomal system with fresh anterior pituitary tissue as carrier. The Sephadex G-75 column (1.6 x 110 cm) was equilibrated with 5% acetic acid. The cross-hatched areas correspond to the effluent volume of standard prolactin.


A
B
C
D
P·I
P·II
0.2
0.1
200
100
1000
800
600
400
50
20
40
60
80
100
120
140
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/ml x--x)
EFFLUENT VOLUME (ml)

alkaline buffers (Fig 27B and C) the profiles of absorbance at 280 mμ displayed broad peaks which corresponded to the NIH reference standard (Fig 27A). However, radioactivity was predominantly present in the material at the void volume, corresponding to high molecular weight material. With an acidic buffer, 5% acetic acid (Fig 27D), two peaks were evident. The faster moving fraction represented the void volume of the column, while the slower moving fraction corresponded to the elution volume of NIH-prolactin. The radioactivity coincided with the absorbance in both fractions. However, fresh pituitary tissue had been used as a hormone carrier only in the isolation procedure for the sample analyzed with the 5% acetic acid buffer, and it is possible that some synthesis occurred because of the presence of intact cells. Hence the data in Figure 27D are open to question.

ii. Polyacrylamide gel electrophoresis

The two components from the gel filtration of labeled prolactin on Sephadex G-100 with 0.05 M NH_4HCO_3 were subjected to gel electrophoresis (Fig 28). The faster moving component, peak I, displayed three bands (staining with Amido black) which corresponded to those present in a standard prolactin, electrophoresed at the same time (Fig 28A). Elution of the radioactivity from a duplicate gel revealed that the

FIGURE 28. POLYACRYLAMIDE GEL ELECTROPHORESIS AT pH 8.9 OF PROLACTIN FRACTION FROM BOVINE PITUITARY RIBOSOMAL SYSTEM.

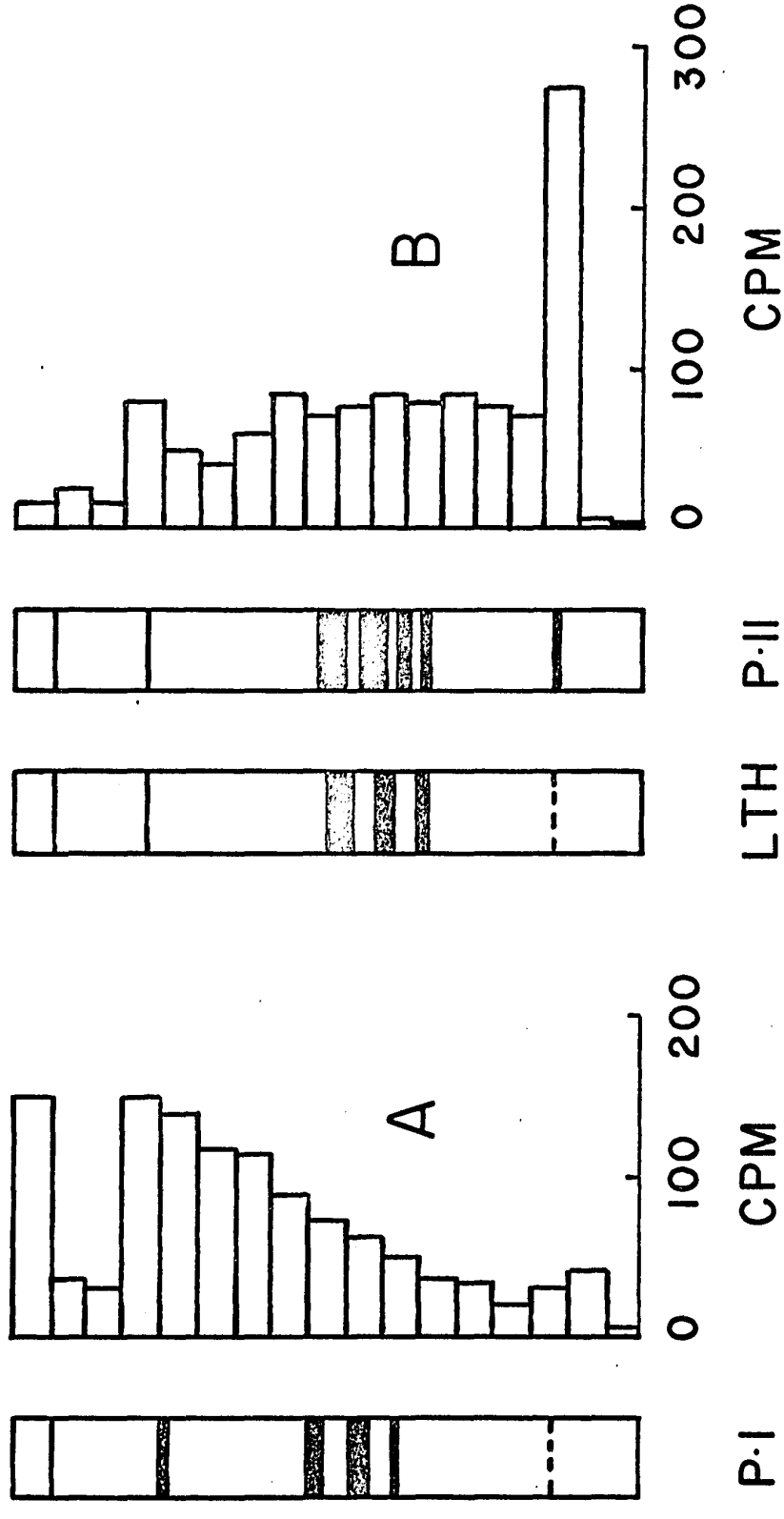
P.1, 1.0 mg of lyophilized material from peak I, tubes 45-70 of Figure 27C.

