IN VITRO PRODUCTION, DISC GEL ELECTROPHORETIC ISOLATION AND PURIFICATION OF RAT PROLACTIN

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY
KENNETH H. KORTRIGHT

1973

This is to certify that the

thesis entitled

In Vitro Production, Disc Gel Electrophoretic Isolation and Purification of Rat Prolactin

presented by

Kenneth H. Kortright

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Physiology

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Date November 9, 1973

ABSTRACT

IN VITRO PRODUCTION, DISC GEL ELECTROPHORETIC ISOLATION AND PURIFICATION OF RAT PROLACTIN

By

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An efficient method for the isolation and purification of biologically and immunologically potent rat prolactin in high yield is herein reported. Adenohypophyses from estradiol benzoate-pretreated female Sprague-Dawley rats were organ cultured for periods of up to eight days. Medium was harvested from these cultures every two days until their termination. The individual batches of medium were separately pooled, centrifuged, and concentrated. Volume reduction was accomplished on an Amicon Diaflo apparatus at 20 PSI N_2 (nitrogen) pressure. Prolactin was isolated from these media by preparative polyacrylamide disc gel electrophoresis. The electrophoretic column employed a 2.5% polyacrylamide stacking gel, 7.5% polyacrylamide resolving gel, with a modified Tris-HCl, Tris-glycine discontinuous buffer system. Column fractions exhibiting absorption peaks at 280 mμ were pooled and dialyzed for 48 hr. The dialyzed fractions were concentrated as before but at 55 PSI N_2 pressure. Following concentration these samples were lyophilized. The recovery of radioimmunoassayable prolactin from the starting medium was 67.6 $\pm$ 3.3% representing a yield of 42.3 $\pm$ 5.8 mg/gm of wet weight of pituitary tissue. The mean biological potency of 3 isolated batches was

29.0 ± .95 IU/mg. The efficiency of this system represents a significant increase in yield of rat prolactin equivalent in potency to the best rat lactogenic hormones reported thus far in the literature. These isolated fractions were compared immunologically and biologically with all other rat prolactins presently available. The gain in efficiency of this system was enhanced by using pituitaries from female rats pretreated with estrogen. Adenohypophyses from young female rats that had been pretreated *in vivo* with estrogen released 41% more prolactin than non-treated females. Moreover, pituitaries from old female rats pretreated with estrogen *in vivo* released 105% more prolactin than young pretreated females, and 69% more than old estrogen-pretreated males. Combining these effects with a starting medium of high prolactin-low total protein ratio organ culture medium, an efficient purification technique, and a high quality and quantity yield, provides an economical method for rat prolactin isolation and purification.

The statistical computations of pigeon crop-sac responses in the bioassays were treated differently than thus far reported. A linear regression equation was developed and precise confidence intervals were set on both standard dose-responses and predictions of unknowns. This method permitted as competent a means as available to account for heterogeneous variance in bioassay responses. Covariate and weighted regression analyses were applied as well but found to be unsatisfactory due to extreme variances encountered in individual pigeon responses. The degree of these variances were correlated with the season of year in which the assays were performed.

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Kenneth H. Kortright

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Physiology

1973

ACKNOWLEDGEMENTS

A statement praising all of those generous and empathetic people who contributed to the development of this chronicle on a biological research problem might better be achieved by a twenty-four hour eulogy. However, in view of tradition and economy, I will penuriously bestow my appreciation to those most deserving.

My gratitude is extended to Dr. Joseph Meites for providing the laboratory space and facilities necessary to work on this problem. Members of his laboratory, past and present, who aided in developing the data herein reported were Dr. Steven W. Clark, Gregory P. Mueller, and Mary K. (Spindler) Vomachka.

The Biochemistry Department's Dr. James L. Fairley, his student Richard Jagger, and the electronics shops' Ben Stutsman were all helpful in their offers of advice and encouragement.

Professor John L. Gill unselfishly interrupted many home and academic commitments to offer aid, advice, and encouragement, in fording the rapids of statistical analysis of the data. To Dr. Gill I offer my heartfelt gratitude.

To Dr. Robert K. Ringer, who pops chicken pituitaries from their in situ confines like blanching almonds, I can only say thank you from from the bottom of my heart.

Job placement, consultation, and encouragement was generously and sincerely offered by a grand renal physiologist. Thank you very much Dr. Collings.

Dr. William L. Frantz contributed greatly to my understanding and applications of the prolactin physiology in this work. Moreover, his wise and considerate advice (personal, academic, and professional) were greatly appreciated.

Thanks are also given to Dr. H. Allen Tucker for reviewing the thesis in Dr. Hafs absence.

Last, but not least, of my guidance committee is the seasoned and crusty philosopher Dr. E. Paul Reineke. Words cannot express feeling for a man you would be proud to call Dad. His pursuance of statistical analyses of thesis data is sometimes extreme, but that is the Sam Brody in his blood. What thanks could mean, Paul, words will never say.

There are two secretaries who have made this all possible. To them, a note in these acknowledgements should be self-explanatory. My warmest gratitude to both of you--Amylou Davis and Nancy Turner. Thanks!

I also have a son, Kristopher Scott, whom I will long to have at my side one day. His existence continues to drive my energies towards being the best man, scientist, and father, I can possibly be.

There is another person that deserves special thanks for her warm and sincere encouragement, during periods when all life seemed cold. To Mrs. Leona (Leigh) A. Dunn, thank you for being you.

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INTRODUCTION

Prolactin History

The seemingly ubiquitous nature of the actions of prolactin, which hails by a variety of aliases (prolactin, galactin, galactopoietic hormone, mammotropin, mammotropic or mammogenic hormone, lactogen, lactogenic hormone, luteotrophic hormone, luteotropin, or paralactin) has recently been reviewed by Bern and Nicoll (1968) and Nicoll and Bern (1972). "Prolactin" (PRL) is the name coined for the first anterior pituitary (AP) protein hormone to be extracted in partially pure form (Riddle et al., 1932a & b, 1933). This active principal has become most prominent in worldwide research interest owing to its multiplicity of actions in numerous vertebrate species (Nicoll and Bern, 1972). Prolactin's role in mammary gland development, function, and tumourigenesis has largely contributed to the interest surrounding this hormone (Meites et al., 1972a, b, & c). To characterize the rapid advancement of the study of PRL, the amino acid analysis of ovine PRL was completed very early by C. H. Li (1949) and sequenced by the same investigator (Li, 1969, 1972), as well as others (Seavey and Lewis, 1971). Recent technological advancements have permitted more efficient isolation, with less molecular damage, of adenohypophyseal protein hormones. These methods will be reviewed under "Methods of Isolation". In order to appreciate the approaches utilized in these isolation studies and their

findings, a resume of the history and physiological roles of PRL is essential. Interpretation of some of these studies may be enhanced by Appendix A, which tabulates the terminology employed in describing the biological actions of PRL.

Prolactin Bioassay History

The existence of PRL, initially called "galactin" for its galactopoietic activity in rabbits (Turner and Gardner, 1931), was demonstrated by Stricker and Grüter (1928, 1929). These workers discovered that some anterior pituitary principal was responsible for the initiation of lactation in previously developed mammary glands of the rabbit, bitch, hog, and cow. It was soon after concluded that this lactational response was not due to the gonad-stimulating hormones (Grüter and Stricker, 1929; and Corner, 1930). Using extracts similar to those of Corner (1930), Turner and Gardner (1931) brought spayed virgin female rabbits into complete mammary development. These findings were confirmed in ovariectomized rabbits by Asdell (1931), and Nelson and Pfiffner (1931). Riddle and Braucher (1931) demonstrated that this same principal could stimulate mucosal epithelial proliferation ("crop milk", Beams and Meyer, 1931) in doves and pigeons. Riddle and Braucher (1931) were also the first investigators advocating the use of pigeon crop-sac proliferation as an assay procedure for the lactogenic principal. Using beef, sheep, and hog pituitaries the lactogenic hormone was extracted, partially purified, named "prolactin", and utilized in developing a preliminary crop-sac bioassay in pigeons (Riddle et al., 1932a & b, 1933). Other reports confirming

the crop-sac, "crop milk" production, assay method soon followed: Lyons and Catchpole (1933a & b), Lyons and Page (1935), McQueen-Williams (1935), McShan and Turner (1936). Refinements of these methods, reporting the later accepted bioassay of PRL, were given by Lyons (1937) and Reece and Turner (1937). The preferred method was based upon intradermal injections of 0.1 ml volumes of test materials into the feather follicles over the crop of fledglings. Following a four day injection regime, using both ventrolateral sides of the crop-sac as test sites, the pigeons were killed. The crop-sac was then removed, cleared of excess fat and muscle tissue, held to the light and subjectively rated upon the area of mucosa epithelial proliferation using a rating index of one to four. The credit for developing this "micro-method" of the pigeon crop-sac assay ($PCSA_m$) was given to Lyons (1937). More recent developments of this technique are given in Tanabe et al. (1954), Grosvenor and Turner (1958), and Kanematsu and Sawyer (1963). The presently accepted and most quantitative version of the crop-sac bioassay, micro-method, is that of Nicoll (1967). Nicoll's bioassay method was used in this study. Systemic injection regimes, although less sensitive an assay than the intradermal method, were reported by Lyons (1937) as the "macro-method" and later modified and refined by Bates et al. (1963), and Nicoll (1969).

Other Assays

A proliferative mitotic activity in the crop-sac had also been demonstrated in response to PRL (Bergman et al., 1940; Dumant, 1965) which coincided with nucleic acid synthesis (Brown et al., 1951; McShan et al., 1950; Bern and Nicoll, 1968). Using this knowledge Damm et al.

(1961) developed a PRL assay in pigeons on the basis of ^{32}P uptake by the crop-sac epithelium. Ben-David (1967) developed a similar assay on the basis of ^{3}H-methyl-thymidine uptake by crop-sac epithelium. More recently, double antibody assays called radioimmunoassays (RIA), employing ^{131}I-or ^{125}I-labeled standards, have been developed. Arai and Lee (1967) and Bryant and Greenwood (1968) developed the ovine prolactin RIA; Bryant and Greenwood also measured bovine PRL with the ovine PRL RIA. Kwa and Verhofstad (1967) and Niswender et al. (1969) developed the rat prolactin RIA, and Bryant and Greenwood (1972) developed the human prolactin RIA. The latest of these assays, which is a measure of both immunological and biological activity of PRL, is the radioreceptor assay (Frantz and Turkington, 1972; Shiu et al., 1973). These receptor assays are capable of measuring multispecies prolactins.

The lactogenic or mammotropic actions attributed to prolactin were based upon the ability of this principal to initiate lactation in developed mammary glands of many species. PRL also initiated sequential development of the lobuloalveolar and ductal components of the mammary tree as demonstrated by Corner (1930), Turner and Gardner (1931), Asdell (1931), Nelson and Pfiffner (1931), Gardner and Turner (1933), Lyons and Catchpole (1933a & b), Lyons (1937, 1941, 1942), Bergman and Turner (1940), Folley and Young (1941), and Gardner and White (1941), as reviewed by Lyons (1941, 1942) and Folley (1952). A review of the methods of assaying for mammotropic, lactogenic, and crop-sac stimulating activities of prolactin is given in Bergman et al. (1940) and Lyons (1941). The latest assay developments for lactogenic activity involve the synthesis of casein by mammary tissues in vitro (Turkington et al., 1965;

Turkington and Topper, 1966; Turkington et al., 1967; Beitz et al., 1969; R. L. Ceriani, 1969; Frantz and Kleinburg, 1970; Frantz et al., 1972; and Turkington, 1972).

The "luteotropic hormone" title attached to PRL derives its name from the ability of this hormone to produce intense luteinization of the ovaries in virgin rats while concomitantly producing full development of the mammary glands (Selye et al., 1933a & b, as reviewed by Riddle et al., 1933). Moreover, Evans and Long (1922) had much earlier indicated a relationship between the anterior pituitary and the nature of the estrous cycles in rats. Dresel (1935) discovered that PRL caused constant dioestrus in mice which was confirmed by Nathanson et al., 1937, and extended to rats (Lahr and Riddle, 1936). Nathanson and Fevold (1938) demonstrated that PRL could cause maintenance of corpus luteal function in normal mice. These ovarian responses were substantiated as prolactin activities by Evans et al. (1941). Koviačic (1962) developed a bioassay for PRL based upon the prolongation of dioestrus in mice. This method was later modified to utilize the corpora luteal cell nuclei count (CLCN) as a more reliable assay parameter (Wolthuis, 1963). A deciduoma assay was reported as yet another bioassay for PRL that same year (Koviačic, 1963). However, it has recently been proposed, and accepted, to drop the "luteotropic" title used as a synonym for PRL (Short, 1972).

Osmoregulation of serum sodium in fish (Ball and Ensor, 1967) and osmolar content of serum in humans (Buckman and Peake, 1973) has been established as a direct physiological role of PRL. Originally, Ensor and Ball (1968) proposed the name "Paralactin" for the PRL molecule in fish

responsible for osmoregulatory actions on serum sodium balance of euryhaline teleosts in fresh water. Ovine prolactin was previously demonstrated to promote freshwater survival of hypophysectomized Poecilia latipinna, a cyprinodont fish (Ball and Olivereau, 1964). The effect of prolactin upon freshwater survival was first demonstrated by Pickford and Phillips (1959) and later confirmed as the single most important factor of the anterior and posterior pituitary principals in freshwater survival of yet another euryhaline teleost, Fundulus heteroclitus (the killifish, Pickford et al., 1965, 1966a,b). She also demonstrated that aldosterone, cortisol, extracts of corpuscles of stannius, hog renin, and parathyroid hormone did not inhibit the rapid fall in serum chlorine nor serum osmolality. Concurrently, Ball and Ensor (1965) demonstrated that ovine prolactin was effective in preventing the rapid decline of serum sodium in hypophysectomized Poecilia latipinna placed in fresh water, which was later confirmed by those same workers (Ball and Ensor, 1967). Their later work demonstrated that serum calcium and potassium were not affected by either hypophysectomy, placement in fresh water or exogenous prolactin treatment. An assay for multispecies prolactin was then developed in hypophysectomized Poecilia latipinna (a cyprinodont fish) in fresh water, measuring the maintenance of serum sodium levels as the response parameter (Ensor and Ball, 1968).

Prolactin Biosynthesis

The essential element in isolating a protein hormone is obtaining a highly potent and concentrated starting material at minimal expense. Therefore, the controls under which prolactin is produced in vivo might

provide a key for obtaining potent starting material for isolation and purification studies. Neural regulation of PRL secretion was initially suggested by Selye (1934) finding that the suckling stimulus causes prolactin release from the adenohypophysis of the rat. Hypothalamic control of prolactin secretion was indicated by *in vivo* work of Desclin, 1950, 1956; Haun and Sawyer, 1960; Meites and Hopkins, 1960; Nicoll, Talwalker, and Meites, 1960; Everett, 1954, 1956; and Talwalker, Ratner, and Meites, 1964. *In vitro* studies which support the *in vivo* work were done by Pasteels (1961a & b) and Meites, Kahn, and Nicoll (1961). These rat adenohypophyseal (AP) organ culture studies provided additional evidence for central nervous system inhibitory control of PRL secretion. PRL secretion occurred autonomously in AP's cultured in these systems. Upon the addition of hypothalamic extracts or co-cultures of hypothalami and AP's, PRL secretion was inhibited (Pasteel, 1961b, 1962; Meites, Kahn, and Nicoll, 1961). Pasteels (1962) compared rat AP PRL release *in vitro* in the presence of hypothalamic and cerebral cortical extracts. PRL release was retarded in the presence of hypothalamic extracts as opposed to little or no inhibition of release under the influence of cerebral cortical extracts. Subsequent studies confirming these findings have been reviewed by Meites and Nicoll (1966), Bern and Nicoll (1968), Nicoll and Bern (1972), and Schally et al. (1968, 1973).

Steroids seem to markedly affect the *in vivo* release of pituitary prolactin. Although gonadal steroids were first thought to inhibit hypophyseal secretions (Moore and Price, 1932), a different picture indeed has since been elucidated. Cytological changes in the anterior pituitary correlated with different phases of the estrus cycle were first

demonstrated in the woodchuck by Rasmussen (1921). Later Charipper and Haterius (1930) confirmed these findings in the rat. Wolfe and Cleveland (1933) reported similar findings in the domestic dog and sow. It was suggested that different cell types and their numbers in the anterior pituitary were correlated with the phase of the estrus cycle (Cleveland and Wolfe, 1932). Severinghaus (1934) reported alterations in eosinophil counts in pituitaries of mature female rats treated with pregnancy urine. This report was soon after confirmed by Nelson (1934) who noted degranulation of adenohypophyseal esoinophiles of male and female rats treated with oestrin. This evidence was demonstrated in the face of his earlier reports of increased chromophobic cell populations with a concomitant decrease in basophiles in pituitaries of animals under oestrin treatment (Nelson, 1933a & b). Nelson's work (1933a) further demonstrated that a 2-fold higher dose of oestrin or testis hormone was required to maintain normal AP cytology in castrate male rats than in females. Adenohypophyseal eosinophile degranulation was demonstrated by Wolfe (1935) using large amounts of oestrin. The first reports demonstrating an increased pituitary PRL content in response to estrone or estradiol was by Reece and Turner (1936) for male rats and for female rats and male guinea pigs by these same authors (Reece and Turner, 1937). Testosterone proprionate and diethylstilbestrol were also shown to augment lactogenic hormone content *in vivo* of female rats and male guinea pigs (Lewis and Turner, 1941). These findings were extended to the male rabbit that responded to estrone treatment *in vivo* (Meites and Turner, 1942). The *in vivo* stimulatory effects of estradiol, estrone, and testosterone on increased AP prolactin content were extended to yet another agent (norethynodrel,

a synthetic progesterone) by Kahn and Baker (1964, 1966), and Kahn et al. (1965) in the female rat. More recently, Nicoll and Meites (1962) demonstrated a direct effect of estradiol upon female rat pituitary prolactin production in organ culture for periods of up to 3 weeks. These observations were confirmed by the demonstration that AP's from female rats pretreated with estradiol (50 µg estradiol benzoate/day for 6 days) (referred to as a "small dose") released more prolactin than non-pretreated female rat AP's in organ culture (Ratner et al., 1962). Gala and Reece (1964) were unable to confirm the direct effect of estradiol on AP prolactin release in vitro, but did reproduce the results of Ratner et al., 1963) using much lower doses of 1 to 10 µg/day for ten days. The increased synthesis and release of prolactin in vitro by AP's from estrogen-pretreated rats was further confirmed by using leucine-4,5-^{3}H incorporation studies (Catt and Moffat, 1967; Macleod et al., 1969).

