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Histologic identification and immunochemical investigation of growth hormone and prolactin in the primate pituitary gland

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HISTOLOGIC IDENTIFICATION AND IMMUNOCHEMICAL INVESTIGATION OF GROWTH HORMONE AND PROLACTIN IN THE PRIMATE PITUITARY GLAND

by

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ABSTRACT

Histologic Identification and Immunochemical Investigation of Growth Hormone and Prolactin in the Primate Pituitary Gland

By Damon Charles Herbert

Whole or hemisected pituitaries from normal juvenile and adult male and female rhesus monkeys and from pregnant and lactating females have been examined by light and fluorescent microscopy. Two different acidophilic cells, a carminophil and an orangeophil, were tinctorially differentiated. With the indirect fluorescent antibody technique, employing guinea pig antisera to ovine PRO and to human GH, it was observed that the carminophils contain prolactin (PRO) and the orangeophils contain growth hormone (GH). Similar observations were made on pituitaries obtained from cynomolgus and capuchin monkeys and from three species of apes; the gorilla, chimpanzee and the orangutan. The number of carminophils was negligible in the juvenile rhesus monkeys of either sex, as well as in the adult capuchin monkeys and the adult male gorilla, juvenile female chimpanzees and the juvenile male orangutan. A marked increase in the number of carminophils was noted in the pituitary of the adult male and female rhesus monkeys, and in the cynomolgus monkeys. During late pregnancy and lactation, 60-80% of the cells within the pituitary were carminophils, while the number of orangeophils was considerably less in pregnant and lactating animals than in normal adult rhesus females. The two acidophils predominated in separate regions of the primate pituitary; the carminophils predominated anteriorly, antero-inferiorly and medially, while the orangeophils were mainly located posteriorly, laterally and at the superior and inferior poles. In intact juvenile

female rhesus monkeys treated with estradiol benzoate (EB), the number of carminophils increased significantly and was similar to the number observed in normal adult female rhesus monkeys. Fewer orangeophils were noted in the pituitaries of the steroid treated animals.

Whole or hemisected pituitaries from normal juvenile and adult male and female rhesus monkeys and from the intact juvenile rhesus females treated with EB were homogenized and studied by radioimmunoassay to determine the concentration of pituitary GH and PRO. The mean concentration of GH was 20.42 μg/mg pituitary wet weight in the juvenile males, 25.67 μg/mg in the juvenile females, 33.75. μg/mg in adult males and 92.0 μg/mg in the adult female rhesus monkeys. In the juvenile male and female monkeys, the mean pituitary PRO levels were 1.01 μg/mg and 0.91 μg/mg, respectively, while in the adult males and females, the concentration of PRO was 2.17 μg/mg and 4.96 μg/mg, respectively. Pituitary and serum PRO concentrations were elevated while pituitary GH levels were lower in the steroid treated animals, as compared to those values obtained in the controls.

The pituitary homogenates from the normal juvenile and adult male and female rhesus monkeys and from the juvenile females treated with EB were studied by agar gel diffusion. The PRO in the homogenates was qualitatively related to but not identical immunochemically to purified ovine PRO, while the GH in the same homogenates was qualitatively identical immunochemically to purified human GH.

It can be concluded from these studies that:

(a) GH and PRO are produced by two separate acidophilic cell types which predominate in different regions of the pars distalis of the primate pituitary.

(b) the carminophils and orangeophils as well as the concentration of primate PRO and GH vary during the life and reproductive cycles of the primate,

and are influenced by the level of circulating sex steroids, and

(c) rhesus monkey GH and PRO are closely related immunochemically to human GH and ovine PRO, respectively.

Histologic Identification and Immunochemical Investigation of Growth Hormone and Prolactin In the Primate Pituitary Gland

To my beloved wife Marge

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LITERATURE REVIEW

Pituitary growth hormone (GH) and prolactin (PRO) have been isolated, purified and characterized from several different mammalian species. The cellular origin of these two hormones has been described in detail in both mammalian and submammalian species by light, immunofluorescence and electron microscopy (155, 156, 226, 278). Specific bioassays and radioimmunoassays for GH and PRO have allowed indepth studies of the concentrations of these two hormones in the pituitary gland and in the circulating plasma of mammals during their life and reproductive cycles. The importance of these two protein hormones in the maintenance of homeostasis in the normal mammalian vertebrate is nearly overshadowed by the vast amount of time and effort expended by numerous investigators who have essentially devoted their lives to the study of GH and PRO.

A. Growth Hormone

In 1889 Pierre Marie (221) was the first to associate enlargement of the pituitary gland with the clinical findings of the disease he described and termed acromegaly. He states "...amongst the lesions...which after what has been observed in other autopsies seem to me to be constant in acromegaly, must be mentioned hypertrophy of the pituitary body with enormous dilation of the sella turcica..." Harvey Cushing in 1909 (51) corroborated Marie's findings and goes on in his review article to outline the clinical manifestations of both hyperpituitarism and hypopituitarism. Cushing, like Marie, observed "that an enlargement of the pituitary body is found in a large majority of cases of both acromegaly and gigantism..." The concept of a growth promoting factor present in the anterior lobe of the pituitary gland was first demonstrated experimentally in 1921

by Evans and Long (84). They reported that rats who received intraperitoneal injections of bovine pituitary homogenates (extracts (PE's)) grew at a faster rate than their litter mates who were untreated and served as controls. A year later, Smith and Smith (324) were able to restore hypophysectomized ($\bar{H}$) tadpoles to their normal size by injecting them with portions of bovine pituitaries which were found to be rich in acidophilic cells. Smith (321) was one of the first investigators to successfully $\bar{H}$ rats and maintain them long enough to conduct a series of comprehensive studies on all the endocrine tissues in the body. He observed that skeletal growth ceased once the pituitary was removed and dwarfism ensued until the animals were given PE's. Feeding the rats fresh pituitaries failed to induce growth while intraperitoneal injection of PE's reversed the effects of $\bar{H}$, as did daily implants of anterior pituitaries into the $\bar{H}$ rats.