A, distribution of the radioactivity along the gel, P.1.

LTH, 100 μg of NIH-prolactin.

P.11, 1.0 mg of lyophilized material from peak II, tubes 71-120 of Figure 27C.

B, distribution of the radioactivity along the gel, P.11.


B
0
100
200
300
CPM
P·II
LTH
A
0
100
200
CPM
P·I

label was not concentrated at the prolactin region but at the origin mainly over a wide region in the direction of the salt front. Electrophoresis of the slower moving component, Peak II, also revealed stained bands corresponding in position to NIH-prolactin (Fig 28B). In a duplicate gel, the radioactivity was widely distributed with a sharp peak at the salt front, probably corresponding to free-amino acids.

iii. Density gradient centrifugation

After centrifugation for 40 hr at 40,000 rpm a sample of NIH-prolactin migrated approximately one-third of the distance of a 5-20% sucrose gradient (Fig 29A). As previously reported for the growth hormone sample, free ^{3}H-Phe applied concurrently with the standard did not bind to the hormone.

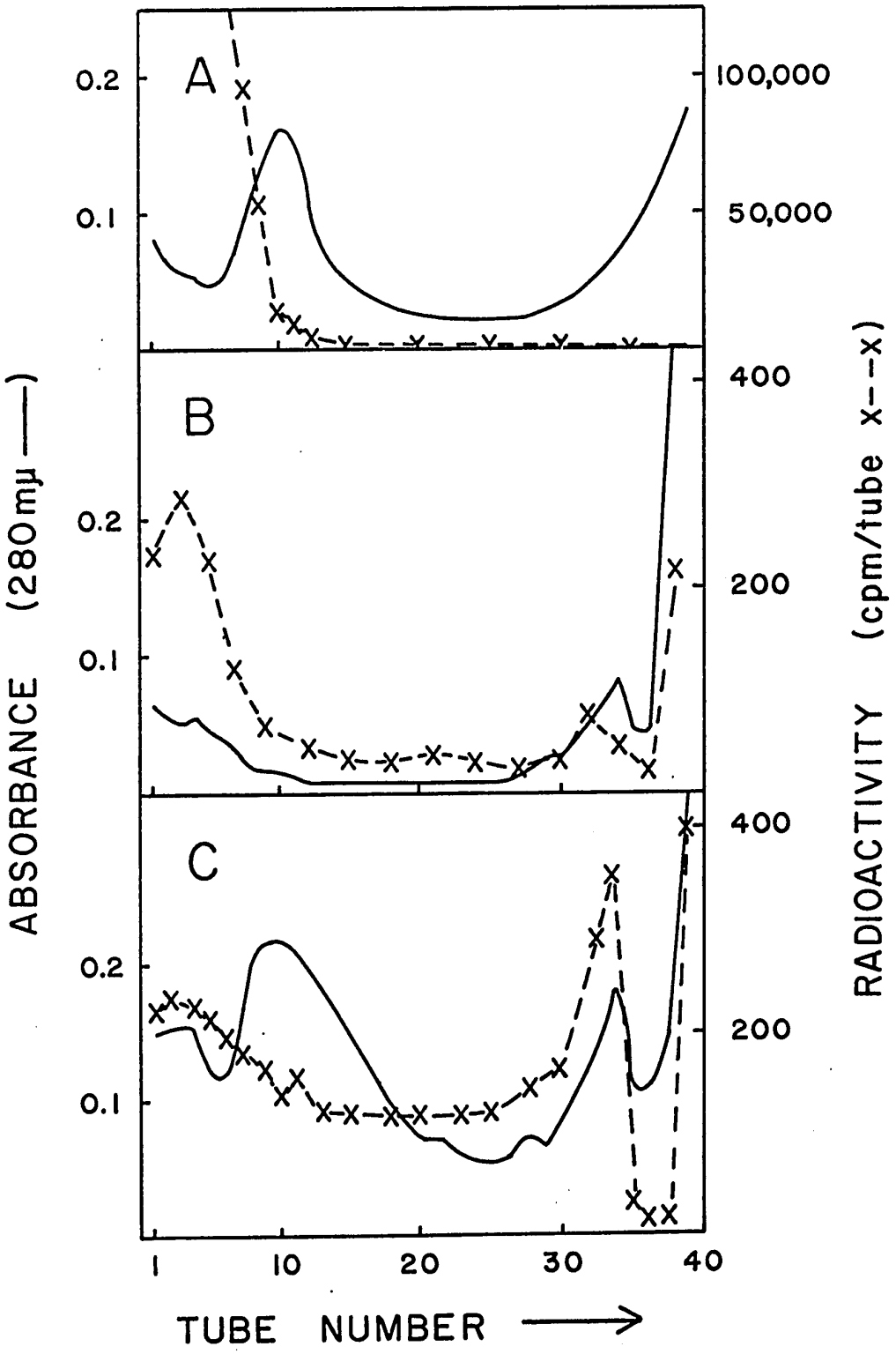
In an attempt to determine the nature of the radioactive material present in the prolactin preparation a sample was subjected to similar sucrose density gradient analysis. No peak corresponding to the monomer of prolactin was evident (Fig 29B). A region of absorbance and radioactivity was present at the top of the gradient, possibly reflecting free amino acids and peptides. An increase in absorbance and radioactivity was also noted near the bottom of the gradient, that is at the interface between the gradient and the 50% sucrose cushion, which might be

FIGURE 29. DENSITY GRADIENT CENTRIFUGATION OF PROLACTIN FRACTION ISOLATED FROM PITUITARY CELL-FREE SYSTEM.