Methods of Isolation

Alterations in the pituitary protein profiles in relation to estradiol and cortisol treatment were achieved through the use of analytical polyacrylamide disc gel electrophoresis (a-PAGE) (Lewis et al., 1965). This a-PAGE method offering higher resolving power than achieved before in analyzing mixtures of proteins was introduced by Ornstein and Davis (1962), Davis (1964), and Ornstein (1964). Soon after its introduction this technique was employed in isolating bovine and human growth hormone and displaying the protein hormone profiles of rat pituitary extracts (Lewis and Clark, 1963). The following year an apparatus for preparative polyacrylamide disc gel electrophoresis (p-PAGE) was demonstrated by

Jovin et al. (1964), which would revolutionize protein purification. Rat prolactin and growth hormone were the first hormones partially purified by the use of this technological advancement (Groves and Sells, 1968). These isolations, as all other prolactin purification procedures before this, extracted the hormone directly from pituitary glands (Kwa et al., 1967, Groves and Sells, 1968, Ellis et al., 1969, Neill and Reichert, 1971, for the rat; Cheever et al., 1969 for the mouse; Lewis et al., 1968 for bovine growth hormone and prolactin; Lewis et al., 1971 for human prolactin). Those utilizing organ culture medium as starting material fared better in their quantity and quality of hormone yields of rat PRL (Catt and Moffat, 1967, Gala, 1970, 1972; and human growth hormone, Kohler et al., 1971). This technique appears to offer at least one of the major steps toward developing a method to economically isolate highly purified and biologically and immunologically potent rat prolactin in high yield. The questions remaining, then, are what are the optimal methods for obtaining good starting material, and how may it be concentrated, fractionated, and processed with minimum loss to yield a large amount of active hormone?

MATERIALS AND METHODS

Experimental Animals

Sprague-Dawley adult male and female rats (Spartan Research Animals, Inc., Haslett, Mich.) were divided according to sex with four per cage and maintained on Purina Rat Chow and water ad libitum. Caging environment consisted of a room temperature of 23°C and a photo period of 14 hr. fluorescent light and 10 hours darkness, with the midpoint of each phase at noon and midnight, respectively. These animals were pretreated daily for five days with subcutaneous injections of 5 µg of estradiol benzoate (Nutritional Biochemical Company, Cleveland, Ohio) in corn oil. On the morning of day six, the rats were killed by decapitation and the pituitary was immediately removed and placed in Medium 199 (Lot #528601, Difco Labs., Detroit, Mich.).

Organ Culture Procedure

The adenohypophysis (AP) was separated from the neurophypophysis, cut into 16 pieces (explants) approximately 1 mm^3 in size, and cultured according to the methods of Fell and Robinson (1929), Chen (1954), as modified by Nicoll and Meites (1963), and Barnawell (1965). The explants were placed on polyester (Dacron) organdy rafts supported by stainless steel benches in 2.0 ml of Medium 199 containing 50 I.U./ml of penicillin (Penicillin G, Potassium, Lot #4440, Nutritional Biochemical Company, Cleveland, Ohio) and 5 µg/ml of insulin (Amorphous Beef Insulin,

Lot #PJ-657, Eli Lilly and Co., Indianapolis, Indiana). Cultures were incubated at 37°C in a humidified atmosphere of 95% O_2, and 5% CO_2, at a pH of 7.4. At the end of every 48 hr. the medium was changed during culture periods of up to 6-8 days. The culture media were collected and pooled within sexes, and samples were taken for protein and prolactin analyses. Pooled medium and 48 hr. interval samples were quick frozen and stored at -20°C to await further treatment as shown in Figure 1.

Concentration of Medium

Optimal Conditions for Concentration: In order to determine the optimal conditions under which volume reduction of organ culture medium should be handled, a series of three conditions were tested. These conditions were determined from previous work on a similar apparatus employed in isolating human growth hormone (Lewis et al., 1969), and advice from Dr. Deal's lab (M.S.U.) on concentration of proteins similar to PRL in molecular weight (T. Massey, personal communication). A test batch of media, 150 ml in volume, was harvested from cultures of 110 AP's from animals varying in size, weight, pretreatment, and condition of health. The original volume of medium (150 ml) was mixed with a magnetic stirring device and Teflon stirring bar at 2°C to insure homogeneity of the mixture. This medium was aliquoted into three portions of 50 ml each. Each batch was separately sampled for protein and prolactin analyses. All of the concentration conditions tested utilized the Amicon Diaflo Apparatus (Model #52, Amicon Corporation, Lexington, Mass.) with a 65 ml capacity and the Amicon UM-10 membrane (Lot #379, 43 mm diam.). This membrane possessed a molecular exclusion limit of approximately 10,000.

All volume reductions were carried out under N_2 pressure at 2°C with rapid magnetic stirring. The three conditions tested were: (I) concentration at 55 PSI N_2, (II) concentration at 20 PSI N_2, and (III) concentration at 20 PSI followed by three washings. The latter washings consisted of restoring the concentrated medium three times to its original volume with cold deionized H_2O and concentrating as before. In no instance was the magnetic stirring rapid enough to cause vortexing of the medium thereby risking protein denaturation.

Actual Concentration Conditions: Media from five cultures, three from cultures of 50 rat AP's each and two from cultures of 25 rat AP's each, were utilized as purification starting material. Each of these samples was separately quick-thawed in a 37°C water bath, with agitation, taking care to remove the tubes from the water bath while still containing ice crystals. This procedure insured maintenance of media temperature at approximately 4°C. Once thawed, the media were separately centrifuged in polycarbonate tubes at 30,000 g's for 30 minutes (Sorval RC2-B, Head #SS-34) at 2°C. Both the supernatant and precipitate were sampled for prolactin content. The supernatant was then concentrated on an Amicon Diafiltration Apparatus (Model #52, Amicon Corporation, Lexington, Mass.) employing an Amicon UM-10 membrane (exclusion limit approx. 10,000, Lot #379, 43 mm dia., Amicon Corp.) at 20 PSI, N_2 pressure with rapid magnetic stirring. These samples were concentrated by a factor of 3.5 leaving a concentrate never below 14.9 ml or above 22.0 ml in volume. Aliquots of the filtrate and concentrate were analyzed for protein and prolactin content while the remaining portions of each were quick frozen and held at -20°C until used. Upon quick thawing, the

concentrates for preparative electrophoresis were made to contain 5% sucrose (wt./vol.) by the addition of dry, highly purified analytical grade reagent (Sucrose, Lot #50C-3080, Sigma Chemical Company, St. Louis, Mo.).

Preparative Disc Gel Electrophoresis

Preparative polyacrylamide disc gel electrophoresis (p-PAGE) was carried out according to the procedures of Jovin et al. (1964). Buchler's "Poly-Prep-200" column was employed with a Tris-HCl, Tris-Glycine discontinuous buffer system at a running pH of 10.3. The equipment, reagents, and buffer systems used in these procedures are tabulated in Appendix B. All stock solutions were prepared in 20 gallon lots and stored at 2°C for periods no longer than sixty days. Analytical polyacrylamide disc gel electrophoresis (a-PAGE) as well as frequent pH checks were used to check the buffer solution consistency. Fresh preparative grade polyacrylamide was weighed out and prepared for gels of each run. The stacking and resolving gels consisted of a 62.5 ml volume of liquid each, and were polymerized at 0-2°C. Following polymerization, the gels were of equal height at 35 mm each and 15.8 cm^3 in area. The top of the stacking gel was washed with upper buffer solution, and then the apparatus was charged with buffers and set-up for the run. Organ culture medium concentrates now containing 5% sucrose were slowly applied to the top of the stacking gel through 1 mm I.D. Tygon tubing with a peristaltic pump. Once applied, preparative electrophoresis was run at 40 ma constant current with a range of 200-400 volts at 0°C throughout. The elution buffer was circulated at a rate of 1.0 ml/minute while fractions were collected every five

minutes, i.e., 5 ml fractionation volumes. The elution buffer was monitored throughout the run at 280 mμ during fractionation and electronically recorded. The fractions were again scanned on a Beckman DBG spectrophotometer at 280 mμ and plotted. Aliquots of each fraction were taken for radioimmunoassay analysis of their prolactin content.

Processing Column Eluants

Dialysis: Fractions to be pooled for dialysis were determined on the basis of the protein elution patterns previously scanned and plotted. The dialysis tubing employed in this work had a 1.73 inch flat width and 1.125 inch inflated diameter (Lot #8-667E, Fisher Sci., Co.) and was boiled for 15 minutes in deionized, double distilled water, rinsed twice and held in 2°C deionized water for immediate use. Fractions were pooled directly into the dialysis bags and varied from 35 to 50 ml in volume depending on the column load. Fraction collector tubes were carefully aspirated free of solution using a pasteur pipette which minimized losses at this point. Each bag was placed in a 12 L., 2°C, circulating bath of deionized water for 48 hours. Aliquots were taken at this point for protein and prolactin analysis.

Concentration of Dialyzed Fractions

The pooled eluants were separately concentrated to a volume of 5-8 ml, irrespective of the starting volume, on an Amicon Diaflow Model 52 apparatus. The Amicon UM-10 membrane was employed at 55 PSI N_2 pressure at 2°C with rapid magnetic stirring. Following volume reduction the filtrate and concentrate were sampled for later analysis. The concentrate

containing two deionized H_2O washes, each of a 1.0 ml volume, of the dia-flow apparatus, was quick-frozen in 100 ml vessels and freeze-dried in a glass column lyophilizer (Model K563000, Kontes Glass Co., Vineland, N.J.). Contents of the lyophilizing vessels were easily removed with a small sterile spatula and placed in screw cap vials (cap loose) in vacuum desiccators containing Drierite and stored at room temperature.

Deionized H_2O washings of each vessel, each of 1.0 ml volume, were lyophilized and desiccated as before. Total dry yields were based upon weighings of the 72 hr desiccated materials, both initial and rinse lyophilization products, on a Cahn Electro Balance (Model G, Cahn Instu-ment Co., Paramount, California). Aliquots were taken for analysis as described in the following section. The remaining materials were stored under vacuum at -20°C.

Analytical Techniques

Bioassay Procedure: The intradermal pigeon crop-sac assay for prolactin reported by Lyons (1937), as modified by Nicoll (1967), was used to determine the biological potency of isolated batches of rat prolactin. The paired assay procedure was employed as described by Nicoll and Meites (1963) using 6-week old white king pigeons (Cascade Pigeon Farm, Cascade, Mich.) weighing 370.7 $\pm$ 8.9 gms (S.E. mean, n=100). Material from each isolated lot was carefully weighed out on a Cahn Electro Balance and dis-solved in 0.9% NaCl solution at a pH of 8.0. Total doses of 1, 3, or 10 µg of lyophilized column fractions were injected intradermally on one side of the crop-sac while a known dose of ovine prolactin (NIH-P-S8, 28 I.U./mg, National Institutes of Health, Bethesda, Maryland) was

injected over the opposite hemicrop. Four levels of ovine prolactin were employed as references in each assay: 1 µg, 3 µg, 10 µg and 20 µg. Assays were conducted over a three day period in which each pigeon was injected intradermally twice daily (each injection volume 0.1 ml), with control and experimental material on days one and two. On day three, approximately 20 hours following the last injection, the pigeons were killed by decapitation. The crop-sac of each pigeon was removed and the mucosal epithelium 4.0 cm in diameter around the center of the injection side on each hemicrop was scraped free using a rounded elevator tipped probe (J. Sklar Mfg. Co., Long Island, N.Y.). The mucosal epithelium from each hemicrop was placed in a pre-weighed foil pan and dried overnight in an oven at 100°C. Following a drying period of 10-12 hours, each pan was placed in a desiccator, brought to room temperature, and weighed to the nearest 0.1 mg on a Mettler Balance (Model H18, Mettler Instrument Corp., Highstown, N.J.). Analysis of the hemicrop responses to the standards and unknowns are described under statistical methods.

Radioimmunoassay Procedure

The rat prolactin radioimmunoassay was performed as originally described by Niswender et al. (1969) and modified as described in K. H. Lu et al. (1971). The reference preparations used in developing the dose response curves were NIAMD-RP-1 at 1/7 ng/µl and KK-RP-1 at 1/7 ng/µl (see results). A comparison of the standards presently used for radioimmunoassay of rat prolactin was made on the basis of biological and immunological activity, and logit transformations (Rodbard et al., 1968;

Midgley et al., 1969) of standard inhibition curves as described in the results section.

Lowry Protein Test

Analysis of sample protein content was performed using the Lowry Protein Assay (Lowry et al., 1951). Bovine serum albumin (BSA), Fraction IV (Lot #A7044R, Grand Island Biological Co., Grand Island, N.Y.) was used as the reference preparation.

Iodination of Rat Prolactin

The radioiodination procedure has been previously described in Niswender et al. (1969) and modified to incorporate 0.25 mC_i of ^{125}I (New England Nuclear, Boston, Mass.). Iodinations were evaluated on the basis of plotting the activity eluted from 0.5 x 15 cm Bio-Gel P-60 (BioRad Laboratories, Rockville Centre, N.Y.) columns developed with 0.05 M phosphate buffered saline at a pH of 7.5. Eluants of one ml each were counted in a manual well type counter (Model #DS-202 (V), Nuclear-Chicago Corporation, Des Plaines, Illinois). Comparisons were made between our preparations and NIAMD-I-1 (courtesy of A. F. Parlow) and H-10-10-B (courtesy of Dr. S. Ellis) rat prolactins.

Analytical Polyacrylamide Disc Gel Electrophoresis

An estimate of purity of the isolated protein fractions was made on the basis of analytical polyacrylamide disc gel electrophoresis (a-PAGE). Preparations used in these analyses were KK-RP-I and H-10-10-B. The KK-RP-I material was one of the first isolated and purified lots of rat

prolactin from this work. Dr. Ellis's preparation, H-10-10-B, was used as the control in this procedure. The electrophoresis of these two ^{125}I-labeled preparations was carried out on 0.5 x 6.5 cm, 7.5% gels, at 220 volts in a continuous buffer system of 1.538 M tris, 0.0713M Na_2 EDTA, and 0.2286 M boric acid. The pH of this system was 8.9 with a running temperature of 0°C. Each gel was layered with approximately 25 µl of the protein hormone iodination medium and transfer solution before the run commenced. Current was applied to the gels at a rate of 1 ma/gel for 15 minutes followed by 3 ma/gel for 67 minutes. The total run time was 82 minutes. Following electrophoresis, the gels were gently removed from the glass tubes, wrapped in Saran wrap, side by side, and X-ray film laid over the gels for a period of 3.0 minutes. The autoradiographs were then developed.

Statistical Analyses

Bioassay Results: The bioassay results were statistically treated by regression analysis employing the following equations:

$$Y = B_0 + B_1 \text{ (Log } X) \qquad \text{Linear Regression Equation}$$

and

$$\frac{Y - B_0}{B_1} = \text{Log } X \qquad \text{Inverse Regression Equation}$$

where:

Y = dried crop-sac tissue response (mg
B_0 = origin of the regression slope on Y axis
B_1 = slope of the regression line
X = dose of prolactin

The standard curves for each bioassay were plotted according to the results of the regression analysis and an equation representing each curve was developed. Confidence intervals were computed for the origin (b_0), slope (b_1), mean levels of the standard (P-S-8, ovine prolactin), and the predictions from the regression equations (using the inverse regression method for unknowns). Equations employed in developing these confidence intervals at the 95% level are given as follows:

Confidence Interval on the Origin @ 95% level

$$b_o = \pm t\ \alpha/2\ (n-2)\ (\sqrt{SS_e/n-2})\sqrt{(1/n) + \bar{x}^2/SS_x}$$

Confidence Interval on the Slope @ 95% level

$$b_1 = \pm (t\ \alpha/2,\ n-2)\ (\sqrt{SS_e/n-2})/\sqrt{SS_x}$$

Confidence Interval on Predictions from the Regression Curve at 95% level

$$g = (b_1^2 - t^2\ \alpha/2,\ n+m-3\ \frac{(SS_e + \sum_{i=1}^{m}(Y_o - \hat{\bar{Y}}_o)^2)}{n+m+3}$$

$$h = (\hat{\bar{Y}}_o - \bar{Y})^2/SS_x = (n+m)\ (g/nm)$$

$$C.I. = \bar{x} + (b_1(\hat{\bar{Y}}_o - \bar{Y}))/g \pm t\ \alpha/2,\ n+m-3\ \frac{(SS_e + \sum_{i=1}^{m}(Y_o - \hat{\bar{Y}}_o)^2}{n+m+3}\ (h/g)$$

Covariate analysis (Finney, 1964) of the crop-sac responses to the levels of reference preparation used did not aid in linearizing the data. Weighted regression analysis (Steele and Torrie, 1960) was applied to the standard dose-response curve (responses to four levels of the reference). This method was found to be inadequate to handle the non-homogeneous variance encountered with all four reference levels. When responses from only three levels of the standards (1, 3, and 10 µg) of bioassay

experiments I and II were statistically treated, the linear regression equation was the only method under which the data could be adequately handled. Bioassay experiment III was also treated with the linear regression analysis and found to possess homogeneous variance over the entire range of the 1, 3, 10, and 20 μg levels of ovine PRL. Tests for homogeneous variance were made with Hartley's (1950) maximum F-ratio test and 10% table points (Beckman and Tietjen, 1973). Using the 10% tables decreases the probability of making a Type II error, allowing higher choice of alpha (α) levels of confidence in the experiment. Any extremely large responses in known reference dose-response curve analyses or pigeon responses to unknowns were tested for being "Significant Outlying Observations" after Grubbs and Beck (1972). If these tests were positive, the data were analyzed both in the presence and absence of the extreme observations.