The production of dwarfism, gigantism and acromegaly in experimental animals was well documented in the literature by the mid-1930's (83). The term 'growth-promoting factor' was rapidly being replaced by the term somatotropin, somatotrophic hormone (STH) or growth hormone (GH). The purification, however, of this hormone was delayed for nearly a decade. C.H. Li and H.M. Evans (202, 203) reported the first purification of GH from acetone-dried bovine pituitaries. The hormone was extracted by salt precipitation and was biologically active in inducting a weight gain in $\bar{H}$ female rats. The hormone was found to be essentially free of contamination by other anterior pituitary hormones, including PRO. At that time a molecular weight (mw) of 44,250 was proposed for bovine GH; however, more accurate analysis have shown that the mw is about 21,000 and that there are 181 amino acids in the molecule (301).

GH's from several species have since been isolated and purified by a variety of chemical procedures. In 1955, Wilhelmi (351) reported that GH from ox, sheep, horse, pig and fish pituitaries (hake and pollack) had been isolated. To some degree or another all of these GH's were active in the H̄ rat except the two fish GH's. These preparations along with bovine GH were assayed in the H̄ killifish, (Fundulus heteroclitus), and were found to induce an increase in the weight and length of the fish. The only other fish GH that has been isolated was by U.J. Lewis in 1972 (187) who reported the purification of shark GH by preparative gel electrophoresis. Two other GH's have been isolated from submammalian pituitaries and these are duck and turtle GH's (260). Both are active in stimulating growth of the epiphyseal cartilage of the tibia in the H rat and appear to be similar in amino acid composition.

GH from the sheep, pig and whale were purified by Papkoff and Li from 1958-1962 (261-263). All three preparations were biologically active in the mammal and were were first reported to have a mw ranging from 40,000 to 47,000. More recent studies on sheep and porcine GH's indicate that their mw in the monomeric form are 24,000 and 22,500 respectively (3). These two hormones along with bovine GH were initially reported to have a mw greater than 40,000 which is the dimer form of the hormone molecule. Wallis has speculated that some GH's such as bovine and ovine GH may be found as dimers in vivo (348). Rat GH was first purified by Reisfield et al. in 1964 (289) and later by Ellis et al. in 1968 (73). The latter preparation was more potent based on its USP units than Reisfield's; however, it appeared to be slightly more contaminated with traces of other anterior pituitary hormones. These investigators reported different mw's for rat GH. Ellis et al. (73) reported a mw of 45,000 while Reisfield et al. (289) calculated it to be 24,000 which is probably the monomeric form of the molecule. Ellis et al. (73) also purified GH from rabbit pituitaries which was quite active biologically

and reported to have a mw of 45,500. A very potent preparation of mouse GH which has an activity of 3.1 USP units/mg in bioassay (317) was isolated by preparative gel electrophoresis in Lewis' laboratory (44). GH from two other mammalian species, the cat (176) and the dog (137, 354), have also been purified. Both were equally as active in promoting growth of cartilage in the tibia of the $\bar{H}$ rat; the cat GH having an activity of 2.9 IU/mg (176) while potency of the dog GH was reported to range from 2.3-3.6 IU/mg (137). The mw of dog GH was 24,000 (137, 354) and for the cat it was calculated to be about 45,000 (176) which is probably the hormone in its dimer form.

Three hormones have been found in the primate which are capable of promoting growth in the $\bar{H}$ rat; human GH, monkey GH and human chorionic somatomammotropin (HCS). Human and monkey GH were isolated independently in 1956 by Li and Papkoff (206) and in 1957 by Raben (280). Later in 1961, Wilhelmi (352) published his protocol for the isolation and purification of primate GH. Li and Papkoff's procedure involved the use of calcium oxide, ammonium salts, anion exchange chromatography and ethanol to extract the GH from lyophilized primate pituitaries (206). The methodology was later modified by the addition of sephadex chromatography to the above extraction procedure (204). The two GH's were of equal potency in bioassay and had quite similar mw's; 27,100 for human GH and 25,400 for monkey GH (192). Their amino acid compositions were also quite similar and both were reported to have phenylalanine at the N-terminus (206) as well as at the C-terminus (192). The amino acid sequence for human GH has been modified several times since Li's initial report in 1956. In 1969 (199) the complete primary structure of human GH showed that 188 amino acids were present in the molecule and the mw was reduced to 21,734. The hormone was also reported to be coiled

and to have two disulfide bridges holding it together. Two years later Niall (241) and Li and Dixon (197) modified the amino acid sequence of human somatotropin by adding two more residues to the peptide chain. The "final" change in the primary structure of human GH was reported by Li in 1972 (196) when glutamine was added in position 69 bringing the total to 191 amino acids. Li was also successful in synthesising a "protein with human growth hormone activity" in 1970 (208). This synthetic protein, however, possessed only 10% of growth promoting activity when compared to highly purified human GH.

Morris Raben in 1957 (280) used a more severe technique to extract GH from acetone-dried primate pituitaries. He treated the glands with hot glacial acetic acid followed by successive precipitations with ether and ethanol. The yield was slightly higher than that obtained by Li and Papkoff (206) but the chemical purity was probably less, since Raben himself states "it is assumed that the product is chemically impure..." (280). When assayed in the $\bar{H}$ rat, both primate GH's were as active as the porcine GH standard, which was prepared using the same procedure. A third major preparation of human GH was reported by Wilhelmi in 1961 (352) and later modified slightly in 1965 (265). GH was extracted from acetone-dried and fresh frozen pituitaries by ammonium sulfate, sodium chloride and hydrogen chloride precipitation. Ten fractions were obtained by this procedure; however, only two had a significant amount of biological activity as measured in the $\bar{H}$ rat (352). The activity of these two fractions ranged from 0.2-1.0 USP units/mg. The yield was about equal to that obtained by Li and Papkoff (206).