(A) 1 mg NIH-prolactin plus 1 μc ^{3}H-Phe.

(B) DEAE-cellulose prolactin fraction isolated from the bovine pituitary ribosomal system.

(C) 1 mg of NIH-prolactin was added to the prolactin fraction from pituitary cell-free system before application to the gradient. Gradients, 5-20% sucrose, were prepared in 0.1 M NH_4HCO_3. Centrifugation was carried out for 40 hr at 40,000 rpm in the SW-41 rotor.


A
B
C
0.2
0.1
100,000
50,000
400
200
ABSORBANCE (280mμ ——)
RADIOACTIVITY (cpm/tube x--x)
1
10
20
30
40
TUBE NUMBER ⟶

due to aggregated material. However when another sample of the labeled prolactin fraction was mixed with standard prolactin prior to layering on the gradient (Fig 29C) no significant increase in labeling associated with the prolactin peak was evident indicating no mixing of aggregated material with the standard. Gel filtration of a number of fractions from this gradient showed the usual pattern for standard prolactin at tube 10. Gel filtration of tube 34 from the interface region revealed a slow moving component, corresponding in position on the gel to the highly labeled material of peak I in Figure 28A. Electrophoresis of the material pelleted to the bottom of the gradient (tube 39) showed only one band of aggregated material which stayed at the origin and did not enter the gel (data not included).

From these attempts to characterize the radioactive material present in the prolactin fraction of the cell-free system, it became apparent that although endogenous prolactin was being isolated in this preparation, the radioactive material did not represent the conventional form of prolactin. Further studies of this problem are underway.

IV. Discussion

A. Biosynthesis of prolactin and growth hormone in slices of bovine anterior pituitary tissue

1. Isolation procedure

The procedures described by Ellis (1961) and Kwa, et al. (1965) for the isolation of large quantities of relatively pure growth hormone and prolactin required only slight modifications for the successful separation of labeled growth hormone and prolactin, from incubates of anterior pituitary slices. Omission of the 0.1 M $(NH_4)_2SO_4$ extraction step (Table IV) resulted in approximately 1.5 times greater yield in mg of growth hormone purified to the Amberlite CG-50 stage. In earlier studies (Rao, et al., 1967), prolactin was recovered from the 1% DOC extract, after dialysis and lyophilization, by the isoelectric precipitation procedure of Cole and Li (1955). Comparison of this method with the DEAE-cellulose column chromatography procedure described by Kwa, et al. (1965) (Table V), but modified to a step-wise elution procedure (Table VI), indicated that the latter method was more suitable for use in these studies, since the variation between duplicate samples was less; 3.2%, compared with 22.8% for the isoelectric precipitation method.

The yields of growth hormone and prolactin obtained from bovine anterior pituitary slices after purification to the Amberlite CG-50 and DEAE-cellulose stages (Table IV and Table VII) showed average values of 8.5 and 12.3 mg/g of wet anterior

pituitary tissue for growth hormone and prolactin, respectively. Yields for growth hormone varying from 2.8 mg for whale (Papkoff and Li, 1958), 4.7 mg for bovine (Ellis, 1961), to 6.0 mg/g for human pituitaries (Li, 1968) have been reported for fresh glands extracted by this method, but including the additional extraction step with 0.1 M $(NH_4)_2SO_4$.

Kwa, et al. (1965) reported a yield of 1 to 2 mg of growth hormone, and 3-4 mg of prolactin, per g wet weight of pituitary material by their method, which also involves separation of granular fraction from the pituitary glands by differential centrifugation, prior to the deoxycholate treatment and DEAE-cellulose chromatography. Jiang and Wilhelmi (1965) reported a yield of 1 mg/gm of bovine prolactin by their procedure which involves an additional isoelectric-precipitation before the DEAE-cellulose step. The much higher yields of prolactin, obtained in our studies, may be due to the presence of a nucleic acid contaminant, mentioned by Kwa, et al. (1965), which is usually excluded by gradient elution of prolactin from DEAE-cellulose. Subsequent rechromatography by gradient elution on DEAE-Sephadex, of the material obtained by stepwise elution, indicated that this prolactin still contained approximately 30% of the nucleic acid contaminant (data not included). Although stepwise in place of gradient elution of prolactin from DEAE-cellulose may have affected the weight yield, the former method had no affect on the radioactivity recovered (Table VI). Whether the pituitaries were obtained

from dairy or feed lot cattle may have also influenced the yield of prolactin.