Radioimmunoassay Results

The standard curve and unknowns (isolated column fractions) were developed in quadruplicate for each point of a fifteen point (level) progression. The assay dose response curves ranged from 143 to 28,600 pg (or 1/7 ng to 200/7 ng), respectively (Appendix Q). The data obtained following radioimmunoassay were analyzed by converting the dose responses to "logits of Y" (Logit (Y) = $\log_e$ (Y/1-Y) where Y = labeled hormone bound to antibody divided by the amount of labeled hormone bound to the antibody in the absence of unlabeled hormone) on a log-log scale producing a linear regression plot after Rodbard et al. 1968, and Midgley et al., 1969. The radioimmunoassay results are reported in terms of nanograms per milliliter (ng/ml).

RESULTS

The method of isolation and purification of rat prolactin developed from the application of techniques previously described is outlined in Figure 1. Further reference to each step of this procedure will be covered in sections of the results.

Organ Cultures

Estrogen Pretreatment: Pituitaries from 30, two-month-old, female Sprague-Dawley rats were cultured following two *in vivo* pretreatment regimens. Fifteen of these animals were pretreated with corn oil in a volume of 1.0 ml/day for five days. The remaining fifteen were treated with 5.0 µg estradiol-benzoate per day in the same volume of corn oil for the same number of days as the controls. The release of prolactin by the adenohypophyses (in micrograms prolactin/AP = µg PRL/AP) of these two groups is graphically shown in Figure 2 and tabulated in Appendix B. After the first two days in organ culture the AP's from estradiol-benzoate pretreated rats had released an average of 106.3 $\pm$ 1.9 (mean $\pm$ standard error of the mean) µg PRL/AP. The controls released only 62.1 $\pm$ 2.2 µg PRL/AP. This difference in µg PRL/AP released constitutes a 71% increase in release of PRL by the pretreated group. An additional two days in culture following a medium change resulted in a smaller prolactin release by both groups. Thirty-two percent more prolactin was released by AP's from the pretreated group of animals (61.0 $\pm$ 1.1 vs. 46.3 $\pm$ 1.1 µg PRL/AP).

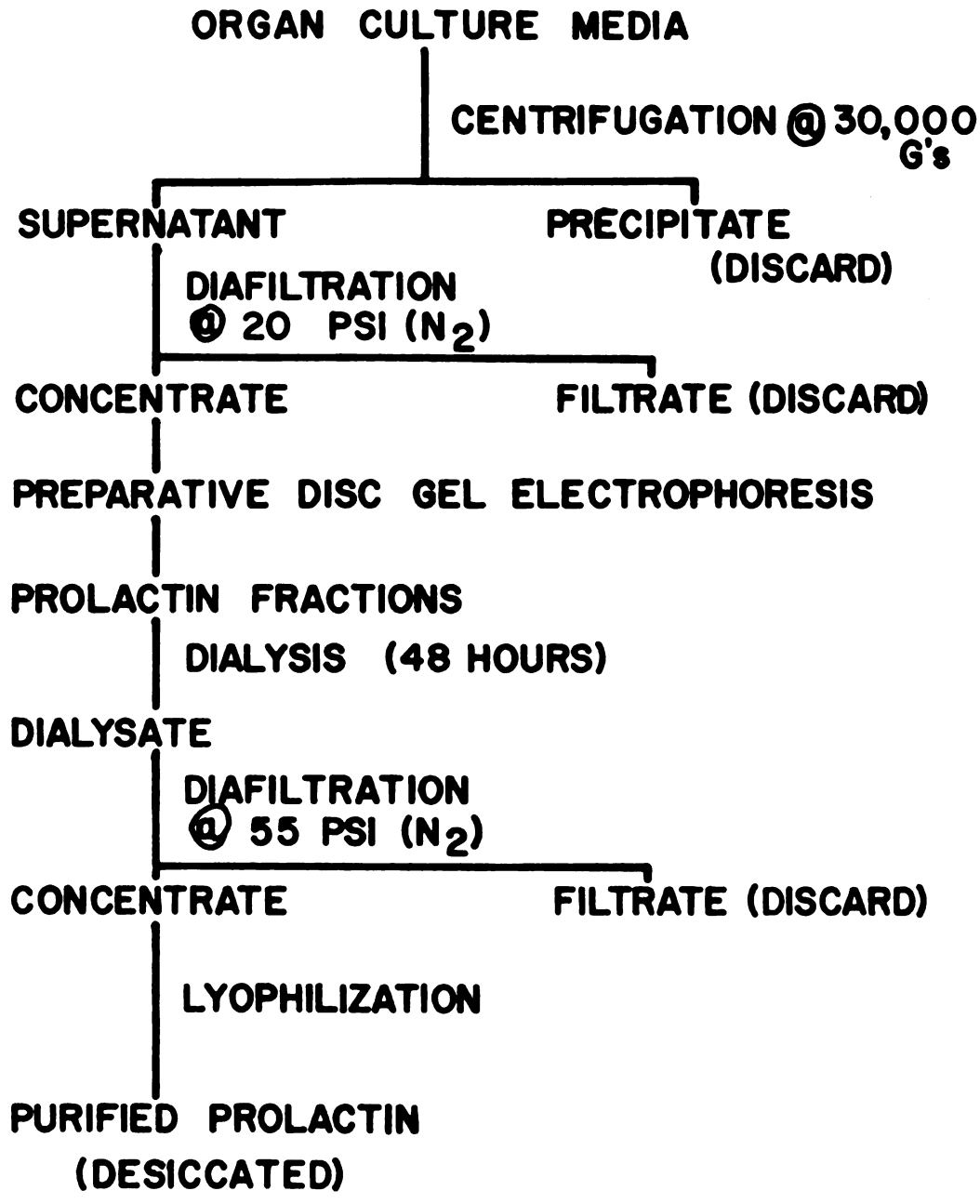

Figure 1. Prolactin Isolation Flow Chart.

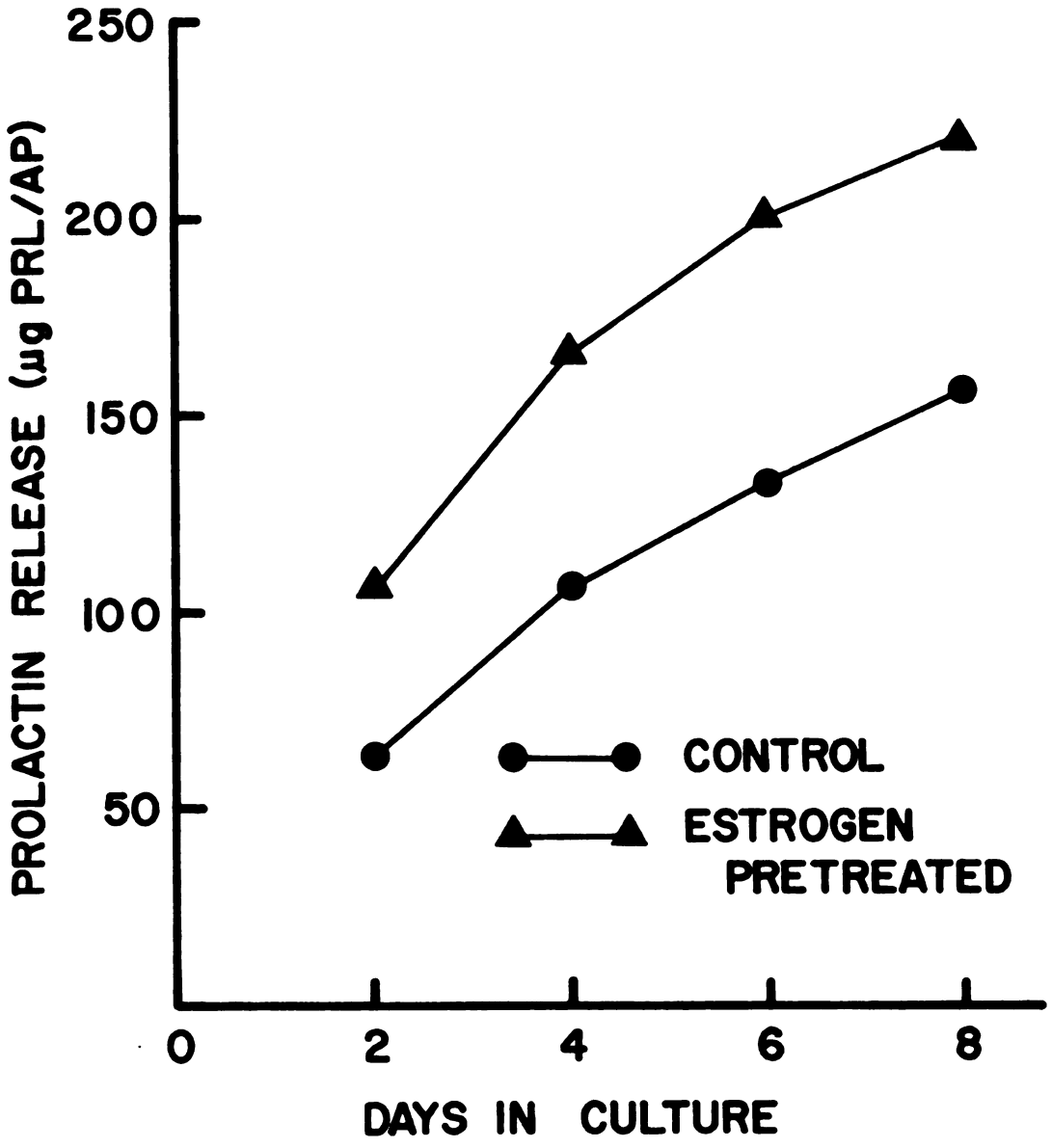

Figure 2. Cumulative yield of prolactin by controls and estrogen treated rats.

Continuing the cultures through day 6 (an additional 2 days in fresh medium) demonstrated a further decrease in PRL release by both groups. AP's from the pretreated group released 46% more PRL than their controls (32.7 ± 1.7 vs. 22.4 ± 0.2 µg PRL/AP) as shown in Figure 2 and Appendix B. Culturing these two groups of AP's for an additional two days in fresh medium (totaling 8 days in culture) revealed a further but smaller decrease in PRL release. AP's from pretreated animals released 19% of their 48 hr release level and the controls 40% (19.9 ± 0.2 vs. 25.1 ± 1.3 µg PRL/AP). Moreover, the non-pretreated group was at this point releasing 26% more PRL/AP than the group pretreated with estradiol-benzoate. The mean total PRL released by the group pretreated with estradiol-benzoate and cultured for 8 days was 220.1 ± 2.8 µg PRL/AP versus 155.9 ± 2.9 µg PRL/AP by the controls. This difference constituted a 41% increased PRL release by the steroid pretreated female rat AP's following 8 days in organ culture.

Effect of Age and Sex: Eleven-month-old male and female, and 3-month-old female Sprague-Dawley rats were pretreated *in vivo* with estradiol-benzoate (5 µg/day for five days in 1.0 ml of corn oil). The release of PRL by their AP's in organ culture was compared. There were ten animals in each group. The cultures were maintained for 6 days with 48 hr changes of fresh medium. The results of this experiment are reported as µg PRL released/AP and are graphically shown in Figure 3 and tabulated in Appendix C. After the first 48 hr in culture the AP's from old females had released an average of 343.7 ± 6.0 µg PRL/AP, whereas the AP's from old males had released 195.8 ± 3.3 µg PRL/AP. AP's from the young females released 181.9 ± 3.7 µg PRL/AP. These values constitute

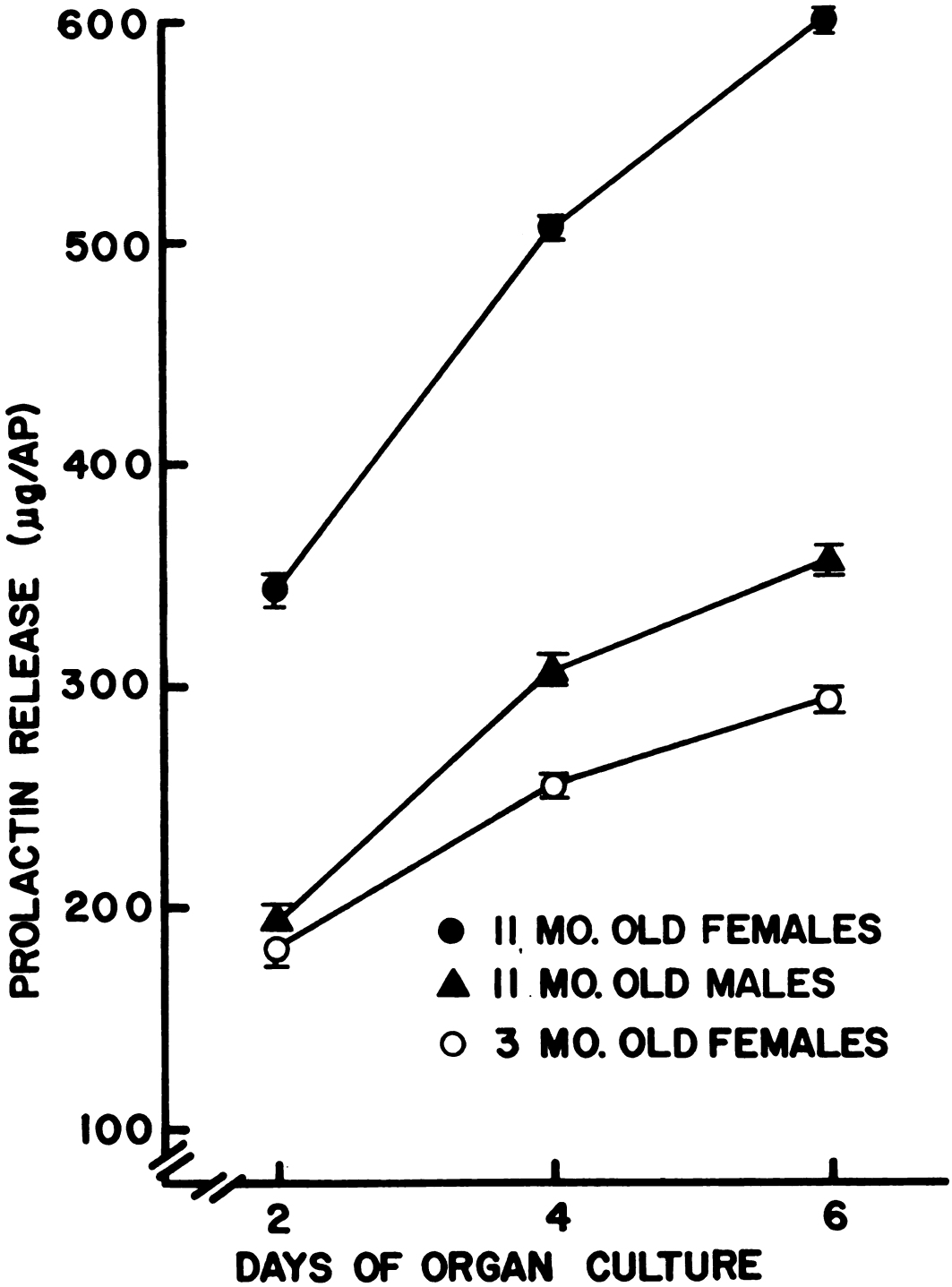

Figure 3. Effects of age and sex on prolactin release *in vitro*.

a difference of PRL release by the AP's from old females, 76% greater than from old males, and 89% greater than from young females. The AP's from old males released only 8% more PRL/AP than those from young females. After two additional days in culture, AP's from old females released 46% more PRL/AP than AP's from old males, and 126% more than AP's from young females. The actual mean values for hormone release after four days in organ culture are 162.1 ± 1.8 µg PRL/AP for AP's from old females, 110.7 ± 1.1 µg PRL/AP for AP's from old males, and 71.8 ± 2.2 µg PRL/AP for AP's from young females. By day 6 the average PRL released by cultured adenohypophyses expressed as µg PRL/AP had declined to 95.1 ± 2.3 for old females, 48.8 ± 1.2 for old males, and 39.2 ± 1.2 for young females. Therefore, AP's from old females had released 95% more PRL than AP's from old males and 143% more than AP's from young female rats. The mean total PRL released by these three groups of estradiol-benzoate pretreated rats were 600.9 ± 7.3, 335.3 ± 4.0, and 293.0 ± 4.7 µg PRL/AP, respectively (Appendix C). The mean cumulative totals of prolactin released by adenohypophyses from these three groups demonstrated that AP's from old females released 105% more PRL/AP than AP's from young females, and 69% more than those from old males.