Other investigators have used a variety of methods to extract and purify primate GH (194). Among these three are noteworthy. Lewis et al. (195) prepared human GH by ultrafiltration. The preparation obtained was as biologically active as the bovine GH standard (0.97 USP units/mg) and by polyacrylamide gel electrophoresis, appeared to be quite homogeneous (186). In 1970 (113) and 1971 (112, 131) Friesen and collaborators published a method of isolating primate GH from culture medium in which pituitary glands had been incubated in the presence of ^{3}H-leucine. Their procedure for isolating human GH (113) involves the separation of the GH molecule and the ^{3}H-labelled proteins from the rest of the material in the culture medium by Sephadex chromatography followed by successive treatment with hot glacial acetic acid, acetone and ether. Monkey GH (112, 131) was isolated from culture medium using a similar method as for human GH (113) except that after Sephadex chromatography, the monkey GH was further purified by affinity chromatography using antibodies to HCS (131) or by immunoprecipitation (112). Peckham reported a method for purifying monkey and human GH in 1967 (273) by the use of Sephadex chromatography, anion exchange chromatography and electrophoresis on a sucrose density gradient. The two hormone preparations were quite homogeneous, as determined by polyacylamide gel electrophoresis and were approximately twice as active as the sheep GH standard (0.96 USP units/mg) in bioassay.

The presence of HCS in the placenta of pregnant human females was first demonstrated by Josimovich in 1962 (168). The hormone resembled human GH in its primary structure and amino acid composition (198, 311), in its mw (198) and in its immunochemical characteristics but was biologically a very weak stimulator of growth in H rats (109). The metabolic activities of HCS in man, however, are

thought to be identical to those observed with human GH (130). A similar hormone has also been isolated from term placentas of rhesus monkeys which has greater growth promoting activity than HCS (116). When the amino acid composition of monkey chorionic somatomammotropin (MCS) human GH and HCS were compared, more similarities existed between the latter two hormones than existed between MCS and human GH.

A significant amount of homology exists between the mammalian growth hormones when a comparison of their primary structures is made. Lewis et al. have recently shown that the amino acid sequence of rat and mouse GH (187), and bovine and ovine GH (308) are nearly identical to each other except for a difference of two amino acids in each case. The primary structure of porcine GH resembles rodent more than ungulate GH's, differing from mouse GH by only eight amino acids (187). All nonprimate GH's show greater structural homology to each other than to human GH (184, 317) which itself is structurally very closely related to HCS (33, 198, 311). Approximately 84% of the amino acids in HCS and human GH occupy identical positions in the primary structural sequence of the two hormone molecules (33, 198, 311). These homologies are significant in that they indicate distinct phylogenetic relationships exist between the GH molecules. There may be a common factor present within all GH's which has survived evolutionary changes and is responsible for the biological activity of the hormone. This concept was first proposed by Wilhelmi in 1955 (351) and based on the structural homologies reported to date, it is being more widely accepted by comparative endocrinologists and biochemists.

Similarities are also present in the electrophoretic mobility of the vertebrate GH's. Nicoll and Licht (250) have examined the polyacrylamide gel electrophoretic columns from selected vertebrate species and have reported that the major slow

moving band closest to the cathode in PE's from all the vertebrates has the most GH activity. The exception may be in PE's from the primates where the GH band appears to run ahead of the PRO band and is thus more electronegative (164, 188, 189).

Besides structural homologies and physico-chemical relatedness, the vertebrate GH's are also immunochemically related to each other. Extensive immunological studies using agar gel diffusion and radioimmunoassay (RIA), principally by Hayashida (142-145, 148, 419), have shown that common antigenic determinants are present in nearly all vertebrate GH molecules. A greater number of antigenic determinants are shared between GH molecules from closely related species than from species more phylogenetically distant from each other. A certain degree of species specificity, however, is still retained regardless of the number of shared antigenic determinants. Immunochemical relatedness has also been observed between human GH and HCS (168), which might be expected since their primary structures are quite similar (33, 198, 311). This immunological relationship has been taken advantage of in the purification of human GH and PRO (131) and in the cellular localization of the source of HCS in the placenta by immunofluorescence (305).

Two bioassays for GH have been published and are still routinely used. Marx et al. in 1942 (222) reported an assay in which $\bar{H}$ female rats were treated daily with GH for a period of ten days. The change in the overall body weight of the animal at the end of the experiment was taken as the endpoint. No minimum effective dose was reported; however, the animals did respond when given a daily dose of 100 μg of crude GH. Parlow et al. (265) have modified the procedure by reducing the number of injections to nine and changing the route of injection

to a subcutaneous administration of the hormone in an effort to improve the reliability and sensitivity of the assay. A more widely used GH bioassay was introduced by Greenspan et al. in 1948 (128). $\bar{H}$ female rats are treated for four days with daily injections of GH and on the fifth day the tibia is removed and the width of the epiphyseal cartilage is measured and used as an indicator of GH activity. The minimum effective dose is 2.5-5 μg/day which makes this procedure considerably more sensitive than that developed by Marx et al. (222). Variability in responses observed from one animal to another along with seasonal variations in responsiveness (139, 222) have been serious disadvantages in these assays and appear to be inherent disadvantages of any bioassay system.

The chondrogenic effect of GH on the tibia has been reported to be an indirect action which is primarily mediated by a substance called somatomedin (57). This substance is taken up by cartilage and its activity appears to be directly related to the presence of GH. The tibia assay, therefore, is a measurement of the ability of GH to stimulate somatomedin secretion which in turn directly acts on the chondrocytes.

Nicoll and Licht (250) have introduced an assay for GH which uses a submammalian species, the intact, juvenile toad, Bufo boreas. The methodology involves the treatment of the experimental animal for ten days with GH and then measuring the changes in the length and the weight of the toad. The latter parameter is generally too unreliable and is rarely used. This assay system may be useful in the future as a method for measuring GH in lower vertebrates, since GH from some species such as the modern bony fishes is inactive in present day mammalian GH bioassay systems (142, 351).

The most reliable and sensitive assay methods for quantitating hormone values in pituitary or plasma are the immunoassays. The only major drawback in any of these procedures, is whether the hormone being assayed is truly the biologically active hormone or is there also present a nonimmunoreactive but biologically active hormone as well. This problem has recently been investigated by Ellis (72) who observed discrepancies in results obtained when assaying the same plasma sample by bioassay in the rat and by RIA. He concluded that the rat GH molecule loses some of its immunoreactivity after it is secreted from the pituitary with little or no change in its biological activity.