After gel filtration, the amount of monomer component recovered was approximately 1.3 and 1.7 mg/gm for growth hormone and prolactin, respectively. By a similar procedure, Li (1968) reported an average yield of 2.8 mg/g of human growth hormone from fresh pituitary glands. The aggregate material represented about 15% of the total protein. Our preparation from slices contained a higher proportion of aggregated material, probably formed during the lyophilization of the material from dilute salt solutions (Squire, et al., 1963). Bioassay or radioimmunoassay estimates on samples taken at different stages in the isolation of the hormones would be of great value since they would give an indication of the specific activity of the preparations.

It was pertinent to carry out the same incubation and isolation procedure with several other bovine tissues as controls (Table VIII). These experiments showed that the anterior pituitary had 3-8 times more label in hormone fractions than non-pituitary tissues. In the case of posterior pituitary and hypothalamus tissue (Fig 3 and 4), gel filtration revealed that the labeled material was not of the same molecular weight as the hormones. These experiments indicated that the incubation and isolation procedure was fairly specific for measuring the synthesis of these two hormones. It also appeared that the anterior pituitary was the sole site of origin of these hormones.

2. Synthesis of growth hormone and prolactin in bovine pituitary slices

Measurement of the relative rates of incorporation of labeled amino acids into the hormones (Fig 5) revealed that the process was more active for prolactin than growth hormone. This difference was more pronounced at 2 hr than at 4 hr. After the longer time interval, the incorporation into protein continued to rise while the radioactivity in prolactin appeared to reach a maximum, and that amount in growth hormone declined. The relative labeling in the two hormones appear to be correlated with the natural abundance of these hormones in the pituitary gland. Prolactin and growth hormone are reported to be present to the extent of 3-4 and 1-2 mg/g wet weight of tissue, respectively. With rat pituitaries, McLeod and Abad (1968b) found equal rates of incorporation into prolactin and growth hormone at 2.5 hr. Although these workers noted a difference in the amount of radioactive hormone released into the media after 6 hr of incubation, the total extent of synthesis was approximately equal for the two hormones.

In our experiments, the rates of release of the hormones into the media at the times chosen, 2 and 4 hr (Table IX), appeared to be quite similar. Although relatively more prolactin appeared after 2 hr, at 4 hr the radioactivity in the media was almost equal for the two hormones. McLeod and Abad (1968b) reported that no significant amount of labeled hormone was found in the external medium after 2.5 hr. At 6 hr, three to four

times more radioactive prolactin than growth hormone was found in the medium. Unfortunately the relative rates of synthesis and release of the bovine hormones were not measured at this time period.

The curious decline in the rate of growth hormone synthesis after 4 hr may be due to a protease present in pituitary extracts. Ellis (1961) reported two proteolytic activities in the pituitary gland at pH's of 4.5 to 5 and 6 to 7. Recently, Ellis, et al. (1968) described another trypsin-like enzyme present as a contaminant in crude growth hormone preparations and active in degrading growth hormone at pH 8-9.

After 4 hr the extent of labeling observed in the growth hormone was approximately 8.5% that in total protein, while the corresponding figure for prolactin was 14%. Assuming a concentration of approximately 160 g of protein per kilogram of fresh anterior lobe tissue (Melchior and Halakis, 1952), and using the yield values reported in the previous section (Table VII), growth hormone and prolactin isolated to the Amberlite CG-50 and DEAE-cellulose stage, respectively, would represent 5.3 and 7.7% of the total protein present in the gland. The rate of incorporation of label into the hormones was therefore not appreciably different than that into total proteins. This is in accord with findings for ACTH and protein reported by Hussa (1968). Hopefully further purification of the hormone preparations should bring closer agreement between these values.

3. Characterization of radioactive material from slice incubations

The identity of the isolated hormones was established on the basis of several physicochemical and immunological characteristics, since no one criterion can be used as proof that a protein is pure.

Growth hormone was eluted as a single peak from Amberlite columns (Fig 1). Gel filtration of this material on Sephadex G-100, in acetate buffer, pH 4.0 (Fig 6) exposed the heterogeneity of this product. Monomer and aggregate components were present which showed no interconversion on rechromatography (Free and Sonenberg, 1966). In some cases, when an increased salt content was evident, conversion of the monomer to a dimer was noted (Dellacha, et al., 1968) (data not included). Upon polyacrylamide gel electrophoresis, both components manifested patterns similar to those of NIH-growth hormone (Fig 10B). The labeled material in each component had a different specific activity, with a higher proportion of ultraviolet-absorbing material in the aggregate. It was difficult to determine what percentage of the radioactivity associated with the polymer was growth hormone, since the aggregated protein does not dissociate readily, especially if it represents degraded material (Ellis, et al., 1968). Gel filtration of the crude growth hormone preparations in alkaline buffers (Fig 7) revealed less heterogeneity (Dellach and Sonenberg, 1964). A possible solution to the isolation of aggregate free growth hormone preparations may be the ultra-filtration technique described recently by Lewis, et al. (1969).

Prolactin was resolved into two to three components on DEAE-cellulose (Fig 2A). This heterogeneity has also been attributed to aggregate formation (Squire, et al., 1963). Gel filtration on Sephadex G-100 in either acid (Fig 8) or alkaline buffers (Fig 9) separated the monomer and aggregate. Like growth hormone these preparations differed in specific activity. The radioactivity associated with the aggregate could not be dissociated by treatment with 8 M urea at pH 4.0 or 8.1 (data not included). Upon gel electrophoresis, the aggregate material from the acidic column had more of the faster moving components, while that from the basic column showed more retarded material. Lewis and Cheever (1965) concluded that formation of new end terminal groups, or loss of ammonia, would result in more acidic, faster moving components; while aggregates produced by alkaline treatment would result in bands migrating behind the major component (Lewis, et al. 1967).