Release of Prolactin and Protein in Culture: The amount of PRL per unit of protein released *in vitro* by adenohypophyses from ten, 3-month-old estradiol-benzoate pretreated female rats are graphically shown in Figure 4 and tabulated in Appendix D. These data are reported in µg PRL/AP released *in vitro*, and milligrams protein/AP (mg PROT./AP) released, and µg PRL/mg PROT./AP released in organ culture over a period of 8 days, at 48 hr intervals. Following 2 days in organ culture these AP's, on

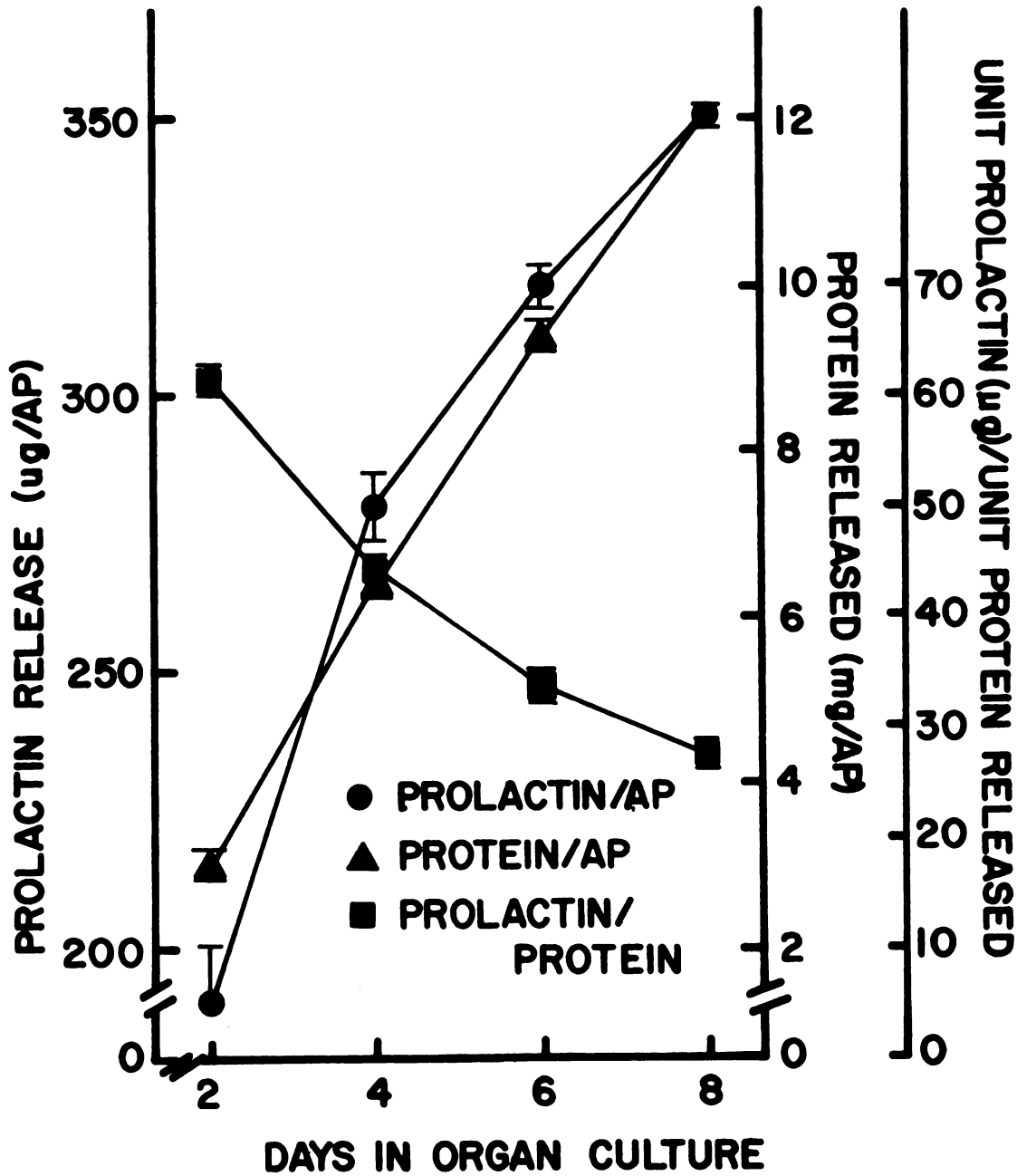

Figure 4. Prolactin and Protein release *in vitro*.

the average, released 190.3 ± 10.9 µg PRL/AP with 3.09 ± 0.02 mg PROT./AP, constituting a mean release of 61.5 ± 3.6 µg PRL/mg PROT./AP. By 4 days in culture (two additional days in fresh medium) the amount of prolactin released had declined to 89.3 ± 6.5 µg PRL/AP. This accounted for a 113% decrease in PRL release while the protein released increased to 3.36 ± 0.04 mg PROT./AP (a 9% increase from the 48 hr level). These data show a release of 26.5 ± 1.8 µg PRL/mg PROT./AP after 4 days in culture. The amount of PRL/unit of PROT. released by these AP's in organ culture thus dropped from the 48 hr level by 57%. These results thus denote a small increase in protein release but a marked decrease in PRL release per AP. Following 6 days in organ culture the PRL and protein released into the medium had declined by 55% and 9%, respectively (40.4 ± 3.1 µg PRL/AP, 3.05 mg PROT./AP). The ratio of PRL/unit PROT./AP concomitantly declined to 13.2 ± 1.0 µg PROL/mg PROT./AP constituting a 50% decline from the 96 hr level and a 79% decline from the 48 hr level. By day 8, the PRL/unit PROT./AP dropped 26% from the 6 day level, 63% from the 4 day level, and 84% from the 2 day level (0.8 µg PROL/mg PROT./AP). This decline was recognized as a 23% reduction from the 6 day level in PRL release but a 4% increase in protein release (31.0 ± 1.2 µg PROL/AP, 3.17 ± 0.06 mg PROT./AP). These data are graphically summarized in Figure 4. They denote a fluctuating but somewhat constant level of release of protein during a marked decline in PRL released by the 8-day organ cultured AP's

Medium Processing

Medium Centrifugation: The organ culture medium from five separate cultures composed of 25, 25, 50, 50, and 50 cultured AP's, respectively,

were quick-thawed and prepared for centrifugation. Since each culture of rat adenyhypophyses was continued for six days with 48 hr interval medium changes, the harvested medium from each change (i.e., 2, 4, 6 day) was sampled and pooled within cultures prior to centrifugation. The samples were immediately prepared for PRL RIA analyses. The remaining five batches of culture medium were centrifuged as previously described. The precipitate which formed during centrifugation amounted to 31.4 $\pm$ 3.1 mg (wet weight) and contained 1.10 $\pm$ 0.37 mg of PRL (Appendix I). This amount of PRL constituted a 3.8 $\pm$ 1.2% loss of the initial radioimmunoassayable PRL content in the starting medium (Figure 5, Appendix I).

Medium Concentration: In order to determine the most efficient conditions under which to concentrate organ culture medium of high PRL content on an Amicon Diaflo apparatus, a series of three conditions were tested. The results of this study are graphically displayed in the form of a histogram (Figure 6) and tabulated in Appendix E. From the data one can observe that a negligible amount of PRL or protein was detected moving through the membrane into the filtrate (Appendix E). However, the concentrates (volume of medium remaining above the filter following concentration) contained 67%, 83%, and 87% PRL resulting from concentration at 55 PSI N_2, 20 PSI N_2, and 20 PSI N_2 with repeated washes, respectively (Figure 5, Appendix E). The amount of protein recovered from the starting medium following concentration, is shown as protein content of the concentrates (Figure 6, Appendix E). These protein recoveries were nearly equal at 47%, 42%, and 48% for concentration conditions of 55 PSI N_2, 20 PSI N_2, and 20 PSI N_2 with repeated washes, respectively. A 36% increase in concentration with an additional 16% PRL recovery resulted from

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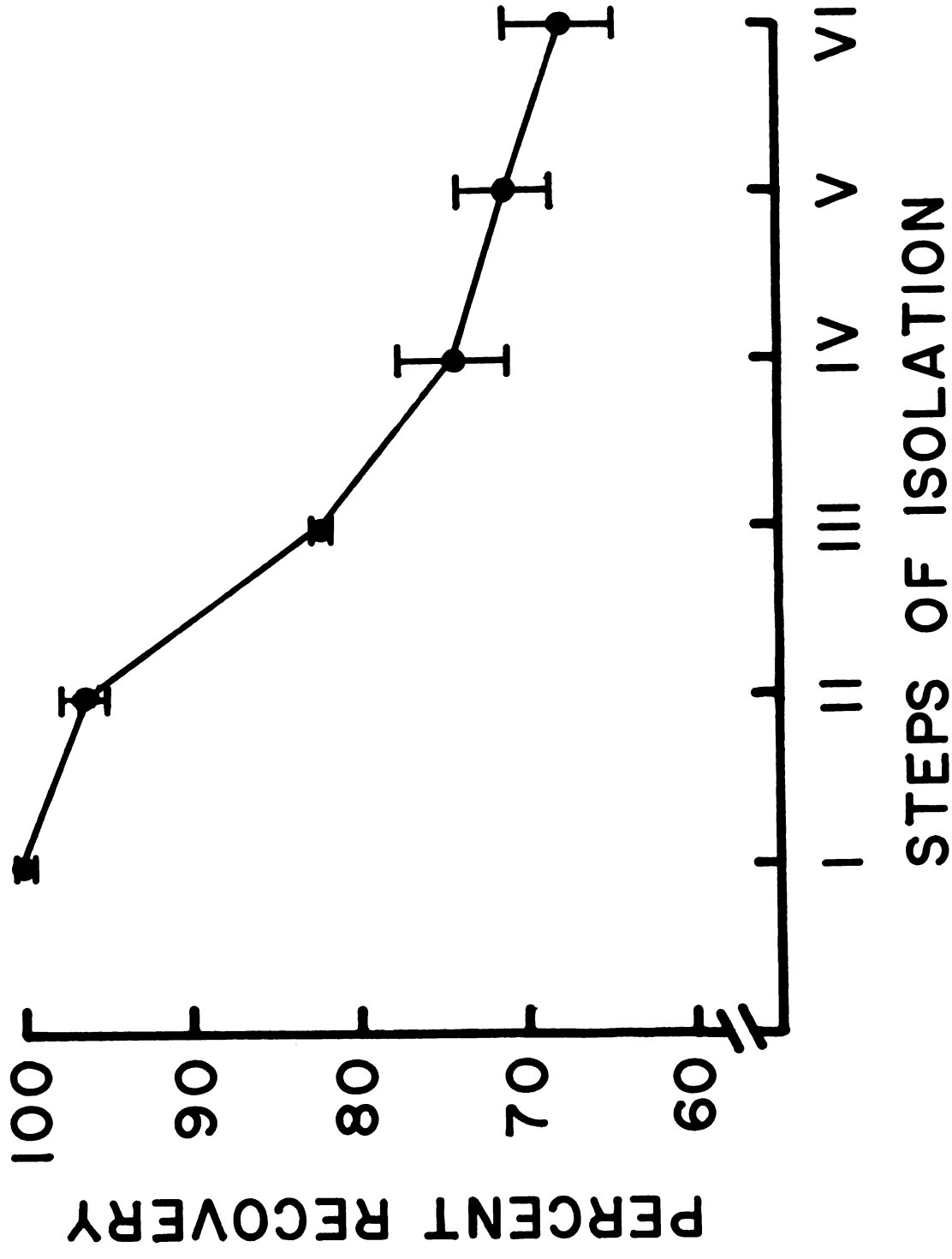
Figure 5. Mean recovery of prolactin at six stages of prolactin isolation.

(1) The steps in sequence represent the following processes:

I. Original medium
II. Medium following centrifugation
III. Medium following diafiltration/concentration
IV. Buffer following preparative electrophoresis/fractionation
V. Buffer fractions following dialysis
VI. Buffer fractions following diafiltration/concentration and lyophilization

(2) Losses are tabulated as losses attributed to that step alone, not from the total as a sequential loss tabulation.

(3) Recoveries in each batch of original medium are tabulated as sequential recovery of the total initial amount of prolactin.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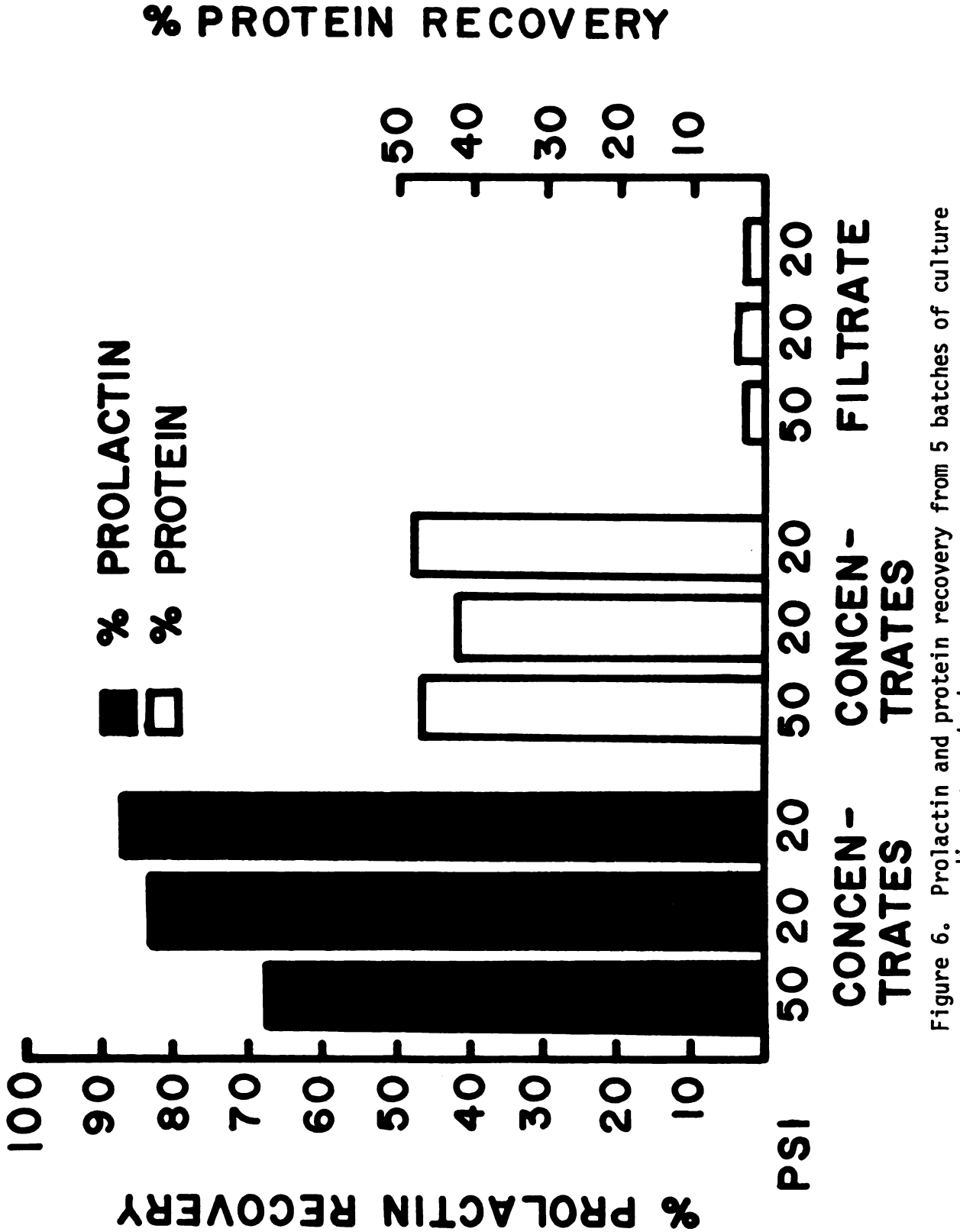

Figure 6. Prolactin and protein recovery from 5 batches of culture medium concentrate.

reducing the concentrating nitrogen pressure from 55 PSI to 20 PSI. However, employing 20 PSI N_2 with repeated washings permitted only a 4% gain in PRL recovery but required almost one additional working day to accomplish. Concentrating the organ culture medium at 20 PSI N_2 permitted 20% and 26% greater recovery of radioimmunoassayable PRL from the starting medium than did 55 PSI N_2. Therefore, the method of choice appears to be volume reduction at 20 PSI N_2 at 2°C with rapid magnetic stirring. It was interesting to note that the protein lost from the medium, during volume reduction, did not appear in the filtrate (Figure 6, Appendix E).

Column Protein Elution Profile: The absorption peaks at 280 mμ of the p-PAGE column elution buffer are graphically shown in Figures 7 and 8 and tabulated in Appendices F and G. Data given in Figure 7 and Appendix F were derived from the column run which provided the most biologically and immunologically potent rat prolactin in this study. A compilation of five column runs, including that in Figure 7 giving the mean protein elution profile for these data, are given in Figure 8 and Appendix G. The PRL fraction appears to be consistently eluted from the column between fractions 15 and 26. This represents a mean elution time beginning 14.7 $\pm$ 1.2 hr after the run began and peaking at 15.2 $\pm$ 1.2 hr. The PRL band was colored dark yellow and could be visually followed through the resolving gel as it moved toward the anode (+) at a rate of 0.13 $\pm$ 0.01 millimeters (mm) per minute (min).

Column Protein and Prolactin Elution Profile: To confirm the location of the PRL band on the protein elution profile, all fractions of the column during one run were assayed for PRL by RIA. The results of this

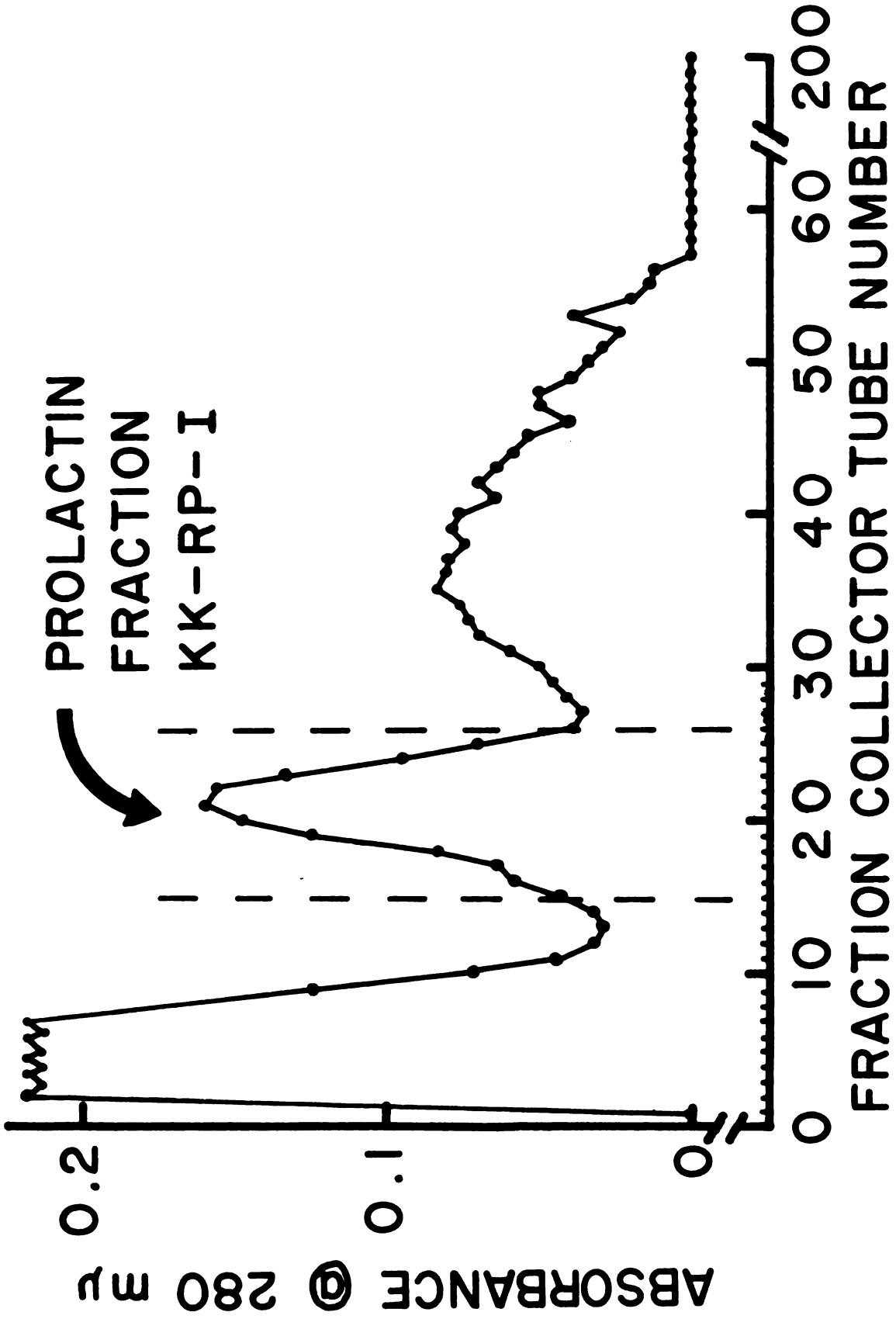

Figure 7. Preparative column protein elution profile.