Three immunoassays have been used to measure plasma and pituitary concentrations of human GH. Read and Bryan in 1960 (287) reported values in human plasma by the hemagglutination technique. GH values in plasma from normal children and adults ranged from 90-600 ng/ml and in all acromegalics studied, the values were greater than 500 ng/ml. Trenkle et al. (335) used complement-fixation to assay the amount of GH in fresh human pituitary glands and reported 21.3 and 31.8 µg/mg wet pituitary weight in each of two glands assayed. These values agree with results obtained when assaying GH in human PE's using the quantitative precipitin test (335) and the tibia bioassay for GH (128). The most sensitive assay for measuring hormone values is the RIA which is capable of detecting less than 1 ng of a hormone in PE's or plasma. The assay was introduced to endocrinology by Berson and Yalow in 1958 (29) when they reported their studies of the disappearance of exogenously administered insulin from the plasma of rabbits. In 1960, they published the first article using RIA to measure hormone (insulin) levels in human plasma, along with a thorough analysis of the technique (356).

Since this initial report, countless articles have been published using RIA to measure hormone values in various biological media. Glick et al. in 1963 (123) reported the first complete and successful study of plasma GH values in man. They observed that the normal levels of GH in adults were between 0-5 ng/ml, while in most acromegalics the values were greater than 50 ng/ml. GH levels have also been measured in the plasma and the PE's of several mammalian species by RIA such as in the rat (302), mouse (317), cat (262), dog (336), pig (218), sheep (58, 157), rabbits (118), beef (258), and monkeys (232). The technique has been useful in immunochemical studies with GH (142-145, 148), and is now an established clinical method for assaying hormones in patients.

Histological studies of pituitaries from patients with acromegaly and from experimental animals and humans with dwarfism led investigators to suspect that GH was secreted by an acidophilic cell type. The earliest reports of hyperplasia of the acidophils in acromegalics were published by Bailey and Davidoff (23) and Cushing and Davidoff (52) who observed this phenomenon in all patients displaying the clinical signs of acromegaly. In an effort to define the etiology of this disease, Cushing and Davidoff (52) concluded "It is these glandular cells (referring to the acidophils) which elaborate or at least hold a hormone... which are almost certainly the cause of acromegaly and gigantism in man." This was the first implication that GH was secreted by the acidophils. In a study by Hewer (158) on a single pituitary taken from a patient with ateliotic dwarfism, the opposite clinical observation was made; only 0.2% of the cells in the gland were acidophils. Smith et al. in 1930 (322) observed that acidophils were absent in pituitaries from dwarf mice as well. Implants of normal mouse pituitaries were observed to restore the growth of the dwarfs (322); however, implants of

pituitaries from dwarf mice failed to induce growth in the same animals (323). These early reports of hyperplasia of the acidophils in acromegalics and the absence of the same cells in dwarfs led Severinghaus in 1938 (310) to conclude "...it seems certain that the growth regulating hormone is secreted by the acidophils of the anterior lobe of the hypophysis."

Prior to the association of GH with the acidophils by Severinghaus (310), a few reports were published regarding the use of specific stains which tinctorially differentiated the acidophils into two separate categories. Cleveland and Wolfe in 1932 (46) combined the two dyes orange-G and erythrosin in a procedure which stained the acidophils orange and red, respectively. Dawson and Friedgood used a modification of Mallory's connective tissue stain and Heidenhain's azan stain (66) to differentiate the acidophils of the rabbit and the cat into orange-G and azo-carmine staining cells. Dawson referred to these two cell types as the "ordinary acidophile" (meaning the orange-G staining cell) and the "modified acidophile" (meaning the azocarmine staining cell) (60). Several investigators such as Brooks (36), El Etreby and Tüshaus (70) and Herlant (154) have also published stains which will differentiate the two acidophilic cell types. Herlant's tetrachrome staining procedure (154) is probably the most widely used. It will differentiate the acidophils into orange-G staining cells and erythrosin staining cells, as well as staining the basophils with either aniline blue or alizarin blue. The various stains have been applied to pituitaries from numerous mammalian species (36, 63, 65, 66, 70, 125, 127, 152, 156, 238, 271, 330), as well as several varieties of birds (281, 282), reptiles (88, 136, 209), amphibians (4, 49, 64) and fishes (17, 75, 78, 300, 306) in an effort to differentiate the two acidophils in these vertebrates.

The clinical and experimental data eventually led the histologists to deduce that the "ordinary acidophile" secreted GH, a fact well accepted amongst endocrinologists long before it was formally stated by Goldberg and Chaikoff in 1951 (125). However, the most conclusive evidence that the acidophils were the cells which secreted GH came in 1960. Leznoff et al. (190) used the immunofluorescent technique of Coons (47, 48) and localized fluorescence in the acidophils of normal humans and patients with acromegaly with fluorescein-labelled antibodies to human GH. This provided the first direct experimental evidence of the cellular source of GH. Similar studies by Beck et al. in 1966 (22) have confirmed Leznoff's finding. Herlant and Pasteels (156) tinctorially differentiated the acidophils in man and by the fluorescent antibody technique demonstrated fluorescence only in the orange-G or alpha cells using antibodies to human GH. Immunofluorescent localization of GH in the acidophilic cells of the pituitary has been reported in other vertebrates such as the monkey (152, 156), sheep (298, 330), ox, mouse, and rat (298), rabbits (312), pig, dog (240), and the salmon (226).

I. General Summary Regarding Growth Hormone

In the last 70-80 years, GH has been thoroughly studied. The biochemists have isolated it from several vertebrate species, have chemically characterized it, have studied its physical properties and have succeeded in producing a synthetic form of the hormone molecule. The experimental endocrinologists have observed numerous similarities in the primary structure and in the immunochemistry of GH's from various vertebrates. Clinicians have observed and described the clinical manifestations of hypo- and hypersecretion of GH, have analyzed its metabolic effects in humans and have developed methods to measure minute quantities of the hormone in patients' plasma. Lastly, the histologists

have determined the cellular origin of GH using several different methods as well as describing in detail the secretory events occurring within the somatotroph.