Although some aggregated material was detected on polyacrylamide gel electrophoresis (Fig 10A and 11), the distribution of radioactivity parallelled the reference standards for both growth hormone and prolactin. This coincidence of radioactivity and protein peaks in gel electrophoresis and column chromatography suggests strongly that the incorporation does indeed represent labeling of the hormone molecules.

The molecular weight of 26,000 found for both the labeled growth hormone and NIH reference standard agrees with the value

reported by Andrews (1966), using the same 0.05 M Tris HCl-0.1 M KCl, buffer (pH 7.5). With a glycine-HCl buffer (pH 3.6), Dellacha, et al. (1968) reported a value close to 21,000 for the minimum molecular weight of growth hormone in presumably nonaggregated condition. These workers showed that the addition of neutral salts led to association of this protein. The value of about 20,000 found for both labeled and standard prolactin is also in agreement with reported values (Squire, et al., 1963, Andrews, 1966). Andrews found the molecular weight of this protein to depend on both concentration and pH.

When tested by immunodiffusion (Fig 13), the two hormones preparations gave single precipitin lines only between the homologous antisera and the antigens. Using anti-bovine serum, no contamination by serum proteins was detected. Both components of prolactin gave precipitin lines when tested with anti-prolactin serum (data not included).

Amino acid composition (Table X) of the Amberlite CG-50 growth hormone preparation showed fairly close agreement with the reported composition. Li (1968) reported no difference in the amino acid content between the aggregate and monomer of human growth hormone. Similarly, the DEAE-cellulose prolactin product displayed reasonably good agreement in amino acid content with a NIH standard.

No free amino acid was detected at the C-terminal end of prolactin, and threonine was the only amino acid at the N-terminal in agreement with published results (Cole, et al., 1957). Phenylalanine and alanine were found as N-terminal

amino acids of growth hormone. Although quantitative determinations were not performed, the NIH standards gave the same results. The presence of two amino acids in the N-terminal end of bovine growth hormone has been reported by others (Li and Ash, 1953; Dellacha, et al., 1968). Although possible branching has been postulated, the nature of the N-terminal end of BGH is still an open question (Dellacha, et al., 1968).

Treatment of ^{3}H-Phe-labeled growth hormone with carboxypeptidase released maximal amounts of labeled phenylalanine, and lesser quantities of alanine (Table II). These findings are in agreement with the C-terminal sequence of bovine growth hormone determined by Wallis (1966) and Santome, et al. (1965) of CyS- Ala- PheOH. The difference in labeling of growth hormone at the N-terminal as compared to the C-terminal (judged by ^{3}H-Phe release) should be viewed with caution as the material used for these estimations was a preparation which had been purified only to the CG-50 stage. As indicated above, when this material is subjected to additional purification procedures, small amounts of contaminating proteins are detected.

4. Effect of the releasing factor, GRF, on growth hormone synthesis and release

The effect of hypothalamic factors on the release of hormones from the anterior pituitary gland is well documented (McCann and Dhariwal, 1966, Schally, et al., 1967). Recently claims based on in vitro systems have appeared that these

factors may play a role in the synthesis, as well as in the release of hormones (Schally, et al., 1968). An attempt to study the effect of these factors on the present system seemed warranted.

Release of prelabeled hormone from pituitaries of both normal and cold-stressed rats (Tables XIII and XIV) exhibited a 1.5 to 2 fold enhancement when GRF was added (significant at $p = 0.05$, Student's t). Abad and McLeod (1968) showed that growth hormone release from pituitaries without the intervention of releasing factors was minimal until 8 hr when an increase was noted. Possibly greater divergence between control and experimental groups might have been attained if longer incubation periods had been used. Puromycin had no effect on the GRF-stimulated release of preformed hormone in the two cases studied. GRF showed no effect on the release of general protein into the media. Samli and Geschwind (1967) reported similar lack of effect of puromycin on the LRF-stimulated release of luteinizing hormone. Watanabe, et al. (1968) however, reported that puromycin inhibited the release of FSH. It would appear that the mechanism of action of releasing factors may not be similar for all hormones.

When added at the beginning of the incubation period, GRF showed no effect on the incorporation of labeled amino acids into growth hormone or protein (Tables XV and XVI). Under these conditions no significant difference was noted in the release of the hormone into the media. These results confirm

those of McLeod and Abad (1968), who studied the effect of SME extracts on the incorporation of radioactive amino acids into rat growth hormone and prolactin. Our results are at variance with those of Schally, et al. (1968), who claimed that GRF stimulated the synthesis of growth hormone in incubated pituitaries from cold stressed rats. In their study, growth hormone activity was measured by the tibia assay but the amounts of growth hormone reported were extremely high when compared with the normal growth hormone content of rat pituitaries (Ellis, 1968, Daughaday, et al., 1968). The results reported in our study are very inconclusive. The experiments illustrate that there is no easily measured effect of GRF on either growth hormone synthesis or release. More work in this area is obviously needed.