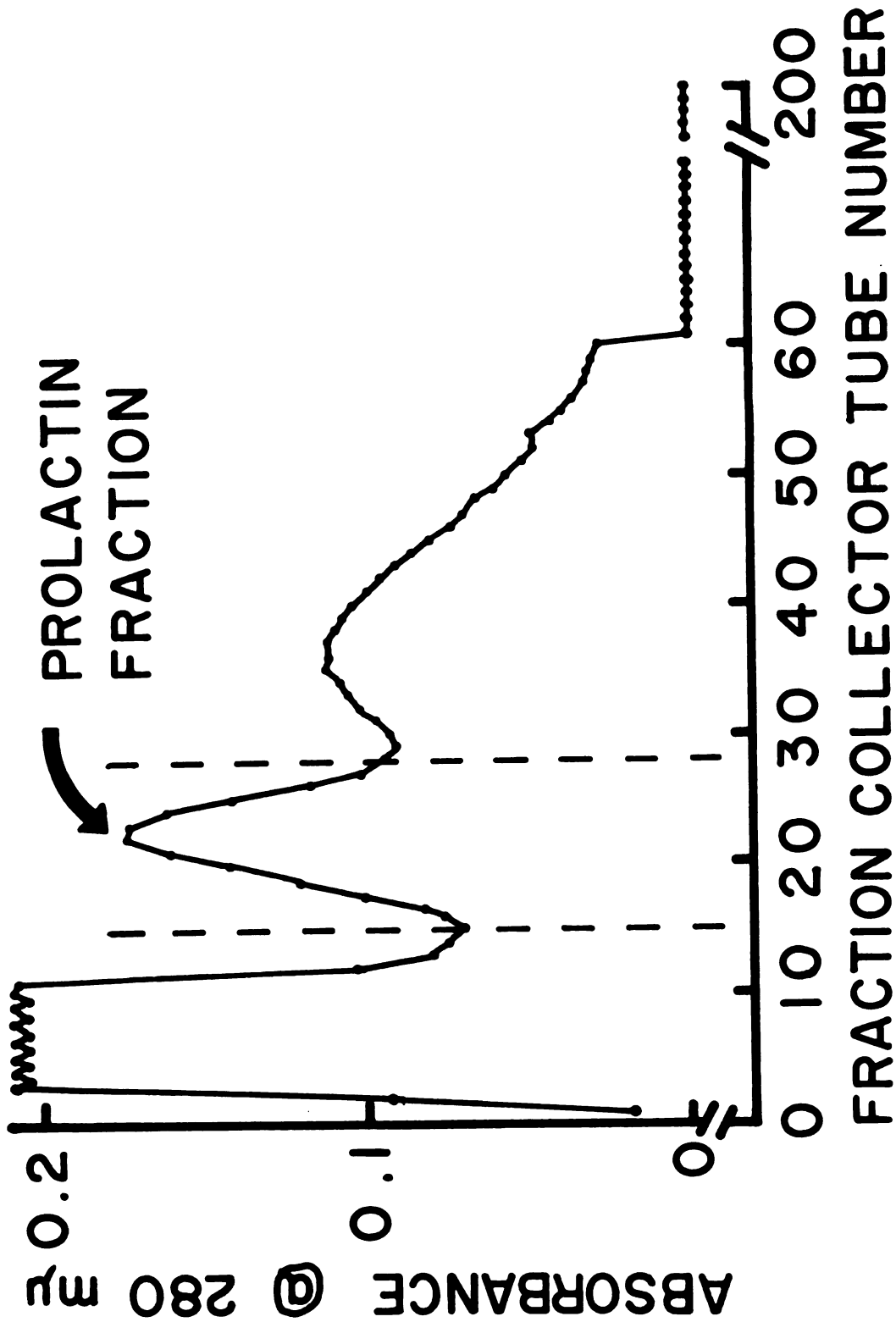

Figure 8. Preparative protein elution profile, mean for five runs.

analysis are shown in Figure 9 and tabulated in Appendix H. There appears to be identical agreement between the protein elution profile (measured at 280 mμ absorption) and the radioimmunoassayable PRL analyses (Figure 9, Appendix H). This confirms the location of the PRL band and by taking only tubes 15 through 26 insures a more pure (uncontaminated by prealbumins or albumins) product. It may be of interest to note that the yellow tag that PRL carries in p-PAGE appeared in all tubes registering a positive rat PRL RIA response. The color may be due to riboflavin. The yellow coloring was removed following 48 hr dialysis in deionized H_2O, but the PRL remained in the dialysis tubing.

Isolation and Purification Efficiency: The efficiency of each of the steps (Figure 1) of this procedure is reported in terms of mean percent loss (and total percent recovery) through each step (Figure 5, Appendix I). From the data collected one can see that the concentration step accounts for the greatest loss of PRL amounting to 14.5 $\pm$ 1.3%. Losses in the other steps ranged from 3.2 to 7.4% (Appendix I). The cumulative mean percent recovery of five complete isolations is 67.6 $\pm$ 3.3%. With the recent advent of Dupont's microfiber continuous flow dialysis and concentration systems it appears one could yet further improve on this efficiency.

Pigeon Crop-Sac Bioassay

The Standard Curves: The pigeon crop-sac bioassay "micro-method" (m-PCSA) was developed with four different levels of NIH-P-S8 (ovine prolactin, 28.0 IU/mg, see Appendix AA). In all three of the bioassays in this study, no dose was evaluated on less than six hemicrops. Some were assayed over as many as nine test sites. Each assay was analyzed by

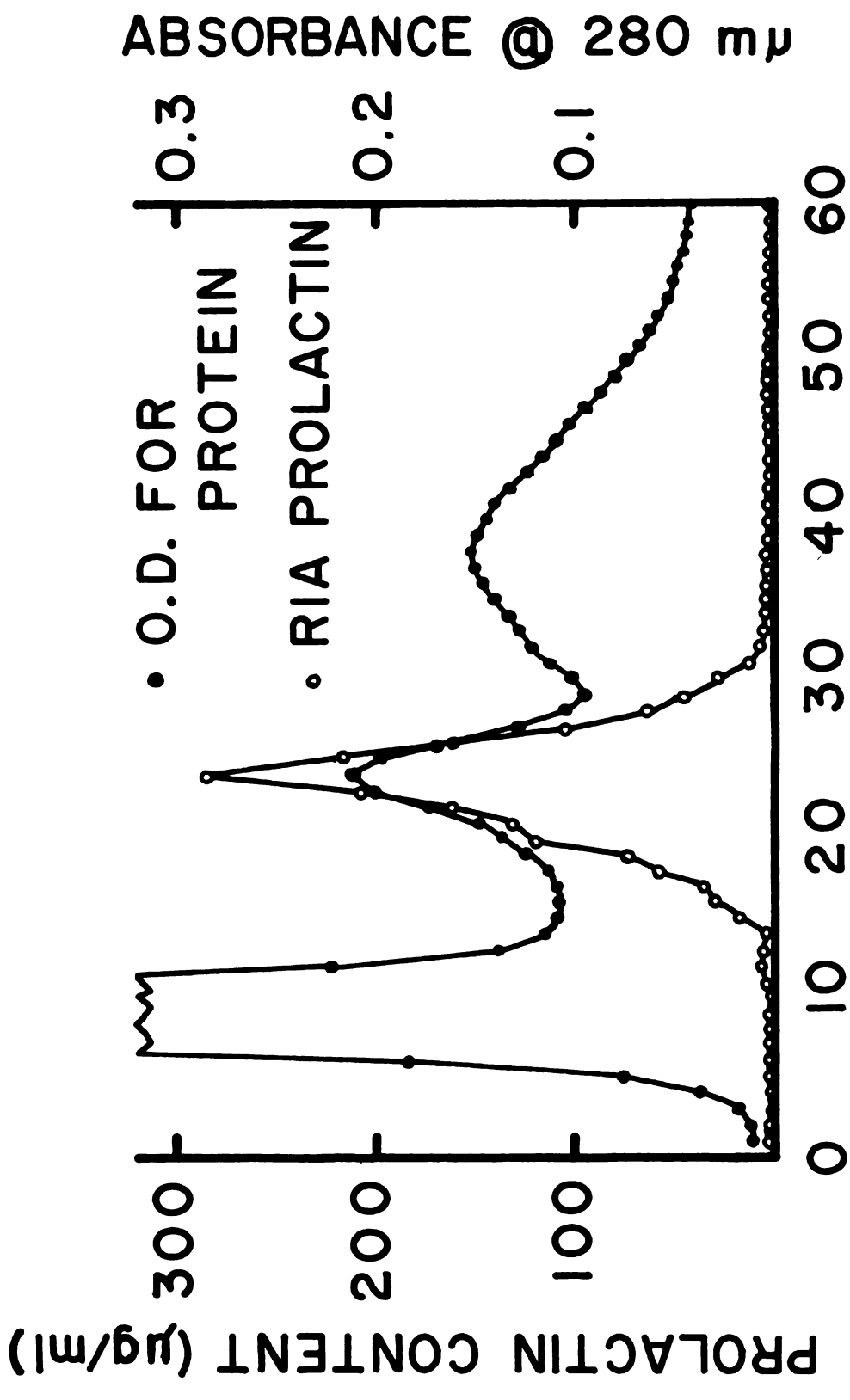

Figure 9. Preparative column protein and prolactin elution profiles.

linear regression analysis as previously described (Steele and Torrie, 1960). The crude data complete with body weights of each pigeon are tabulated in Appendices J, K, L, and M. The dose-response curves are graphically shown in Figure 10. The characteristics of each curve are summarized in Appendices J and K. The slopes of experiments I, II, and III are 13.9, 13.4, and 7.8, respectively (Appendix K). The bioassay correlation coefficients of experiments I, II and III are 0.85, 0.79, and 0.87. This indicates a close relationship between the crop-sac response and the log of the dose of prolactin (Appendix K). An index of precision used for comparing pigeon crop-sac bioassays in the past is lambda (λ). Lambda represents the standard deviation of Y on X (analyzed by linear regression analysis) divided by the slope of the curve (Bliss, 1952). Each of these indices falls well under those reported by the National Institutes of Health (Appendix AA) and represents a very reliable assay. Those λ values are 0.256 (Exp. I), 0.311 (Exp. II), and 0.287 for experiment III. Each of the pigeon crop-sac responses were analyzed by covariate analysis with body weight and body weight to the three-quarter power (Finney, 1964). The results of these analyses did not aid in linearizing the pigeon responses and therefore are not reported. Weighted regression analyses were also run on each of the bioassay dose-response curves after Steele and Torrie (1960). The heterogeneous variance (tested by Hartley's F-max test (1950) encountered in experiment one and two were too great to be dealt with by this method (J. L. Gill, personal communication). Therefore, a method of eliminating the heterogeneous variance which existed in experiments one and two, but not three, was sought. If the 20 µg dose level responses were dropped from the

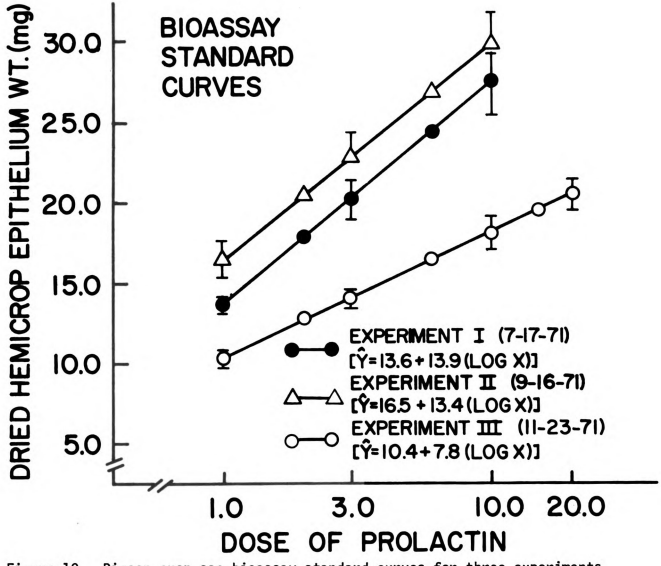

Figure 10. Pigeon crop-sac bioassay standard curves for three experiments.

regression analyses of bioassays one and two (as judged by Hartley's F-max test (1950)) homogeneous variance was obtained. Should heterogeneous variance have been retained, these data would have been biased and indicated acceptance of false responses as being accurate estimates of hormone potency (Type II error). Therefore, the 20 µg dose level of the standards in experiments one and two were deleted from these analyses.

Seasonal Pigeon Responsiveness: The slopes of the standard curves denote a variation with regard to the time of the year they were run (Figure 10, Appendix M, and K). Note that the slope of the dose-response curve in experiment three (Figure 10) is markedly less than either of those of one and two. Experiment three was run in November whereas experiments one and two were run in July and September.

Potencies of the Rat Prolactins: The isolated column fractions of rat prolactin as well as other rat prolactins were assayed over no fewer than six hemicrops each. The results of these analyses are tabulated in Appendices N, O, P, and Q. The individual crop-sac responses and the body weights of each of the animals yielding those responses are given in Appendix P. The estimate of the potency of each rat prolactin is tabulated in Appendix O. The mean relative potency judged from the mean of the crude responses, transformed by the inverse method of regression analysis, is given under the column marked $\bar{x}$ (mean). Confidence intervals set upon each of these estimates at the 95% level of probability, are also tabulated (Appendix O). Using the transformed mean of the crude observations, the highest estimates of potency for each batch tested is: 29.5 IU/mg for KK-RP-I, 25.2 for KK-RP-2, 27.1 for KK-RP-II, 27.0 for KK-RP-3, 26.4 for KK-RP-4, 29.5 for NIAMD-RP-1, 38.4 for NIAMD-I-L, and 25.5 for

H-10-10-B. Clearly, the most potent rat prolactins tested as judged from these responses are NIAMD-I-1, KK-RP-I and NIAMD-RP-1, respectively. A more accurate measure of potency is obtained by transforming each of the crude responses by the regression analysis, inverse method, and computing a new mean. However, since the log of a number is a non-linear treatment, one risks injecting error in converting this log response into international units per milligram (I.U./mg) of relative potency (by taking the antilog). This risk is a reasonable one if the variance of an assay is homogeneous and reasonably small. Therefore, in the cases where unknowns were tested in bioassay experments one and two, assays with greater variation within doses, the potency evaluation will carry a larger confidence interval. It is therefore desirable to choose the potency estimates which show the smallest confidence intervals and are more accurate estimators of hormonal activity. The relative potency evaluations of the transformed data indicate the most biologically active hormones are KK-RP-4, H-10-10-B, NIAMD-I-1, and KK-RP-I (Appendix O).

Radioimmunoassayable Potency and Dose-Response Curves: The radioimmunoassay dose response curves are reported in Appendices Q and R. Each assay was run with fifteen points in quadruplicate. The responses shown in Appendix Q are the mean responses for each level. A summary of these data showing the mean amount of prolactin (in nanograms, ng) required to give a 50% binding point, by least squares regression analysis, is shown in Appendix R. The smaller the amount of hormone required to reach a 50% binding point, the greater its potency. The regression slopes, intercepts, and correlation coefficients are also shown. It is interesting to note that all of the RIA dose-response curves have a

correlation coefficient of 0.99. Those assays showing the steepest slopes (obviously the most sensitive assays) are NIAMD-RP-1, NIAMD-I-1, KK-RP-I, KK-RP-II, and H-10-10-B, respectively (Appendix R). From these results the most active rat prolactins appear to be (in decreasing order): NIAMD-I-1, KK-RP-I, NIAMD-RP-1, KK-RP-3, KK-RP-II and -2, and H-10-10-B (Appendix R) in descending order of activity.

Summary of Potency Evaluations: A summary of the biological and immunological (radioimmunoassayable) potencies of the isolated column fractions is given in Appendices S and T. Appendix S gives the biological potency in terms of the transformed mean of the crude data showing the highest estimates of each. The mean relative potency of transformed responses is shown in Appendix T. Comments as to which is a more effective estimate of biological potency will be given under Relative Comparisons of the Rat Prolactins. The biological and immunological potencies of the five isolated column fractions in contrast to those of NIAMD-I-1, RR-1, and H-10-10-B are shown in Appendices U and V. The transformed mean of the crude bioassay responses is shown in Appendix U with the radioimmunoassayable potencies. Means of the transformed bioassay responses are tabulated with the radioimmunoassayable potencies in Appendix V. From these data it appears that the isolated column fractions KK-RP-I, -II, -III are equally as potent as NIAMD-RP-1 (also see C.I.'s in Appendix Y). The most potent of these rat prolactins is NIAMD-I-1, presently used for iodination in the NIH-RIA kits.