B. Prolactin

In 1928, Stricker and Greuter (331) first demonstrated that there was a factor in the pituitary gland which influenced lactation. They observed lactation in adult psuedopregnant female rabbits following administration of PE's while no response was observed in juvenile female rabbits. At about the same time Evans and Simpson (86) noted mammogenesis in juvenile and adult female rats bearing implants of bovine or rat pituitaries. Chronic administration of anterior lobe extracts also produced the same effect (82). Riddle and Braucher in 1931 (293) reported that bovine PE's stimulated growth of the crop sac of doves and pigeons as well as inducing crop milk formation. The investigators were unsure as to which factor in the PE's was inducing the observed responses, causing them to remark "It is not known whether the principle activating the crop-gland is the growth, the sex maturity, or a third and now unknown anterior pituitary hormone..." A year later Riddle and his associates (291) isolated the third, unknown anterior pituitary hormone from ovine and bovine pituitaries. The procedure involved extraction and precipitation with acid, alkaline-ethanol and ethanol at several different pHs. "The hitherto undefined pituitary principle which is essential for lactation..." was termed prolactin and became the first anterior pituitary hormone to be isolated in relatively pure form. Riddle et al. (291) reported that their new hormone induced lactation in adult male and female guinea pigs and in adult female rabbits as well as stimulating the crop sac of pigeons and doves. The same investigators reported in 1933 (292) a more detailed account of the extraction and purification procedure for obtaining PRO from the previously

mentioned species and from porcine pituitaries as well. Within a few years, other investigators also reported methods for purifying ovine PRO (205, 211, 350) including W.R. Lyons (211) and C.H. Li et al. (205).

Chemical analysis of ovine PRO was begun by Li et al. (205) who reported in 1941 a mw of 25,000 for ovine PRO and 26,500 for bovine PRO. More recent chemical analyses of ovine PRO, however, have shown that the mw is slightly less than first reported, and is now calculated to be 22,550 (201). There are 198 amino acids in the hormone molecule with three disulfide bridges joining together separate portions of the molecule (200, 201). Eppstein (81) has studied the chemistry of porcine PRO and reports a mw of 25,000 which is similar to that observed for ovine and bovine PRO by Li (201, 205).

PRO from a few other mammals besides the sheep, beef and pig have been purified along with PRO's from three submammalian vertebrates. The first preparation of rat PRO was obtained by Kwa (177) from rat pituitary tumors. The tissue was processed by differential centrifugation and DEAE cellulose chromatography and yielded a product which was later reported (178) to have an activity of 15.6 IU/mg. Two other groups of investigators, Groves and Sells (129) and Ellis et al. (73, 74) have also prepared rat PRO preparations which have an activity of 15 IU/mg and 28 IU/mg respectively in the pigeon crop sac bioassay. Ellis et al. (74) have calculated the mw of rat PRO to be about 22,000. Kwa and Verhofstad (180) later purified the first mouse PRO from estrogen-induced pituitary tumors in mice. A few years later, Lewis' group (73) prepared a very active preparation of mouse PRO by preparative gel electrophoresis. The activity was reported to be 25 IU/mg in bioassay, with a mw of 23,000 similar

to rat PRO (74). Ellis et al. (73) have also purified rabbit PRO from a side fraction off their purification procedure for rabbit GH. The activity, however, is only 6 IU/mg. PRO from two species of fishes, the carp and the pollack, have been extracted by Emmart and Wilhelmi (80). Neither preparation is active in the crop sac bioassay or in maintaining survival of the H Killifish Fundulus in fresh water, which is a well documented activity of mammalian PRO's (28). The third submammalian PRO to be extracted is shark PRO (184) which Lewis reports to be a very crude preparation at this time.

The complete isolation and purification of primate PRO was achieved by Friesen and colleagues (111-114, 117, 131, 162, 164) in the early 1970's. The apparent low concentration of PRO present in normal human pituitaries, based on histological data (127) and recent experimental data by Friesen's group (132), coupled with the misconception that primate GH and PRO activities resided on the same hormone molecule (194) were two principle reasons for the difficulties surrounding the purification of the hormone. Several investigators made unsuccessful attempts to purify human PRO before Friesen and colleagues succeeded. Chance et al. (42) prepared a crude fraction of PRO from acetone-dried human pituitaries by isoelectric precipitation. The activity of the hormone in the crop sac bioassay was only about one third as active as a highly purified ovine PRO which was prepared in the same manner. During the purification of human GH by acid and salt precipitation, Wilhelmi's group (265, 352) observed that GH and PRO activity, as measured by bioassay, were always present in the same fractions. All PRO preparations were contaminated with GH to varying degrees. Further purification of human PRO by Freeman and Wilhelmi (106) using DEAE chromatography failed to remove the GH contaminant which in some fractions was as high as 1.5 IU/mg.

Various ratios of PRO/GH activity were obtained, ranging from 15-30:1.

Apostolakis in 1965 (5) isolated what was thought to be purified human PRO by acetone extraction and Sephadex chromatography. Two PRO preparations were isolated and found to be quite active in the crop sac bioassay; however, each had a significant amount of GH contamination. The PRO/GH ratios of Apostolakis' human PRO preparations were generally higher than Wilhelmi's (265, 352), suggesting that the latter preparations were not as pure as Apostolakis'. Several reports indicate that the Apostolakis PRO molecule may be modified human GH. Stephenson and Greenwood (329) and Laron and Apostolakis (182) have shown that in agar gel diffusion studies a reaction of identity occurs between purified human GH and PRO when using antiserum to Apostolakis' human PRO. In RIA studies, an equal or greater amount of cross-reactivity is observed between antiserum to human GH and Apostolakis' PRO than with the same antiserum and purified human GH itself (329). Immunofluorescent studies with anti-Apostolakis human PRO serum causes fluorescence of all the acidophils in the anterior lobe (35) without distinguishing between GH and PRO secreting cells. The results suggest that cross-reactivity exists between GH within the somatotrophs and the anti-PRO serum. Pasteels in 1967 (267) extracted PRO from the culture medium in which fetal human pituitaries had been incubated. Acetone acid extracts of the medium were only slightly active in bioassay. Further purification using Sephadex and DEAE cellulose chromatography as well as preparative gel electrophoresis eventually yielded a product which had an activity of only 2.95 IU/mg (268).