B. Biosynthesis of growth hormone and prolactin in a bovine pituitary cell-free incubation mixture

1. Evaluation of isolation procedures

In dealing with the minute amounts of labeled material, formed in a cell-free incubation mixture, the addition of an extraneous source of the protein to act as a carrier during the isolation seems advisable. Since the use of purified hormone for this purpose was not feasible, a crude anterior pituitary powder was employed instead (Table XVII).

Treatment with RNAse after the incubation period, but prior to the isolation procedure, decreased the recovery of labeled growth hormone. Leaver (1966) has presented evidence for the

existence of a growth hormone-RNA complex in acetone-dried human pituitary glands which were subjected to alkaline treatment. The present Amberlite CG-50 growth hormone preparation may therefore have contained a small percentage of bound RNA. With prolactin, on the other hand, the amount of radioactivity recovered remained the same after RNAse treatment, suggesting the absence of a prolactin-RNA complex.

2. Components of the incubation mixture

The incorporation of labeled amino acids into hormones and general proteins in the pituitary preparation was similar to that reported for other mammalian cell-free systems (Peterman, 1964, Von der Decken, 1967). The requirements for ATP, GTP, magnesium and an amino acid mixture had been established previously (Adiga, et al., 1966) for pituitary proteins. Growth hormone and prolactin did not respond to the same extent as general proteins to the omission of GTP and the amino acid mixture.

The presence of both ribosomes and pH 5 enzyme was necessary for efficient incorporation into both hormones and protein. However, the pH 5 supernatant factor was sometimes inhibitory for hormone synthesis. The addition of pH 5 supernatant factor alone to ribosomes resulted in very active incorporation into the prolactin fraction. The inhibitors of protein synthesis, puromycin and RNAse, caused reduction of incorporation into all fractions, as was expected.

3. Optimal conditions

Although optimal conditions had been established for the synthesis of ACTH and protein in the pituitary system, a study of the optimal requirements for the synthesis of growth hormone and prolactin proved of value. As anticipated, the extent of incorporation of isotope into the hormones was dependent on the ribosome concentration (Fig 16). The prolactin fraction appeared to respond to an increased ribosome content more than did the growth hormone fraction. However, the label in growth hormone represented 2% of that in total protein, while that in the prolactin fraction accounted for 10-15%. This large discrepancy suggested that the radioactivity associated with the DEAE-cellulose fraction was due to other components in addition to prolactin.

The magnesium optimum (Fig 17) was the same for all processes and similar to that reported by Adiga, et al. (1966). The optimal concentration of Mg^{++} in the pituitary system was estimated to be 9 mM, while that of bovine liver was approximately 6 mM.

The three activities displayed diverse requirements for pH 5 enzyme (Fig 18). The optimal for growth hormone synthesis was 5 mg/ml, while that for the prolactin fraction was 2-3 mg/ml. Adiga, et al. (1966) reported an optimal at 1 mg of pH 5 protein/ml for ACTH, and about 2-3 mg/ml for protein. Gospodarowicz and Laporte (1968) found maximum incorporation of ^{3}H-Pro into pituitary proteins in the presence of 4 mg of

supernatant protein. Since the products in each case represented contaminating material as well as the hormone, these results must be viewed with caution. These findings illustrate that the conditions which are optimal for the biosynthesis of one hormone are not necessarily optimal for the synthesis of another.

Addition of pH 5 supernatant factor (Fig 19) to the incubation mixture caused added stimulation of incorporation into growth hormone only when the concentration of pH 5 enzyme was suboptimal. The presence of this factor did appear to protect growth hormone somewhat from degradation (Fig 20B).

The time course for amino acid incorporation into the proteins was very similar to that reported by others (Von der Decken, 1967), with maximal activity occurring at 30-40 minutes. This period is much shorter than that reported by Adiga, et al. (1966), who were still able to measure an increase in labelling of protein after 90 minutes. Gosporadowicz and Laporte (1968) demonstrated that the rate of incorporation was dependent on the concentration of supernatant protein present. Since the standard system of Adiga, et al. contained 1 mg/ml protein while the optimal system for growth hormone synthesis contained 5 mg/ml, this may help to explain the discrepancy. The decrease in synthesis after 45 min was not noted in either the Adiga or Gosporadowicz systems. Factors such as proteases or RNAse may contribute to these differences.

4. Biosynthetic efficiency of the pituitary ribosomal system

The present studies confirm and extend previous work which showed that ribosomes from pituitary tissue could incorporate radioactive amino acids into TCA-precipitable protein and ACTH (Adiga, et al., 1966). The value of 3.6% (Table XXII) for the efficiency of incorporation of labeled amino acids into pituitary hormones is slightly higher than the 2% previously reported for experiments with ^{3}H-proline. The Leu and Phe used in the present research are more abundant in proteins. In the pituitary system, 3.5 and 7.0% of the label incorporated into protein could be attributed to growth hormone and prolactin, respectively. These values represent 0.1 to 0.3% of the total radioactivity employed.

These calculations illustrate some of the problems inherent in the study of hormone synthesis. Since incorporating activity is low, amino acids of very high specific activity are required, great care is necessary to be certain that the association of radioactivity with a protein represents true incorporation into the molecule.

5. Biosynthetic activity of bovine pituitary polysomes

As was expected, preparation from pituitary containing predominantly heavier polysomal-components was more active than a mixed ribosome preparation (Table XXIII).