Relative Potencies of the Rat Prolactins: If one takes the biological and radioimmunoassayable potency of NIAMD-RP-1 (presently accepted national standard for RIA's) and divides it into each of the other

prolactin values, respectively, a relative index for comparison is yielded. These indices are shown in Appendix W and X. As in previously described tables, the biological potencies are shown as transformed means (Appendix W) and mean of the transformations (Appendix X). These data indicate that the biological potencies of NIAMD-I-1, KK-RP-I, and NIAMD-RP-1 are the highest. The immunological activity indices confirm these latter data as being the hormones utilizing the least amount of protein to obtain a 50% binding point (Appendix W). The index of biological potency per unit of immunological potency again shows the same three or four hormones as being the most potent, namely NIAMD-I-1, KK-RP-I, NIKND=RP-1, and KK-RP-II.

A summary of the potency estimates determined in this work is reported in Appendix Y and Z. The 95% confidence intervals on the biological potency estimates, taken from Appendix O, are also shown. These values were chosen as being those highest estimates of potency with smallest range of confidence intervals (i.e., the best of the estimates). The biological potency of the isolated column fractions is equivalent in all respects to those of the National Institutes of Health standards. On the basis of the mean alone, a rating of greater to lesser biologically potent rat prolactins is: NIAMD-I-1 with 28.5 IU/mg; KK-RP-I and II with 27.7 IU/mg; KK-RP-3, with 26.9 IU/mg; KK-RP-4 with 26.4 IU/mg, H-10-10-B (25.5IU/mg) and KK-RP-2 (25.2 IU/mg), and NIAMD-RP-1 with 24.2 IU/mg. In viewing the biological potency per unit of immunological potency, however, the same hormones rate the highest (Appendix Z), namely, NIAMD-I-1 at 1.64, KK-RP-I at 1.25, KK-RP-3 at 1.04, NIAMD-RP-1 at 1.00, and KK-RP-II at 0.99. These preparations are the most potent rat prolactins analyzed in this study.

DISCUSSION

A rapid and efficient method of fractionation and purification of AP organ culture medium has been developed for the isolation of highly potent rat prolactin. The pretreatment of old female rats with estradiol-benzoate increased the harvestable prolactin content of the organ culture medium 76.3% (mg PRL/gm AP) over that of non-pretreated groups. This yield was realized in the form of 42.2 $\pm$ 5.8 mg PRL/gm AP over a period of eight days in organ culture. However, since the medium harvested for preparative isolation was done over a six day period, the PRL released in the last 48 hr interval was calculated as being 9.5% (on the basis of mg PRL/gm AP) more than the yield for six days (based on releases reported in Appendix B and D, averaged). In contrast to the 42.2 $\pm$ 5.8 mg PRL/gm AP yield by estradiol-benzoate pretreated old female AP's, the AP's from pretreated young females produced only 17.3 $\pm$ 3.2 mg PRL/gm AP. Furthermore, AP's from non-pretreated animals yielded only 10 mg PRL/gm AP. These yields denote the AP's from old pretreated rats being 76.3% greater than non-pretreated and 59% greater than pretreated animals. These values not only agree but are 10% to 20% greater than yields formerly reported (Ratner et al., 1963; Gala and Reece, 1964). Estrogens were previously reported to have less of an effect on male than female rats in maintaining AP cytology following castration (Nelson, 1933a). The present results agree with these data (Figure 3; Appendix C) in comparing relative AP PRL content in response to *in vivo* estrogen treatment reported for male rats

(Reece and Turner, 1936) and female rats (Ratner et al., 1963; Gala and Reece, 1964; Chen and Meites, 1970). Estrogen pretreatment *in vivo* prior to AP organ cultures have been shown to markedly enhance prolactin release *in vitro* (Ratner et al., 1963; Gala and Reece, 1964; Catt and Moffat, 1967; MacLeod et al., 1969; and Chen and Meites, 1970). Moreover, the most effective dose in raising both PRL content of the AP and release *in vivo* was previously reported to be 5.0 μg estradiol-benzoate (Chen and Meites, 1970). However, these workers treated the animals longer than five days and would have realized a greater AP PRL content had they not done so. By removing the AP's at six days the best of all possible worlds was created. This permitted harvesting AP's with higher PRL content just prior to peak release responses to steroid treatment (i.e., less *in vivo* loss of hormone sought for release *in vitro*).

The release of PRL per unit of protein *in vitro*, indicated that the early culture medium contained a higher prolactin to total protein ratio (Figure 4, Appendix D). Therefore, early culture medium, 48 hr and 96 hr, was the more valuable for purposes of preparative electrophoretic resolution of the protein mixture.

Workers prior to this have used extraction directly from the pituitaries themselves or untreated AP's in organ culture (Kwa et al., 1967; Groves and Sells, 1968; Ellis et al., 1969; Gala, 1970, 1972; and Neill and Reichert, 1971). Catt and Moffat (1967), although using a less effective estrogen pretreatment, were the first to report the use of culture medium from AP's of estrogen-pretreated animals for the electrophoretic isolation of rat prolactin. Their yields were reportedly very low and only involved small amounts of labeled hormone. The yields of rat

prolactin (reported in mg PRL/gm AP) to date have been 0.5-2.3 by Kwa et al., 1967, 0.8 to 1.0 by Ellis et al., 1969, 7.0 by Groves and Sells (1968), and 27.1 by Gala, 1972. All of these systems report yields of rat prolactin in varying quantity and quality. The biological potencies reported by these workers were (given in IU/mg) 51.0, 28.0, 15.2, and 24.9, respectively. The assays used to evaluate this parameter are varied and no definitive statistical treatment of the data is shown or described. Moreover, although Gala (1972) recently reports a new potency of 28.4 IU/mg, there are little data to support his claim. Subsequently, Gala (1972, personal communication) reported to me that his isolated prolactin showed only one-third of the radioimmunoassayable potency as that of NIAMD-RP-1. It should be noted that the NIAMD-RP-1 he used as a reference is the same reference employed in the present work.

The efficiency of the system developed in this work gains much of its strength from pre-electrophoretic concentration and preparative electrophoresis fractionation. Although these steps contribute the greatest losses of PRL from the initial medium, they have been effectively minimized to mean percent losses of 14.6 $\pm$ 1.3 and 7.4 $\pm$ 3.0, respectively. The overall present recovery of PRL in the medium was 67.6 $\pm$ 3.3% and marks a distinct advance in protein hormone isolation work (Appendix I).

The pigeon crop-sac assays were carefully treated with a variety of statistical tools to insure the most accurate reports of relative potencies. The pigeon responses for the dose-response curves were analyzed for homogeneous variance using Hartley's F-max test (1950). Bioassay experiments one and two were found to contain marked heterogeneous variance whereas experiment three did not. Experiment three showed homogeneity

across all four dose levels of the standard. In an attempt to locate the source of heterogeneous variance in experiments one and two, analyses were run on extreme responses for each dose. These analyses were tests for outlying observations introduced by Grubbs and Beck (1972). Examples of such observations are the 10.9 and 62.8 mg responses to the 20 µg level of standard in experiment one (Appendix J and L). Both observations were significantly non-normal with respect to the mean of all of the 20 µg level pigeon crop-sac responses using analysis of outlyers. However, in ignoring these two observations during linear regression analysis of the dose-response curve of experiment one, the slope was markedly altered indicating bias. The possibility of committing a type II error was ripe. Moreover, Hartley's analysis for homogeneous variance validated the existence of heteroscedasticity. The remaining alternative was to drop the 20 µg dose level from regression analyses of bioassays one and two or drop the assays all together. Deletion of the 20 µg dose level in both assays eliminated the heterogeneous variance and therefore permitted use of these curves in analyses of rat prolactin potencies. The unknowns analyzed in these assays which may have otherwise been biased are noted in Appendix N. Precise determination of confidence intervals by the inverse regression analyses after Ostle (1963) permitted a realistic method of evaluating the predictions of relative potencies of the unknowns tested in each bioassay. This method involves the use of a non-linear log-antilog transformation. Any large variances in responses to unknowns are demonstrated by large confidence intervals for those potency evaluations. Therefore, the choice of whether or not to accept the log transformation of the crude mean or the mean of the log transformations of the individual

analyses of each unknown is accurately estimated by the breadth of the confidence intervals (C.I.'s). The choices of those responses demonstrating the greatest biological potencies per unknown with the smallest C.I.'s are summarized in Appendices Y and Z. These data clearly indicate that the isolated column fractions KK-RP-I, -3, and II are equipotent to the best references available (NIAMD-RP-1, NIAMD-I-1).

The slopes of the bioassays show a definite alteration with respect to the time of the year the assays were run (Figure 10, Appendices J, K, and L). These results indicate a steeper slope for assays run in the summer or early fall (bioassays I and II). It seems probable that the pigeon crop is less responsive in the winter since the Columbiformes do not normally breed during this season. The observation that the bioassay slope varies with season is confirmed and supported by previous work in the literature (Kanematsu and Sawyer, 1963).

The radioimmunoassay (RIA) procedure was used as yet another measure of the potency of these rat prolactins (after Niswender et al., 1969). Each of the dose-response curves was statistically analyzed by the most current logit response of Y versus log of the dose least squares regression method after Rodbard et al., 1968, and Midgley et al., 1969. These potency evaluations, based on the amount of hormone required to obtain a 50% binding point in each assay, confirmed the estimates of the bioassays regarding which rat prolactins are the most potent (Appendices Y and Z). If one develops an index of relative potency, as previously described, these conclusions are yet again supported. Therefore, the most potent rat prolactins, as judged from the index of biological activity per unit

of radioimmunoassayable (immunological) activity, are: NIAMD-I-1, KK-RP-I, KK-RP-3, KK-RP-II, and NIAMD-RP-1, respectively (Appendix Z).

An evaluation of the purity of the rat prolactins isolated in the present work stems from three sources. Two of these are the biological and radioimmunological potencies determined on a common solution of carefully prepared and fully solubilized hormone for each rat prolactin. The third parameter was the autoradiographic patterns developed from ^{125}I-labeled rat prolactins subjected to analytical polyacrylamide disc gel electrophoresis. Only one band of activity was found in the gels containing KK-RP-1 and H-10-10-B. These data indicate that there are no detectable labeled contaminants of the rat prolactins analyzed.

SUMMARY AND CONCLUSIONS

A procedure whereby rat prolactin was isolated and purified in higher yield than reported previously, was herein developed. The biological and immunological (radioimmunoassayable) potency of these purified lots of rat prolactin are equivalent to the best rat prolactins reported in the literature. A description of this procedure precedes a brief statement regarding the pretreatment, age, and sex of rats chosen as pituitary donors for the organ cultures. Organ culture medium of high prolactin to total protein ratio was centrifuged, concentrated, and fractionated by preparative polyacrylamide disc gel electrophoresis. Prolactin rich fractions were dialyzed for 48 hr, concentrated and lyophilized to provide the final product. Biological and radioimmunoassays were made on the isolated column fractions and compared with assays run at the same time on reference preparations of rat prolactin supplied by the National Institutes of Arthritic and Metabolic Diseases. A further test of purity was made by electrophoretic-autoradiographic analysis of the ^{125}I-labeled reference and column isolated rat prolactins in the present study. High yields of rat prolactin were achieved by culturing adenohypophyses from estradiol-benzoate pretreated, old female rats. Precise confidence intervals (C.I.'s) were determined on each of the biological assay parameters and predictions of the rat prolactin relative potencies. Regression analyses by the inverse procedure were utilized to achieve the narrowest C.I.'s

possible in precisely evaluating biological activities of these rat prolactins.

APPENDICES

APPENDIX A

TERMINOLOGY FOR THE BIOLOGICAL ACTIONS OF PROLACTIN

Term	Biological Activity
Lactogenic Hormone "Lactogen"	Initiation of milk secretion
Mammotropin, Mammotrophin or Mammogenic Hormone	Sequential development of ductal and lobuloalveolar components of the mammary tree
Galactopoietic "Galactin"	Maintenance and augmentation of milk synthesis
Pigeon crop-sac stimulating principle "Prolactin"	Proliferation of the pigeon crop-mucosal epithelium to produce "crop milk" in doves and pigeons
Luteotropic hormone, "Luteotrophin or Luteotropin"	Maintenance of corpus lutea and pregnancy in hypophysectomized rat and mice
Osmoregulatory principal or "Paralactin"	Maintenance of serum Na^+ levels in fresh water by hypophysectomized teleosts
	Red Eft water drive

APPENDIX AA

ENDOCRINOLOGY STUDY SECTION

Pituitary Hormone Distribution Program

Specifications for: PROLACTIN, OVINE, NIH-P-S8

PROLACTIN ACTIVITY: Prolactin activity was determined by the pigeon crop-sac weight method (1). The unweighted geometric mean potency, obtained from 5 replicated assays, was 28.38 I.U./mg. *The results of the individual assays are listed below.

Assay Number	Relative Potency I.U./mg*	95% Limits	Assay Lambda	Mean Relative Potency
1	33.53	16.27-69.11	0.339	
2	20.38	7.86-52.88	0.432	
3	23.19	45.52-11.81	0.325	28. I.U./mg*
4	25.54	9.89-65.96	0.433	
5	39.28	20.05-76.95	0.317	

*Results are expressed in terms of 2nd International Standard for Prolactin

CONTAMINATION WITH OTHER PITUITARY HORMONES:

GROWTH HORMONE ACTIVITY: GH was determined by the 10-day body weight gain test in female hypophysectomized rats (2) using the subcutaneous injection modification (3). The unweighted geometric mean potency, obtained from 2 replicate estimates, was 0.0033 USP (or International) units/mg. The results of the individual assays are listed below.

Assay Number	Relative Potency USP units/mg	95% Limits	Assay Lambda	Mean Relative Potency
1	0.0038	0.0025-0.0058	0.159	
2	0.0028	0.0016-0.0050	0.223	0.0033 USP units/mg

APPENDIX AA (continued)

FOLLICLE STIMULATING HORMONE ACTIVITY: FSH activity was determined by the HCG-augmentation method (4). A total dose of 8000 micrograms of NIH-P-S8 failed to elicit a significant response. Therefore, this preparation is judged to contain a maximum of 0.008 NIH-FSH-S1 units/mg. (one unit = activity on one mg of NIH-FSH-S1).

LUTEINIZING HORMONE ACTIVITY: LH activity was determined by the ovarian ascorbic acid depletion assay of Karg (5) and Parlow (6). A dose of 4000 micrograms failed to elicit a significant response in this assay. Therefore, NIH-P-S8 is judged to contain a maximum of 0.0003 NIH-LH-S1 units/mg. (one unit = activity in one mg of NIH-LH-S1).

THYROID STIMULATING HORMONE ACTIVITY: TSH was determined by the thyroidal P-32 uptake method in baby chicks (7). A total dose of 4000 micrograms failed to elicit a significant response. Therefore, NIH-P-S8 is judged to contain a maximum of 0.0005 USP units/mg.

OTHER ACTIVITIES: Not tested

STABILITY: A 1% solution, in 0.9% saline, will retain full potency for one week when kept under refrigerated conditions, and for longer periods when stored in the frozen state.

NOTE: NIH-P-S8 is not sterile and is not intended for human use.

Support by the National Institute of Arthritis and Metabolic Disease (Grant AM03598) is gratefully acknowledged.

NIH-P-S8 was prepared and characterized for the Endocrinology Study Section by Dr. L. E. Reichert, Jr.

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APPENDIX B

PROLACTIN RELEASED BY ADENOHYPOPHYSES FROM PRETREATED AND NON-PRETREATED FEMALE RATS

VALUES IN µg. PROLACTIN/AP

Trtmt.		Days of Organ Culture				Total Product
		2	4	6	8	
CONTROLS		50.8	40.7	22.1	18.7	132.3
		60.3	38.6	21.8	17.3	138.0
		73.2	45.8	22.0	24.2	165.2
		58.9	46.3	22.7	29.0	156.9
		85.7	46.8	21.9	26.2	180.6
		65.1	48.3	20.8	17.7	151.9
		58.0	55.7	23.0	23.1	159.8
		56.7	53.2	22.8	31.2	163.9
		62.0	44.5	22.9	27.0	156.4
		63.3	45.0	22.4	29.5	160.2
		61.8	47.1	22.5	30.6	162.0
		52.3	41.5	23.7	33.7	151.2
		58.8	49.3	22.2	24.6	154.9
		60.2	47.0	22.3	21.5	151.0
		64.3	44.3	23.6	22.6	154.8
	$\bar{x}$	62.1	46.3	22.4	25.1	155.9
	S.E.	2.2	1.1	0.2	1.3	2.9
PRETREATED		123.7	53.8	32.5	19.0	229.0
		110.6	70.3	45.3	18.7	244.9
		89.7	60.2	40.7	20.9	211.5
		105.8	59.1	39.0	20.7	224.6
		106.1	64.5	33.1	19.3	223.0
		107.2	62.0	22.9	20.1	212.2
		106.7	59.7	21.7	19.9	208.0
		103.1	64.3	35.6	19.8	222.8
		104.2	61.5	30.2	21.1	217.0
		101.5	54.2	27.6	21.0	204.3
		108.6	67.1	31.0	18.3	225.0
		99.3	57.9	31.9	20.6	209.7
		108.2	60.9	41.4	19.7	230.2
		107.0	58.3	28.2	19.6	213.1
		112.3	61.8	29.3	19.9	226.7
	$\bar{x}$	106.3	61.0	32.7	19.9	220.1
	S.E.	1.9	1.0	1.7	0.2	2.8

APPENDIX BB

ELECTROPHORESIS REAGENTS

Solution	Compound	Lot #	Concentration	Company
Stack Gel	Prep-Cryl (Acrylamide)	3819	2.5%	Canalco[1]
	Bis	3801	1.25%	Canalco
Stack Gel Buffer	Trizma Base (Tris)	120C-5220	0.2355	Sigma[2]
	TEMED	3800	0.1%	Canalco
Stack Gel Buffer ---- titrated to pH of 7.2 ± .2 with concentrated H_3PO_4				
Resolving Gel	Prep-Cryl (Acrylamide)	3819	7.5%	Canalco
	Bis	3801	0.9%	Canalco
Resolving Gel Buffer	Trizma (Tris)	120C-5220	1.50M	Sigma
	TEMED	3800	0.4%	Canalco
Resolving Gel Buffer titrated to pH of 8.9 ± .2 with concentrated HCl				
Polymerizing agents for:				
Stack Gel	Riboflavin	89B-2060	4.0 mg %	Sigma
Resolving Gel	Ammonium Persulfate	701598	280 mg %	Fisher,Sci.[3]
Reservoir Buffers:				
Upper Buffer	Trizma Base(Tris)	120C-5220	0.0522M	Sigma
pH = 8.9 ± .2	Glycine	702-1	0.0524M	Eastman Kodak[4]
Lower & Elution Buffer	Trizma Base(Tris)	120C-5220	0.10M	Sigma
pH = titrated to pH of 8.1 ± .2 with concentrated HCl				
Membrane Buffer: 4 times the concentration of the lower and elution buffer with the same pH (titrated with HCl)				