In 1970, Friesen et al. (111, 113) were successful in isolating a radioactive protein from the culture medium in which human pituitaries were incubated in the presence of ^{3}H-leucine. This protein could not be precipitated by antibodies to

human GH and was thought to be human PRO. Dr. Hayashida informed Dr. Friesen (June, 1970) that primate (rhesus monkey) GH and PRO could be distinguished immunochemically using antibodies to ovine PRO and antibodies to human GH, based on immunofluorescent and precipitin studies in agar gel performed by this author for this doctoral thesis. In 1971, Friesen et al. (114, 162) reported that antibodies to ovine PRO were able to precipitate the radioactive protein presumably PRO, which would not cross-react with anti-human GH serum. Following the purification of monkey PRO and after raising antibodies to this molecule, Friesen et al. (114) precipitated an even greater amount of the radioactive protein with anti-monkey PRO than with antiserum to ovine PRO. This was the first purified primate PRO which was shown to be immunochemically distinct from human GH. Human PRO has also been extracted and purified from culture medium in which pituitaries from patients with adenomas of the anterior lobe had been incubated (117, 162). In 1972, Friesen and co-workers (164) published a detailed account of the purification of human PRO by more conventional techniques. The starting material was the discarded supernatant from the final step in Raben's procedure (280) for the purification of human GH. The supernatant was precipitated and placed on Sephadex, DEAE and CM cellulose chromatographic columns. The final product was highly purified primate PRO with an activity of 30.5 IU/mg in bioassay and a GH contamination of only 0.4%. The complete amino acid composition and mw determination of Friesen's human PRO has not been reported to date. Preliminary data show that the N-terminal amino acid is leucine (164), and the C-terminus is cystine, which is the same as in ovine PRO (184).

Lewis et al. (188) have prepared the only other purified human PRO preparation. The extraction procedure was carried out on frozen pituitaries using high

speed centrifugation, ultrafiltration and Sephadex and DEAE cellulose chromatography. The hormone obtained has 28 IU/mg of PRO bioactivity, with a 2% GH contamination (319). A mw was calculated to be about 22,000 (188) which is nearly identical to that calculated by Li et al. (199) for human GH.

Two different preparations of monkey PRO have been isolated. Peckham and Nicoll in 1971 (275) extracted monkey PRO from culture medium in which pituitaries from lactating female rhesus monkeys were incubated for 30 days. A crude preparation was obtained following chromatographic procedures and was examined by disc and density gradient electrophoresis. The highest ratio of PRO:GH activity obtained was 10,000 IU/mg (probably on the basis of the pigeon crop sac assay). The actual biological activity was not reported; however, the GH contamination was calculated by Friesen's group (164) to be about 10%.

The most highly purified monkey PRO has been isolated by Friesen et al. (111, 112, 114, 131, 132). The initial isolation of the hormone was from culture medium obtained after incubating pregnant and lactating female rhesus monkey pituitaries in the presence of H-leucine. The PRO was removed from the culture medium by passage through a Sephadex column and then by immunoprecipitation using antibodies to ovine PRO (112). A greater amount of PRO was precipitated by antibodies raised to a rather crude preparation of monkey PRO than with anti-ovine PRO serum. The crude preparation of monkey PRO was obtained by passing culture medium in which pregnant monkey pituitaries were incubated, first through a Sephadex column and then through an affinity chromatography column on which antibodies to HCS were attached (114, 131, 132). Approximately 99.5% of the GH in the medium attached to the anti-HCS column and was removed. The activity of this initial crude preparation was between 1.8-4.9 IU/mg (114, 132)

as measured by the pigeon crop sac bioassay and had only 0.02% contamination with GH. Preparations with slightly more biological activity have been obtained by affinity chromatography, with the most active preparation having an activity of 13 IU/mg (131). No chemical analyses have been conducted on Friesen's monkey PRO although it is speculated that the mw will be very similar to that reported for monkey GH (112).

A lactogenic hormone, chorionic somatomammotropin, has also been isolated from the placenta of humans (109) and monkeys (116). This hormone resembles GH in its chemical structure, metabolic activities and immunologic properties (see above), and has also been found to possess some degree of PRO-like activity. HCS exhibits a significant amount of lactogenic but only a lesser amount of crop sac activity (168). The latter response has been calculated to be about 15% of that observed with purified ovine PRO (195).

Only a few studies have been conducted comparing the structural relationships and homologies among the PRO's due to the paucity of purified PRO preparations available. Seavey and Lewis in 1971 (307) reported a difference of only two amino acids between ovine and bovine PRO. Bovine PRO has one less amino acid in its overall composition than ovine PRO reducing the total to 197 amino acids. According to Lewis (184) the homology between these two hormones is 99%, while that observed between ovine and porcine PRO is 85% and between ovine and human PRO is 75%. No studies have been published comparing the primary structures of rat and mouse PRO or human and monkey PRO. A comparison of HCS and ovine PRO shows that 25% of the total amino acids of the two molecules are identical (33), while the overall homology between the two protein hormones is 60%.

Comparative electrophoretic studies using polyacrylamide gel electrophoresis on PE's from several vertebrate species indicate that the band with the greatest amount of PRO activity is more electronegative and migrates faster than the presumed GH band (237, 252). The exception to this seems to occur with the primate PRO's. Human PRO appears to migrate more slowly and is more electropositive than GH by acrylamide gel electrophoresis (164, 188, 189).

A few comparative immunochemical studies have been conducted using anti-sera to PRO. Results obtained with agar gel diffusion studies (140, 150, 152, 234, 319), RIA (37, 38, 150, 167, 249, 285, 318) and the quantitative precipitin test (78, 80) indicate more species specificity exists between the vertebrate than was observed between the vertebrate GH's. In addition, no clear cut phylogenetic relationships exist for PRO either within or between the classes of vertebrates. This relative lack of extensive cross-reactions among PRO's from various species may be related to the multiplicity of biological activities that have been attributed to PRO (28, 247). Bern and Nicoll (28, 247), Meites and Nicoll (228), Riddle (290), and Lyons and Dixon (214) have published detailed reviews of all the biological activities of PRO in each class of vertebrates. They point out that a certain degree of species specificity is present when considering the bioactivity of the vertebrate PRO's but to a lesser degree than is present in the immunoactivity of the proteins

For nearly every biological activity observed with vertebrate PRO's, there exists a specific assay system to measure changes in the level of the hormone. A few bioassays are routinely used to quantitate levels of PRO in PE's and plasmas and these are worth noting. The most widely used bioassay for pro is the pigeon crop sac bioassay. Riddle et al. (291, 292) first introduced the systemic pigeon crop sac assay following the initial isolation of ovine PRO (291). The assay involves

injecting either juvenile or adult pigeons subcutaneously or intramuscularly with PRO daily for four days and observing the change in the weight of the crop-gland at the end of the experiment. Lyons in 1937 (211) improved the pigeon crop sac assay by introducing the local crop sac assay which is about 1000 times more sensitive. The hormone is injected either intradermally or subcutaneously directly over the crop sac itself daily for two days. The crop-gland then is removed and examined visually to determine whether stimulation occurred. Nicoll (245, 246) has introduced a major modification in each of the two crop sac assays in an effort to reduce individual variability in response among pigeons, to make the assay more sensitive, to reduce nonspecific inflammatory reactions which is a constant problem with the local crop sac assay (12, 245) and to provide a means to accurately quantitate the endpoint.