A number of studies have been concerned with the correlation of the size of the polysomal aggregate with the length of protein being synthesized. Hemoglobin chains (mol. wt.

17,000) are optimally synthesized on polysomes containing five to six ribosomes (Wettstein, et al., 1963) and immunoglobulin light chains (mol. wt. 20,000-23,000) on polysomes containing seven to eight ribosomes (Williamson and Askonas, 1967). The current hypothesis is that the maximum aggregate size of a functional polysome should be compatible with that of the synthesized protein.

From the above studies it was anticipated that the biosynthesis of bovine growth hormone and prolactin, with molecular weights of 26,000 and 21,000, respectively, would be most active with polysomal aggregates containing seven to eight units. In fact, it was found that the synthesis in both cases was most active with polysomes containing six to seven ribosomal units. The labeling obtained with polysomes of smaller aggregate size may reflect the completion of nascent peptides. The rather similar results obtained for general protein synthesis suggested that the average molecular weight of the mixed proteins synthesized in the cell-free system was likewise in the approximate range of 20,000-30,000. Gel filtration of the mixture of soluble proteins synthesized in the pituitary system did indeed show predominantly proteins of this weight range.

6. Characterization of radioactive products isolated from bovine pituitary cell-free incubation mixture

Since no single characteristic is sufficient to establish the identity and purity of a protein, the radioactive products

were examined by several techniques.

a. Growth hormone

The labeled biosynthetic product was precipitated by 50% $(NH_4)_2SO_4$ and eluted from an Amberlite CG-50 column at the same pH and ionic strength as the standard (Fig 14). Subsequent gel filtration of the preparation (Fig 23) established that the labeled material was of the same molecular weight as growth hormone; it contained a small proportion of peptides and free amino acids, but no aggregated material. When the growth hormone was mixed with NIH carrier hormone, reprecipitated by $(NH_4)_2SO_4$ and rechromatographed, the radioactivity was coincident with the absorbance at 280 mμ of the hormone standard.

Upon polyacrylamide disc gel electrophoresis, the isotope migrated to the same position as that of the endogenous hormone present in the preparation and of the standard hormone, run concurrently (Fig 24). Regions of labeled material corresponding to free amino acids and to aggregated material were also detected. When subjected to density gradient centrifugation (Fig 25), the labeled material moved to the same position as standard growth hormone. The peak was symmetrical both with respect to optical density and radioactivity, and its position corresponded closely to that of authentic bovine growth hormone. It seems likely that free amino acids and peptides would be separated by this method (Fig 25A).

The radioactive growth hormone was incubated with rabbit anti-bovine growth hormone serum, and the antigen-antibody complex was separated from the unreacted material by gel filtration. As is shown in Figure 26C, the labeled hormone became bound to globulin. Addition of nonlabeled NIH-bovine growth hormone during incubation inhibited the labeled material from complexing with globulin, as would be anticipated for a specific antigen-antibody reaction. By addition of excess antibody it was possible to show binding of 40% of the labeled material (Fig 26E). Incubation of the labeled growth hormone with a rabbit anti-bovine serum showed no formation of globulin complexes (Fig 26F), indicating that the binding to the anti-growth hormone serum was specific.

Accordingly it is concluded that a protein has been synthesized with the same molecular weight, electrophoretic mobility, and immunological properties as bovine growth hormone. Although fragments of growth hormone could be bound by anti-growth hormone, it is unlikely that they would show the same electrophoretic mobility or sedimentation on a sucrose gradient as growth hormone. It remains important to investigate the distribution of radioactivity within the molecule. Separation of a tryptic digest of the labeled material on an ion-exchange column such as that described for human growth hormone by Li (1960) could give valuable information, if adequate radioactive material

could be accumulated.

b. Prolactin

Similar criteria were applied to radioactive prolactin eluted from a DEAE-cellulose column (Fig 2 and 15). After gel filtration in basic buffers (Fig 27), the majority of the radioactivity was eluted at the void volume, corresponding to the aggregated material seen earlier with prolactin isolated from slices. Although an increase in absorbance at 280 mμ was noted in the region of molecular weight corresponding to prolactin the related radioactivity was insignificant. A peak of isotope in the prolactin region was present only in a sample from a cell-free incubate that had been isolated with fresh pituitary tissue as carrier (Fig 27D).

Polyacrylamide gel electrophoresis (Fig 28) disclosed that although endogenous prolactin was present, the observed radioactivity did not coincide with the hormone. Density gradient centrifugation on sucrose (Fig 29) indicated that this labeled fraction was composed of low and high molecular weight material, possibly representing fragments and aggregates of prolactin, but no material corresponding to the monomer. From these experiments, it was concluded the radioactive material isolated from the ribosomal system by the present procedure was not the usual form of prolactin.

Many speculations arise regarding the possible fate of the prolactin. First, the isolation procedure, although satisfactory with material from slices, may not be suitable for isolating the minute amounts of material in ribosomal systems. Bovine prolactin is exceptionally soluble, even at its isoelectric point, and is much less stable than ovine prolactin (Bates, personal communication).