(1) Canal Industrial Corporation, Rockville, Maryland
(2) Sigma Chemical Company, St. Louis, Missouri
(3) Fisher Scientific Company, Chicago, Illinois
(4) Eastman Kodak, Organic Chemicals Division, Rochester, New York

APPENDIX C

EFFECTS OF AGE AND SEX ON PROLACTIN RELEASE IN VITRO BY RAT ADENOHYPOPHYSES

Trtmt.	VALUES IN µg. PROLACTIN/AP Days of Organ Culture			Total Product
	2	4	6	
OLD FEMALES	331.0	163.2	81.5	575.7
	352.7	159.7	91.7	604.1
	346.1	168.0	93.6	607.7
	362.8	163.2	101.3	627.3
	329.6	155.4	96.3	581.3
	365.4	157.5	97.8	620.7
	305.8	170.2	87.6	563.6
	348.3	159.6	107.9	615.8
	332.5	154.3	98.5	585.3
	362.7	170.1	95.0	627.8
$\bar{x}$	343.7	162.1	95.1	600.9
S.E.	6.0	1.8	2.3	7.3
OLD MALES	196.3	106.5	48.6	351.4
	183.4	108.5	42.4	334.3
	198.1	105.6	45.8	349.5
	191.6	109.3	44.7	345.6
	177.9	111.6	54.6	344.1
	197.1	113.5	49.2	359.8
	202.3	109.7	47.1	359.1
	201.5	116.3	51.2	369.0
	216.1	110.7	50.3	377.1
	193.7	115.3	54.1	363.1
$\bar{x}$	195.8	110.7	48.8	355.3
S.E.	3.3	1.1	1.2	4.0
YOUNG FEMALES	182.0	61.3	32.6	275.9
	195.7	63.5	33.0	292.2
	187.1	65.7	38.8	291.6
	162.3	71.5	39.1	272.9
	185.2	71.9	39.6	296.7
	168.3	72.6	41.3	282.2
	179.6	79.8	40.1	299.5
	201.8	81.3	42.5	325.6
	175.3	80.2	43.3	298.8
	181.6	70.4	42.2	294.2
$\bar{x}$	181.9	71.8	39.2	293.0
S.E.	3.7	2.2	1.2	4.7

APPENDIX D

PROLACTIN AND PROTEIN PRODUCTION

IN VITRO

Days in Organ Culture	Prolactin Content (µg.)	Protein Content (mg)	Prolactin/Protein Ratio (µg/mg)
	169.7	3.28	51.7
	192.9	3.13	61.6
	235.9	3.10	76.1
	168.4	3.03	55.6
2*	154.1	3.10	49.7
	184.2	3.03	60.0
	260.6	3.06	85.2
	193.0	3.06	63.1
	191.0	3.10	61.6
	153.2	3.01	50.9
$\bar{x}$	190.3	3.09	61.6
S.E.	10.9	0.02	3.6
	105.6	3.25	32.5
	94.8	3.33	28.5
	113.4	3.71	30.6
	87.0	3.36	25.9
4*	66.6	3.33	20.0
	61.2	3.28	18.7
	94.2	3.33	28.3
	68.4	3.36	20.4
	121.8	3.33	36.6
	79.8	3.33	24.0
$\bar{x}$	89.3	3.36	26.5
S.E.	6.5	0.04	1.8

APPENDIX D (continued)

Days in Organ Culture	Prolactin Content (µg.)	Protein Content (mg)	Prolactin/Protein Ratio (µg/mg)
	50.2	3.11	16.1
	45.1	3.09	14.6
	35.2	3.14	11.2
	29.8	3.06	9.7
6*	36.1	3.06	11.8
	41.5	3.09	13.4
	30.0	2.89	10.4
	31.7	3.11	10.2
	45.5	3.00	15.2
	59.3	2.98	19.9
$\bar{x}$	40.4	3.05	13.2
S.E.	3.1	0.02	1.0
	27.9	3.17	8.8
	31.0	3.20	9.7
	37.1	3.20	11.6
	36.6	3.17	11.5
8	26.1	2.90	9.0
	30.6	3.00	10.2
	30.6	3.09	9.9
	32.6	3.55	9.2
	31.4	3.33	9.4
	26.3	3.10	8.5
$\bar{x}$	31.0	3.17	9.8
S.E.	1.2	0.06	0.3

*Following organ culture, medium samples were taken for prolactin and protein analyses. Total production/AP was calculated from the results of the analyses and on the basis that each compartment holding one AP contained 2.0 ml of medium.

APPENDIX E

CONCENTRATION OF ORGAN CULTURE MEDIUM UNDER DIFFERENT CONDITIONS

Pressure	Sample	Medium Content						
		Volume[a] (ml)	Prolactin[b] mg	Prolactin[b] %	Protein[c] mg	Protein[c] %	Prol./Prot'n µg/mg	Percent Concentration
(PSI)		50	14.0	100	151.6	100	92.8	
55	Conc.[e]	21.0	9.4	67	70.8	47	132.8	143
	Filtr.[f]	33.9	0.1	0	5.1	3	15.7	
20	Conc.	19.5	11.7	83	64	42	181.2	195
	Filtr.	35.8	0.1	0	5.4	4	12.9	
20	Conc.[g]	23.5	12.2	87	72.5	48	167.9	181
20	Filtr. #1	30.5	0	0	4.4	3	2.3	
20	Filtr. #2	30.0	0	0	2.7	2	0.3	
20	Filtr. #3	30.0	0	0	1.3	1	0.8	
20	Filtr. #4	32.2	0	0	0.8	0.5	1.3	

(a) Each initial test volume was 50.0 ml
(b) Expressed as NIAMD-RP-1 equivalents
(c) Lowry (1951) Protein test utilizing BSA as a standard
(d) Nitrogen pressure in lbs/in^2 with chamber held at 2°C
(e) Volume of media in concentrate sample above filter
(f) Volume of media in filtrate sample below filter
(g) Following removal of #1 filtrate after initial concentration, three additional concentrations were accomplished by adding 30.0 ml deionized to the concentrated medium

APPENDIX F

ABSORBANCE (ABS.) FOR THE PROTEIN ELUTION PROFILE OF PREPARATIVE FRACTION KK-RP-I

Tube No.	*Abs.	Tube No.	Abs.	Tube No.	Abs.
1	0.007	24	0.095	47	0.050
2	0.218	25	0.070	48	0.050
3	1.300	26	0.040	49	0.040
4	1.900	27	0.038	50	0.035
5	2.000	28	0.041	51	0.030
6	1.700	29	0.046	52	0.025
7	0.720	30	0.050	53	0.040
8	0.255	31	0.060	54	0.020
9	0.125	32	0.070	55	0.015
10	0.062	33	0.073	56	0.013
11	0.045	34	0.076	57	0.007
12	0.033	35	0.083	58	0.005
13	0.030	36	0.081	59	0.002
14	0.033	37	0.080	60	0.000
15	0.043	38	0.075	1	
16	0.058	39	0.079	200	0.000
17	0.063	40	0.076		
18	0.086	41	0.065		
19	0.125	42	0.070		
20	0.148	43	0.065		
21	0.160	44	0.058		
22	0.158	45	0.053		
23	0.134	46	0.040		

*Absorbance (Abs.) taken at 280 mμ absorption.

APPENDIX G

MEAN ABSORBANCE (ABS.) OF THE PREPARATIVE COLUMN ELUANTS

Tube No.[a]	Abs.[b]	Tube No.	Abs.	Tube No.	Abs.
1	0.038	24	0.161	47	0.070
2	0.093	25	0.141	48	0.066
3	0.379	26	0.118	49	0.060
4	0.671	27	0.102	50	0.056
5	0.874	28	0.094	51	0.051
6	0.990	29	0.091	52	0.048
7	1.165	30	0.092	53	0.048
8	1.183	31	0.097	54	0.041
9	0.796	32	0.102	55	0.038
10	0.492	33	0.105	56	0.035
11	0.211	34	0.107	57	0.032
12	0.103	35	0.112	58	0.031
13	0.078	36	0.111	59	0.029
14	0.075	37	0.111	60	0.027
15	0.071	38	0.109	1	
16	0.076	39	0.107	200	0.000
17	0.082	40	0.104		
18	0.100	41	0.099		
19	0.120	42	0.095		
20	0.142	43	0.090		
21	0.161	44	0.085		
22	0.174	45	0.080		
23	0.173	46	0.073		

(a) Each fraction collector tube contained a volume of 5.0 ml collected at a rate of 1.0 ml/minute.

(b) Absorbances read from duplicate measures of the solution in each tube from five different column runs. Readings taken at 280 mμ on a Beckman DBG.

APPENDIX H

PREPARATION COLUMN PROTEIN AND PROLACTIN ELUTION PROFILES

Tube Number	Abs.*	Prolactin Content (µg/ml)
1	0.010	0
2	0.010	0
3	0.017	0
4	0.037	0
5	0.076	0
6	0.183	0
7	0.855	0
8	1.995	0.03
9	1.995	0.16
10	1.650	0.26
11	0.650	1.71
12	0.225	6.74
13	0.138	4.71
14	0.114	4.84
15	0.108	17.14
16	0.107	28.00
17	0.108	35.49
18	0.113	58.29
19	0.123	75.29
20	0.136	118.29
21	0.147	130.97
22	0.171	161.14
23	0.201	205.71
24	0.212	284.57
25	0.196	216.00
26	0.161	164.57
27	0.127	104.57
28	0.103	63.94
29	0.094	44.24
30	0.100	27.75
31	0.112	11.96
32	0.121	7.86
33	0.127	7.07
34	0.133	6.04
35	0.139	4.78

*Absorbance (Abs.) at 280 mµ.

APPENDIX H (Continued)

Tube Number	Abs.*	Prolactin Content (µg/ml)
36	0.145	3.12
37	0.150	2.19
38	0.151	1.56
39	0.148	1.59
40	0.144	1.59
41	0.139	1.27
42	0.132	1.20
43	0.123	0.96
44	0.116	0.86
45	0.109	0.85
46	0.102	0.79
47	0.094	0.64
48	0.086	0.66
49	0.079	0.62
50	0.073	0.59
51	0.067	0.49
52	0.061	0.66
53	0.057	0.61
54	0.053	0.58
55	0.051	0.66
56	0.048	0.55
57	0.045	0.60
58	0.044	0.52
59	0.043	0.45
60	0.042	0.47
Buffer Control	0	0

*Absorbance (Abs.) at 280 mµ.

APPENDIX I

PROLACTIN LOSSES DURING EACH STEP OF THE ISOLATION SYSTEM

Step[1] Number	Volume (ml)	Prolactin Content µg/ml	Prolactin Content Total (mg)	Mean % Loss[2]	Mean % Recovery[3]
	100.0	436.8	43.7		
	61.0	301.2	18.4		
I	86.9	163.2	14.2	0	100
	144.6	436.8	63.2		
	65.0	700.8	45.5		
	100.0	432.0	43.2		
	61.0	281.6	17.2		
II	86.5	157.2	13.6	3.8	96.2
	144.6	432.0	62.5	± 1.2	± 1.2
	61.0	705.6	43.0		
	14.9	2550.0	38.0		
	25.5	592.0	15.1		
III	19.3	608.0	11.7	14.6	81.6
	17.0	2985.0	50.7	± 1.3	± 0.7
	17.2	2102.4	36.2		
	52.2	610.4	31.9		
	52.8	268.8	14.0		
IV	52.2	217.8	11.4	7.4	74.2
	61.4	649.2	39.9	± 3.0	± 3.1
	50.0	720.0	36.0		
	82.1	373.8	30.7		
	73.0	187.2	13.7		
V	65.1	172.0	11.2	3.2	71.0
	68.5	585.0	40.1	± 1.8	± 2.7
	59.5	531.0	31.2		
	6.4	4099.2	26.2		
	12.0	1132.8	13.6		
VI	3.2	3432.0	11.0	3.4	67.6
	9.1	44006.4	40.0	± 1.9	± 3.3
	9.4	3081.6	29.0		

(1) The steps in sequence represent the following processes:
- I. Original medium
- II. Medium following centrifugation
- III. Medium following diafiltration/concentration
- IV. Buffer following preparative electrophoresis/fractionation
- V. Buffer fractions following dialysis
- VI. Buffer fractions following diafiltration/concentration and lyophilization

(2) Losses are tabulated as losses attributed to that step alone, not from the total as a sequential loss tabulation.

(3) Recoveries in each batch of original medium are tabulated as sequential recovery of the total initial amount of prolactin.

APPENDIX J

SUMMARY OF THE DOSE RESPONSE CURVE RESPONSES FOR THE PCS BIOASSAY

TOTAL DOSES/ EXPERIMENT	NUMBER OF HEMICROPS(1)	MEAN RESPONSE(2) (mg)	RESPONSE RANGE (mg)	SLOPE / ORIGIN(4)	r(4)
Experiment I(3)					
1.0 µg	8	13.5 ± 0.8	8.8-16.9		
3.0 µg	7	20.6 ± 1.2	16.2-25.4		
10.0 µg(5)	7	27.4 ± 2.0	22.8-37.0	13.9 / 13.6	0.85
20.0 µg(5)	8	31.5 ± 5.4	10.9-62.8		
Experiment II(3)					
1.0 µg	9	16.5 ± 1.2	10.0-21.0		
3.0 µg	9	23.0 ± 1.4	17.2-28.5		
10.0 µg(5)	8	29.9 ± 2.0	22.3-37.6	13.4 / 16.5	0.79
20.0 µg(5)	9	34.3 ± 3.6	21.1-58.4		
Experiment III(3)					
1.0 µg	9	10.4 ± 0.8	7.2-14.3		
3.0 µg	9	14.2 ± 0.5	11.7-17.1		
10.0 µg	8	18.3 ± 1.0	15.5-23.1	7.8 / 10.4	0.87
20.0 µg	9	20.7 ± 0.9	18.1-25.8		

(1) Average and specific assay animal body weights are tabulated in Appendix L and M.

(2) Consult Appendix L for specific responses from which these means were computed. Graphic representation of these standards are shown in Figure 8.

(3) Dates of each experiment are noted in Appendix L.

(4) Slopes, origin and rank order correlation coefficients computed by regression analysis, coding the dose of prolactin in logorithms.

(5) This dose level and the crop-sac responses dropped from the analyses due to heterogeneous variance as defined by Hartley's F-max test (1950).

APPENDIX K

LINEAR REGRESSION ANALYSIS OF THE PIGEON CROP-SAC BIOASSAY DOSE-RESPONSE CURVES

	Origin (b_0)	95% C.I.	Slope (b_1)	95% C.I.	Correlation Coefficient	Precision (λ)
Experiment I						
	13.6	11.6-15.6	13.9	9.9-15.6	0.85	0.256
Experiment II						
	16.5	13.7-19.2	13.4	9.0-17.8	0.79	0.311
Experiment III						
	10.4	9.0-11.8	7.8	6.2- 9.4	0.87	0.287

APPENDIX L

DOSE RESPONSE DATA FOR THE PCS BIOASSAYS STANDARDS

EXPERIMENT	CROP-SAC RESPONSES (mg) TOTAL DOSES OF TEST MATERIAL 1.0	3.0	10.0	20.0[1]	Assay Date
I	8.8	16.2	22.8	10.9	
	12.5	16.9	23.6	21.6	
	12.9	20.2	24.4	22.3	
	13.3	20.3	25.0	31.2	
	13.8	22.5	26.1	33.1	7/17/71
	14.3	22.9	32.9	34.1	
	15.2	25.4	37.0	36.0	
	16.9	-	-	62.8	
$\bar{x}$	13.5	20.6	27.4	31.5	
$SE_{\bar{x}}$	0.8	1.2	2.0	5.4	
	Mean body weight of 30 assay birds--314.3 ± 8.2 gms.				
II	10.0	17.2	22.3	21.2[1]	
	14.4	18.6	23.1	27.6	
	14.4	19.4	26.1	28.3	
	14.9	21.7	29.8	28.3	
	16.4	22.8	31.4	34.5	9/16/71
	17.8	25.3	33.3	37.5	
	18.6	26.0	35.6	38.6	
	19.7	27.5	37.6	40.5	
	21.9	28.5	-	58.4	
$\bar{x}$	16.5	23.0	29.9	34.3	
$SE_{\bar{x}}$	1.2	1.4	1.4	3.6	
	Mean body weight of 36 assay birds--460.6 ± 12.9 gms.				
III	7.2	11.7	15.5	17.5	
	7.2	12.1	15.7	18.1	
	9.1	13.8	15.9	18.7	
	9.9	14.0	17.6	19.6	
	10.8	14.3	17.9	20.6	11/23/71
	11.0	14.6	18.3	21.7	
	12.0	14.9	22.1	22.0	
	12.1	15.3	23.1	22.3	
	14.3	17.1	-	25.8	
$\bar{x}$	10.4	14.2	18.3	20.7	
$SE_{\bar{x}}$	0.8	0.5	1.0	0.9	
	Mean body weight of 36 assay birds--330.1 ± 7.7 gms.				

(1) These responses dropped from the analyses due to heterogeneous variance as defined by Hartley's F-max test (1950).