In vivo and in vitro bioassays have been developed to measure the lactogenic and the mammogenic activity of PRO. In 1942, Lyons (213) developed an in vivo assay using hysterectomized and ovariectomized virgin female rabbits which were treated with a single intraductal injection of PRO following priming of the mammary gland with subcutaneous injections of steroids. After five days, an attempt was made to express milk and a positive response was recorded only if milk could be expressed. Chadwick in 1963 (40) improved the assay by modifying the procedure and the endpoint so that greater quantitation of results could be attained.

In vitro assays have been used successfully to assay PRO in plasma by using either a semiquantitative or quantitative endpoint. In 1957 Elias (71) was the first to culture mouse mammary glands and to maintain them for a suitable period of time for experimental study. The technique was used as an assay for PRO in 1968 by Mishkinsky et al. (233) who employed explants of rat mammary tissue and

cultured these in a synthetic medium containing various hormones for the maintenance of the culture along with varying concentrations of PRO. The glands were examined histologically at the end of 48 hours and graded in a semiquantitative manner, using the extent of lobulo-alveolar growth as an endpoint.

Recently, several investigators have developed in vitro assay systems to measure PRO in human plasma samples (101, 102, 174, 210, 337). Frantz and Kleinberg (102, 174) used mammary tissue from midpregnant female mice while Forsyth and Myres (101) used tissue from pseudopregnant rabbits to develop their respective bioassays. In both systems, the tissue was incubated in the presence of human serum or plasma and then examined at the end of the experiment for histological changes in the mammary tissue. The responses observed were scored based on the extent of development of the mammary tissue using certain histological criteria and the activity was then expressed in terms of an equivalent response obtained with an ovine PRO standard. Turkington (337) and Loewenstein et al. (210) have used more quantitative biochemical endpoints in their in vitro mouse mammary bioassays for human PRO. Turkington (337) measures the total amount of casein synthesized when mammary tissue is incubated with human plasma while Loewenstein et al. (210) compute the changes in the activity of the enzyme N-acetylactosamine which are induced by the PRO in the plasma samples. In both assays the values obtained are expressed in terms of an equivalent response observed with an ovine PRO standard. The in vitro bioassays are quite sensitive and relatively simple to perform; however, a significant amount of variability is present within the assay system to reduce its usefulness. Shiu et al. (313) have recently developed a new radioreceptor bioassay for PRO. The PRO receptors are isolated from the cell membranes of mammary tissue taken from midpregnant rabbits

and incubated together with ^{125}I-labelled PRO and unlabelled PRO. A competitive binding reaction occurs between the labelled and unlabelled PRO for the membrane bound receptors. The amount of ^{125}I-labelled PRO which remains bound to the receptors after the incubation expires is taken as the endpoint of the assay. A standard curve representing the inhibition of the bound ^{125}I-labelled PRO by unlabelled PRO is obtained.

RIA systems have been developed to measure PRO in PE's and plasma from several mammals. Arai and Lee in 1967 (7) published the first RIA for ovine PRO in an effort to assay plasma concentrations of PRO in normal, pregnant and lactating ewes. Bryant and Greenwood (37) have also reported a RIA for ovine PRO which can be used to assay PRO in goat plasma since goat PE's parallel the ovine PRO standard curve. Levels of PRO in plasma and PE's from rats (179, 181, 254), mice (318), pigs (285), and cattle (167, 285) have also been reported using RIA.

Several immunoassays have recently been reported which have been used to measure circulating levels of PRO in humans. The first RIA system was a heterologous assay using antiserum to monkey PRO, and ovine PRO as both the standard and the tracer (132). This system, however, was too insensitive to measure PRO in plasma samples. Friesen and colleagues (163) later published a homologous RIA using antiserum to monkey or human PRO, either of the primate PRO's as the tracer and a serum sample taken from a postpartum patient as the standard. The limitation of the assay system is that the amount of PRO present in the serum used as the standard, which had been previously assayed by Frantz and Kleinberg (102, 174) using an *in vitro* bioassay system, has been calculated and expressed in terms of ovine PRO equivalents. Even though a crude monkey PRO preparation was parallel to the standard curve, it has never been used as the standard *per se*.

The results of Friesen's work have led to a clearer understanding of PRO physiology in man. Slight fluctuations in daily plasma PRO values are observed throughout the entire menstrual cycle; however, surges of the hormone during the preovulatory, ovulatory or postovulatory phase of the cycle are not present (163). Plasma values in normal males range from 0-28 ng/ml while in females they vary from 0-30 ng/ml (163) with a mean of 5.0 and 8.1 for healthy ambulatory males and females, respectively (115). A progressive increase in plasma PRO is observed during pregnancy with a peak at term of 207 ng/ml (110), while a steady decrease in fasting values of PRO occurs following parturition in both lactating and non-lactating females (110, 163, 340). Very high PRO values are found in plasmas from neonates and from patients with galactorrhea, while very low concentrations are present in most acromegalic patients (163).