Treatment with 0.01 M mercaptoethanol was shown by Lewis and Cheever (1965), to change the mobility of prolactin in polyacrylamide gel electrophoresis. Only proteinaceous material was found at the origin when the hormone stood in 0.01 M mercaptoethanol for 16 hr at room temperature. Preparation of the cell-free system components and the incubation procedure required a minimum of 9-10 hr. During this time prolactin is subjected to 0.12 M mercaptoethanol at 5°. The presence of this reagent may have been the cause of some of the aggregated material observed in the isolated endogenous prolactin.

The requirement for B-mercaptoethanol or reduced glutathione has been established for other amino acid incorporating systems. However, as suggested by Kondo, et al. (1968) these sulfhydryl compounds may interfere with the formation of disulfide bonds. Bovine prolactin has six half cystine residues which form only intra disulfide linkages (Dixon and Li, 1964). A cystine residue forms a disulfide loop at the C-terminus of prolactin.

One must also consider that prolactin was extracted from the pituitary residue at pH 8.4, within the active pH range of the proteolytic enzyme described by Ellis, et al. (1968b) in pituitary extracts.

Synthesis of prolactin may require membrane-bound ribosomes such as are necessary for thyroglobulin. Morais and Goldberg (1967) were able to demonstrate synthesis of a thyroglobulin-like molecule only with a microsomal preparation. Ribosomes prepared by 1% DOC treatment were not active. Isolation procedures for prolactin usually employ alkaline alcohol (Bates and Riddle, 1935; Ellis, 1961; Jiang and Wilhelmi, 1965) or DOC (Kwa, et al., 1965) in order to free prolactin from granular material. These agents are surface activating, solubilizing lipids or lipoproteins in membranes. Preliminary studies had shown that microsomes incorporated ^{3}H-Leu into a protein fraction which was precipitated at pH 5.7. Unfortunately this material was not characterized further.

Another possible explanation of the failure to demonstrate synthesis of prolactin in a cell-free system may have related to the sex and physiological state of the animal used. Growth hormone synthesis is usually greater in males, while females show higher incorporation into prolactin (Catt and Moffat, 1967). Feed lot steers were the usual source of pituitary material in the present studies. Estrogen treatment is known to considerably increase the

prolactin content of the gland. Catt and Moffat (1967) were able to demonstrate rapid incorporation of ^{14}C-amino acids into prolactin *in vitro* with pituitaries of estrogen-treated rats. It would be ideal but unfortunately not feasible to obtain pituitaries from young dairy cows post-partum, or just after suckling. The prolactin-producing tumor transplantable in rats, described by Furth (1966) and Kwa, et al. (1967) would also be an excellent source of tissue for such studies.

It is also possible that prolactin is formed from a precursor molecule, by enzymatic cleavage. Such is the case with insulin, which originates from a larger precursor molecule (Steiner and Oyer, 1966). The *in vitro* studies reported by McLeod and Abad (1968) lend possible support for a precursor of prolactin. After 6 and 10 hr of incubation of rat pituitaries with isotopic amino acids, labeled material accumulated at or near the origin on polyacrylamide gel electrophoresis. After longer incubation period, this material disappeared. At approximately the same time the amount of labeled prolactin and growth hormone increased.

7. Critical evaluation

Sargent (1967), describing the ideal system for a study of biosynthesis in a cell-free system, lists the following characteristics for the protein under study:

> Firstly, it should be present in the system in relatively large amounts so that given a suitable isolation method, good yields of the pure protein may be obtained. Secondly, suitable assays for level of protein in the system offer considerable advantage during the isolation procedure, and enzymic assays or immunochemical assays, where possible, should be applied. Detailed studies of the site of incorporation of radioactive amino acids into the protein are greatly facilitated if the amino acid sequence of the protein is known. In the absence of such information a protein with a relatively small molecular weight will be much easier to work with than one with a large molecular weight.

With this statement in mind, the present system is not ideal. Although growth hormone and prolactin are the most abundant hormones in the pituitary gland, they account for only 3 and 5% respectively, of the total protein present. The isolation method described in this study is time consuming but with several purification steps involved, the chances of the final product being contaminated with extraneous radioactivity, are minimized (at least in the case of growth hormone). Radioimmunoassays, or biological assays at various stages of purification would have greatly facilitated these studies.

The yield of ribosomes from pituitary tissue was low; only 0.2 to 0.4 mg r-RNA/g of tissue. However, the ribosomes were very active and fairly stable in protein synthesis. A superior system for basic studies of pituitary hormones may be the transplantable rat pituitary described by Furth (1966).

The entire sequence of human growth hormone has been determined (Li, et al., 1966). Portions of the sequence of bovine growth hormone have been resolved and the primary structure should be completed in the near future (Santome, et

al., 1968). In contrast, only 4 to 5 residues of the C- and N-terminal amino acid sequences are known for prolactin. Although the molecules are relatively small as protein go, difficulties are encountered because of the tendency of these proteins to form aggregates.

The present studies have described the optimal conditions for the biosynthesis in a bovine pituitary cell-free system of a protein which is indistinguishable by physical, chemical and immunological properties from bovine growth hormone. Additional evidence should include investigation of the extent of labeling throughout the molecule. Using this system it may be possible to study more basic mechanisms involved in the synthesis of this hormone, such as mRNA.

Several explanations have been offered for the failure to demonstrate incorporation of labeled amino acids into prolactin with this ribosomal system. Pending development of methods to obtain aggregate free prolactin, and on further sequence analysis of this hormone these would be worthwhile projects to pursue in an attempt to determine the mode of synthesis of this hormone.

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