APPENDIX M

BODY WEIGHTS OF ASSAY BIRDS EMPLOYED IN DEVELOPING THE DOSE RESPONSE CURVES

TOTAL DOSES OF THE STANDARDS (μg)

EXPERIMENT	1.0	3.0	10.0	20.0	Assay Date
I	346	292	260	292	
	365	360	342	220	
	392	268	356	296	
	320	276	320	340	7/17/71
	264	241	330	264	
	344	318	361	326	
	244	341	344	316	
	300	-	-	390	
$\bar{x}$	321.9	299.4	330.4	305.5	
$SE_{\bar{x}}$	17.8	16.0	12.9	18.0	
	Mean of the total of 30 assay birds--314.3 ± 8.2 gms.				
II	449	591	449	456	
	551	457	441	411	
	329	541	452	601	
	431	457	548	402	
	553	480	649	473	9/16/71
	413	341	459	473	
	471	271	539	340	
	437	453	471	487	
	433	405	415	453	
$\bar{x}$	451.9	448.8	491.4	455.1	
$SE_{\bar{x}}$	69.0	102.1	24.6	71.5	
	Mean of the total of 36 assay birds--460.6 ± 12.9 gms.				
III	310	290	295	290	
	325	275	290	320	
	295	320	285	350	
	295	458	330	405	
	270	360	360	390	11/23/71
	310	365	370	400	
	400	325	360	335	
	280	325	305	360	
	340	370	285	240	
$\bar{x}$	318.1	346.0	320.0	340.8	
$SE_{\bar{x}}$	39.2	57.0	11.8	68.1	
	Mean of the total of 36 assay birds--330.1 ± 7.7 gms.				

APPENDIX N

SUMMARY TABLE OF THE BIOASSAYS OF ISOLATED COLUMN FRACTIONS AND PROLACTIN STANDARDS

MATERIAL	TOTAL DOSE (μg)	NUMBER OF HEMICROPS	MEAN[5] RESPONSE (mg)	RESPONSE RANGE
KK-RP-I[1]	1.0	6	13.5 ± 1.4	10.0-18.9
KK-RP-I[2]	3.0	6	14.3 ± 0.9	12.1-16.4
KK-RP-I[1]	10.0	6	27.8 ± 2.4	22.4-40.4
KK-RP-2[3]	1.0	6	15.9 ± 1.6	10.5-21.7
KK-RP-II[2,4]	3.0	6	14.0 ± 1.5	9.0-19.0
KK-RP-2[3]	10.0	6	29.3 ± 2.1	23.0-35.6
KK-RP-3[3]	1.0	6	16.3 ± 1.6	10.7-22.1
KK-RP-3[3]	10.0	6	29.7 ± 2.2	22.9-36.8
KK-RP-4[3]	10.0	6	29.6 ± 2.9	20.2-37.1
NIAMD-RP-1[2]	3.0	6	14.3 ± 1.8	10.1-22.0
NIAMD-I-1[2]	3.0	6	15.2 ± 1.8	11.0-23.4
H-10-10-B[2]	3.0	6	13.8 ± 0.7	11.5-15.8

(1) Analyzed in Bioassay Experiment I, consult Appendix L and Figure 8 for the Standard Curve.

(2) Analysed in Bioassay Experiment III, consult Appendix L and Figure 8 for the Standard Curve.

(3) Analyzed in Bioassay Experiment II, consult Appendix L and Figure 8 for the Standard Curve.

(4) Repurified KK-RP-3 with a second complete run.

(5) Responses from which these means were derived are tabulated in Appendix P.

APPENDIX O

BIOLOGICAL POTENCY EVALUATIONS OF THE RAT PROLACTINS IN IU/mg

Hormone	$\bar{x}^{(1)}$	C.I.	$\bar{x}^{(2)}$	C.I.	λ
KK-RP-I	27.4	25.8 - 45.3	24.4	10.0 - 38.0	0.256
KK-RP-I	29.5	13.7 - 53.2	27.7	12.8 - 49.6	0.287
KK-RP-I	29.3	16.6 - 74.0	26.0	14.8 - 64.2	0.256
KK-RP-2	25.2	11.6 - 53.6	30.5	14.2 - 63.1	0.311
KK-RP-II	27.1	11.1 - 51.0	27.7	11.5 - 53.0	0.287
KK-RP-2	25.1	13.2 - 80.2	25.5	13.6 - 82.6	0.311
KK-RP-3	27.0	8.5 - 49.2	24.2	9.8 - 44.4	0.311
KK-RP-3	26.9	14.0 - 90.6	27.1	14.5 - 93.4	0.311
KK-RP-4	26.4	13.8 -104.3	32.2	17.1 -147.9	0.311
NIAMD-RP-1	29.5	10.9 - 60.0	24.2	8.7 - 45.6	0.287
NIAMD-I-1	38.4	15.9 - 81.7	28.5	10.8 - 56.6	0.287
H-10-10-B	25.5	12.3 - 41.4	28.6	14.2 - 47.7	0.287

(1) The crude mean transformed by inverse linear regression (Ostle, 1963).

(2) The mean of the inverse linear regression transformed crude responses (Ostle, 1963).

APPENDIX P

BIOASSAY RESPONSES FOR THE ANALYSIS OF ISOLATED COLUMN FRACTIONS AND PROLACTIN STANDARDS

MATERIAL	TOTAL DOSE	CROP-SAC RESPONSE (mg)	MEAN RESPONSE	BODY WEIGHT (9 mos.)	MEAN BODY WEIGHT
KK-RP-I (1)	1.0 µg	10.0 10.0 13.6 13.9 14.8 18.9	13.5 ±1.4	292 342 244 268 292 264	283.7 ±13.9
KK-RP-I (2)	3.0 µg	12.0 12.1 13.7 14.2 16.4 17.4	14.3 ±0.9	275 295 400 360 370 320	336.7 ±47.9
KK-RP-I (1)	10 µg	22.3 22.4 24.1 30.4 30.6 37.3	27.8 ±2.4	316 340 341 260 346 344	324.5 ±13.6
KK-RP-2 (3)	1.0 µg	10.5 13.7 14.5 16.3 18.7 21.7	15.9 ±1.6	480 413 441 541 402 553	471.7 ±64.4
KK-RP-II (2)	3.0 µg	9.0 11.8 12.6 15.3 16.3 19.0	14.0 ±1.5	310 325 240 458 290 330	332.6 ±78.9
KK-RP-2 (3)	10.0 µg	23.0 23.7 28.4 32.2 32.9 35.6	29.3 ±2.1	453 649 471 551 473 340	489.5 ±103.4

(1) Footnotes for this table are the same as Appendix N

APPENDIX P--Continued

MATERIALS	TOTAL DOSE	CROP-SAC RESPONSE (mg)	MEAN RESPONSE	BODY WEIGHT (9 mos.)	MEAN BODY WEIGHT
KK-RP-3 (3)	1.0 µg	10.7 14.2 15.2 17.6 18.0 22.1	16.3 ±1.6	449 473 341 459 471 487	446.7 ±53.3
KK-RP-3 (3)	10.0 µg	22.9 24.5 28.7 31.3 34.2 36.8	29.7 ±2.2	452 329 456 449 457 453	432.7 ±50.9
KK-RP-4 (3)	10.0 µg	20.2 22.3 28.5 34.7 34.8 37.1	29.6 ±2.9	548 433 271 405 411 415	
NIAMD-RP-1 (2)	3.0 µg	10.1 10.6 12.2 14.5 16.4 22.0	14.3 ±1.8	310 320 290 270 365 390	324.2 ±45.4
NIAMD-I-1 (2)	3.0 µg	11.0 12.2 13.7 14.0 16.9 23.4	15.2 ±1.8	290 340 405 285 325 360	334.2 ±45.1
H-10-10-B (2)	3.0 µg	11.5 12.4 13.0 14.4 15.7 15.8	13.8 ±0.7	295 360 295 360 400 325	339.2 ±41.6

APPENDIX Q

DOSE RESPONSE CURVE RESULTS OF COLUMN FRACTIONS AND PROLACTIN STANDARDS IN THE RADIOIMMUNOASSAY (RIA)

DOSES AT EACH POINT IN THE RIA (ng) (1)

MATERIAL	0.143	0.286	0.572	0.858	1.144	1.430	2.145	2.86
KK-RP-I	97.9	91.7	87.4	83.5	77.0	73.6	66.7	54.4
KK-RP-II	91.8	90.1	87.0	84.9	84.4	77.8	70.4	61.3
KK-RP-2	116.6	107.4	104.1	102.9	99.3	94.2	83.5	79.3
KK-RP-3	108.4	107.0	103.7	101.6	95.7	87.6	79.4	71.3
KK-RP-4	110.1	104.5	100.7	97.7	94.0	91.8	83.5	77.1
NIAMD-RP-1	95.5	94.6	92.3	87.5	80.1	78.4	70.7	58.9
NIAMD-I-1	90.8	92.3	87.7	81.7	74.2	68.4	55.4	47.8
H-10-10-B	90.1	93.9	93.7	90.4	85.2	82.9	73.4	67.1

DOSES AT EACH POINT IN THE RIA (ng) (1)

MATERIAL	4.29	5.72	7.15	8.58	11.44	14.30	28.6
KK-RP-I	48.9	33.6	28.1	24.4	20.2	19.2	12.3
KK-RP-II	48.1	43.9	35.1	28.9	25.3	22.2	14.5
KK-RP-2	64.0	55.6	48.2	44.3	39.4	35.1	29.0
KK-RP-3	60.0	50.0	46.4	41.9	37.1	32.9	27.4
KK-RP-4	64.7	53.6	49.2	43.8	37.8	34.8	26.9
NIAMD-RP-1	59.5	36.0	28.7	26.0	20.4	15.5	8.0
NIAMD-I-1	33.6	27.3	23.7	18.5	16.3	18.0	9.1
H-10-10-B	53.9	47.8	41.4	34.3	34.5	25.3	17.6

(1) Dose response curves developed using 1/7 ng dilutions of standard doses of 1,2,4,6,8,10,15,20,30,40,50,60,80,100,200 ng.

APPENDIX R

FIFTY PER CENT BINDING VALUES OF RADIOIMMUNOASSAY ANALYSIS OF COLUMN FRACTIONS AND PROLACTIN STANDARDS

MATERIAL	50% Bndg.[1] POINTS (ng)	MEAN 50% BINDING POINT (ng)	INTERCEPT (b_o)	SLOPE	COR. COEF.(r)
KK-RP-I	3.55 3.52 3.21 3.11	3.35 ± 0.11	1.4666	-2.7844	.99
KK-RP-II	4.34 4.30 4.06 4.22	4.23 ± 0.06	1.6951	-2.6833	.99
KK-RP-2	4.11 4.28 4.24 4.33	4.24 ± 0.05	1.7063	-2.6573	.99
KK-RP-3	4.17 3.95 3.90 3.78	3.95 ± 0.08	1.6531	-2.6351	.99
KK-RP-4	4.64 4.51 -	4.52 ± 0.07	1.7521	-2.5912	.99
NIAMD-RP-1	3.70 3.79 3.95 3.28	3.68 ± 0.14	1.7145	-2.9854	.99
NIAMD-I-1	2.67 2.60 2.67 2.64	2.64 ± 0.02	1.2066	-2.8702	.99
H-10-10-B	5.19 5.36 5.18 5.00	5.18 ± 0.07	1.8802	-2.6322	.99

(1) From four assays in quadruplicate.

APPENDIX S

SUMMARY TABLE OF THE ANALYSES OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITY OF ISOLATED COLUMN FRACTIONS

Preparation	Biological (1) Potency (IU/mg)	Immunological (2) Potency (ng)
KK-RP-I	29.5	3.35 ± 0.11
KK-RP-II	27.1	4.23 ± 0.06
KK-RP-2	25.2	4.24 ± 0.05
KK-RP-3	27.0	3.95 ± 0.08
KK-RP-4	26.9	4.25 ± 0.07

(1) The reference preparation used consistently throughout all assays at identical total doses was NIH-P-S8, ovine prolactin with a potency of 28.0 I.U./mg. These responses are crude means transformed by inverse linear regression analysis.

(2) Fifty percent binding point developed from the means of four assays computed by regression analysis. NIAMD-RP-1 was used as the reference comparison in all cases.

APPENDIX T

SUMMARY TABLE OF THE ANALYSES OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITY OF ISOLATED COLUMN FRACTIONS

Preparation	Biological (1) Potency (IU/mg)	Immunological (2) Potency (ng)
KK-RP-I	27.7	3.35 ± 0.11
KK-RP-II	27.7	4.23 ± 0.06
KK-RP-2	30.5	4.24 ± 0.05
KK-RP-3	27.1	3.95 ± 0.08
KK-RP-4	32.2	4.52 ± 0.07

(1) The reference preparation used consistently throughout all assays at identical total doses was NIH-P-S8, ovine prolactin with a potency of 28.0 I.U./mg. These are means of the inverse regression analysis transformed crop-sac responses.

(2) Fifty percent binding point developed from the means of four assays computed by regression analysis. NIAMD-RP-1 was used as the reference comparison in all cases.

APPENDIX U

COMPARISON OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITIES OF COLUMN FRACTIONS WITH AVAILABLE RAT PROLACTINS

Preparation	Biological Potency (I.U./mg) (1)	Immunological Potency (ng)
KK-RP-I	29.5	3.35 ± 0.11
KK-RP-II	27.1	4.23 ± 0.06
KK-RP-2	25.2	4.24 ± 0.05
KK-RP-3	27.0	3.95 ± 0.08
KK-RP-4	26.4	4.52 ± 0.07
NIAMD-RP-1	29.5	3.68 ± 0.14
NIAMD-I-1	38.4	2.64 ± 0.02
H-10-10-B	25.5	5.18 ± 0.07

(1) Crude means transformed by inverse linear regression analysis after Ostle (1963).

APPENDIX V

COMPARISON OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITIES OF COLUMN FRACTIONS WITH AVAILABLE RAT PROLACTINS

Preparation	Biological Potency (I.U./mg) (1)	Immunological Potency (ng)
KK-RP-I	27.7	3.35 ± 0.11
KK-RP-II	27.7	4.23 ± 0.06
KK-RP-2	30.5	4.24 ± 0.05
KK-RP-3	27.1	3.95 ± 0.08
KK-RP-4	32.2	4.52 ± 0.07
NIAMD-RP-1	24.2	3.68 ± 0.14
NIAMD-I-1	28.5	2.64 ± 0.02
H-10-10-B	28.6	5.18 ± 0.07

(1) Means of inverse linear regression analysis transformed crop-sac responses.

APPENDIX W

RELATIVE COMPARISONS OF THE ISOLATED COLUMN FRACTIONS AND RAT PROLACTIN STANDARDS

Preparation	Biol. Index (1)	Immunol. Index (2)	$\frac{\text{Biol. Pot.}}{\text{Immunol. Pot.}}$ (3)
KK-RP-I	1.00	0.91	1.10
KK-RP-II	0.92	1.15	0.80
KK-RP-2	0.85	1.15	0.74
KK-RP-3	0.92	1.07	0.86
KK-RP-4	0.90	1.23	0.73
NIAMD-RP-1	1.00	1.00	1.00
NIAMD-I-1	1.30	0.72	1.81
H-10-10-B	0.86	1.41	0.61

(1) This index was developed by dividing all biological potencies by that of the national standard for rat prolactin, NIAMD-RP-1. The crude means, transformed by inverse linear regression analysis, were used to make these computations.

(2) This index was computed by dividing all RIA fifty percent binding means by that of the national standard for rat prolactin, NIAMD-RP-1.

(3) An index computed by dividing the biological index by the immunological index.

APPENDIX X

RELATIVE COMPARISONS OF THE ISOLATED COLUMN FRACTIONS AND RAT PROLACTIN STANDARDS

Preparation	Biol. Index (1)	Immunol. Index (2)	Biol. Pot. / Immunol. Pot. (3)
KK-RP-I	1.15	0.91	1.26
KK-RP-II	1.15	1.15	1.00
KK-RP-2	1.26	1.15	1.10
KK-RP-3	1.12	1.07	1.05
KK-RP-4	1.33	1.23	1.08
NIAMD-RP-1	1.00	1.00	1.00
NIAMD-I-1	1.18	0.72	1.64
H-10-10-B	1.18	1.41	0.84

(1) This index was developed by dividing all biological potencies by that of the national standard for rat prolactin, NIAMD-RP-1. The means of the inverse linear regression transformed crop-sac responses were used to make these computations.

(2) This index was computed by dividing all RIA fifty percent binding means by that of the national standard for rat prolactin, NIAMD-RP-1.

(3) An index computed by dividing the biological index by the immunological index.

APPENDIX Y

SUMMARY OF BIOLOGICAL AND IMMUNOLOGICAL ACTIVITIES OF COLUMN FRACTIONS WITH AVAILABLE RAT PROLACTINS

Preparation	Biological Potency (I.U./mg) $\bar{X}$ (2)	C.I. (1)	Immunological Potency (ng)
KK-RP-I	27.7	12.8 - 49.6	3.35 ± 0.11
KK-RP-II	27.7	11.5 - 53.0	4.23 ± 0.06
KK-RP-2	25.2	11.6 - 53.6	4.24 ± 0.05
KK-RP-3	26.9	14.0 - 90.6	3.95 ± 0.08
KK-RP-4	26.4	13.8 -104.3	4.52 ± 0.07
NIAMD-RP-1	24.2	8.7 - 45.6	3.68 ± 0.14
NIAMD-I-1	28.5	10.8 - 56.6	2.64 ± 0.02
H-10-10-B	25.5	12.3 - 41.4	5.18 ± 0.07

(1) C.I. @ 95% level by linear regression analysis, inverse method.

(2) Means and 95% confidence intervals (C.I.'s) from Appendix O. These means were chosen as being the highest estimate of biological potency but with the most reliable 95% C.I.'s.

APPENDIX Z

SUMMARY OF RELATIVE COMPARISONS OF THE ISOLATED COLUMN FRACTIONS AND RAT PROLACTIN STANDARDS

Preparation	Biol. Index (1)	Immunol. Index (2)	Biol. Pot. / Immunol. Index (3)
KK-RP-I	1.14	0.91	1.25
KK-RP-II	1.14	1.15	0.99
KK-RP-2	1.04	1.15	0.90
KK-RP-3	1.11	1.07	1.04
KK-RP-4	1.09	1.23	0.89
NIAMD-RP-1	1.00	1.00	1.00
NIAMD-I-1	1.18	0.72	1.64
H-10-10-B	1.05	1.41	0.74

(1) This index was developed by dividing all biological potencies by that of the national standard for rat prolactin, NIAMD-RP-1.

(2) This index was computed by dividing all RIA fifty percent binding means by that of the national standard for rat prolactin, NIAMD-RP-1.

(3) An index computed by dividing the biological index by the immunological index.

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