Other investigators have also reported plasma values of PRO in humans using heterologous and homologous RIA systems (see section C-24, Abstracts of the IV International Congress of Endocrinology). Nokin et al. (255) have reported values in patients using a human serum sample as the standard, antiovine PRO serum, and ovine PRO as the tracer while Jacobs et al. (166) used a mixed heterologous RIA system with antiovine PRO serum, porcine PRO as the tracer and human serum as the standard. Sinha et al. (319) have recently reported the first completely homologous RIA system in which human PRO is used as both the standard and the tracer (unlike Friesen's RIA (163)) along with an antiserum to human PRO. The results reported for mean PRO values in adult males and females are significantly higher than observed by Friesen et al. (115); however, the range of values observed by Sinha et al. (319) is similar to that previously reported by Friesen's group (110, 115, 163). The other homologous RIA for human PRO was developed by

Bryant et al. (39) who used an assay system with human PRO prepared from culture medium in which fetal pituitaries had been incubated. The inconclusive results along with a 1% GH contamination in their hormone preparation led the investigators to state "The radioimmunological studies reported as an example of either the sophisticated use of the radioimmunoassay technique or its frank abuse."

Besides changes in the plasma concentrations of PRO in mammals during different stages of their reproductive cycles, marked changes in the cytology of the pituitary have also been observed. As early as 1909, Erdheim and Stumme (see Floderus (92)) observed an increase in the number of chromophobes present in pituitaries of pregnant human females and coined the term "pregnancy cells." Severinghaus (309), however, states that these cells are specialized acidophils which arise from chromophobes and are only found during pregnancy. Studies made on pituitaries obtained from pregnant female guinea pigs revealed an increase in the number of stainable acidophilic cells (173). In rats, however, no cytological changes were observed during pregnancy until just prior to parturition when the number of acidophils began to rise and continue to increase throughout lactation (87). Rats treated with estrogenic steroids were observed to have a higher concentration of acidophils in their pituitaries than in pituitaries taken from untreated control animals (16). The histologic observations showing an increase in the number of acidophils during late pregnancy, and lactation, and following estrogen treatment agree with the experimental data in which concomitant rises in pituitary PRO levels occur under the same physiological and experimental conditions (161, 230). These early observations led investigators to conclude that PRO was secreted by an acidophilic cell type (87, 304); however, they failed to differentiate which of the two acidophils was the lactotroph. Several different stains which differentiate the

two acidophils were developed in the 1930's and applied successfully to selected species from nearly all vertebrate classes (see preceding page). Notably, A.B. Dawson and H.B. Friedgood (66) reported a staining procedure in which the acidophils were differentiated into orange-G and azocarmine staining cells. In 1939, Dawson referred to the latter cell type as the "modified acidophile" (60) which he later states, in his comprehensive study of the cytology of the female cat pituitary, to be the cell type responsible for PRO secretion (62).

The most definitive method developed to identify the histophysiology of endocrine cells is the fluorescent antibody technique of Coons (47, 48). This method has been used to demonstrate the cellular source of PRO in several mammalian and submammalian species. The results obtained corroborate the experimental findings of Dawson and others. Emmart et al. (79) were the first to use immunofluorescence with anti-PRO serum. They localized fluorescence in the acidophils of pituitaries from normal rats and cats (79) as well as in tumor cells present in the anterior lobe of the rat pituitary (76). Herbert and Hayashida (152) and Pasteels et al. (271) have used antibodies to ovine PRO on primate pituitaries and reported fluorescence only in the carminophils of pars distalis of normal males and females in glands from pregnant and lactating females as well. Other investigators have applied the immunofluorescent techniques to several different mammalian species (264, 298, 312, 330) as well as a few submammalian vertebrates (1, 75, 78, 225, 226, 344). In all these studies, antibodies to ovine PRO were used and fluorescence was present in one of the two pituitary acidophilic cell types, except in the cyclostome pituitary (1), where no fluorescence was observed.

1. General Summary Regarding Prolactin

Like GH, a comprehensive study of PRO has been made. It was the first hormone to be isolated and purified from the mammalian pars distalis. More biological activities are observed with PRO than with any other vertebrate hormone. Innumerable assay systems have been devised to demonstrate these activities and/or to measure changes in concentrations of PRO under various experimental conditions in several vertebrate species. A significant amount of species specificity is found when analyzing the chemistry, bioactivity and immunoactivity of this hormone. Finally, the morphologists have elucidated the cellular source of PRO and have documented an array of changes occurring within the lactotroph under normal, experimental and pathological conditions.

C. Growth Hormone and Prolactin in Primates

The existence of a separate PRO in primates was suggested by the findings of clinicians, experimental endocrinologists and anatomists prior to its biochemical isolation and purification. In the interim, doubt was cast by many scientists in regards to its existence as a separate hormone molecule distinct from GH, especially during the early and mid-1960's.

Cells which stained faintly with eosin and were thought to be chromophobes were observed only during pregnancy in the anterior lobe of humans by Erdheim and Stumme in 1909 (see above). These cells, which were termed "pregnancy cells", were the dominant cell type present at parturition. According to Severinghaus (309), they are a special type of acidophil which may resemble chromophobes due to their highly active secretory pattern. Rasmussen (283, 284) failed to observe pregnancy cells in human pituitaries obtained during pregnancy and lactation as well as noting no changes in the number of chromophobes or acidophils during these two

physiologic periods. He did observe a marked increase in the size and weight of the pituitary during both pregnancy and lactation but could not account for this on the basis of cellular hyperplasia. Floderus (92), however, in his study of the human pituitary implied that changes observed in the pituitary during pregnancy can be attributed to the presence of pregnancy cells. Romeis (see Purves (278)) observed changes in the pituitary of pregnant females and described an increase in the number of cells which resembles Erdheim and Stumme's pregnancy cells (278). Even though these results contradict Rasmussen's observations, they help to explain the probable cause of the weight increase found in the pituitary during pregnancy and lactation.

In 1935, Lyons and Page (217) reported PRO activity in the urine of lactating females. They observed a marked stimulation of the crop sac in pigeons following injection of the urine and concluded that as much PRO is excreted daily in the patients as can be extracted from bovine pituitaries. Lyons (212) later showed that bioassayable amounts of PRO were present in the urine of normal males and neonates, which may account for the formation of "witches' milk" in newborns. Meites and Turner in 1941 (229) extracted a bioassayable PRO molecule from the urine of normal males and females and from pregnant and lactating patients. The amount of PRO present per day in the urine of lactating females varied between 4-12 IU depending on the amount of milk produced.

Coppedge and Segaloff in 1951 (50) extracted PRO from the urine of normal humans and from patients with a variety of endocrine disorders. The extracts were assayed in the pigeon crop sac test and normal daily excretion rates of 0-305 IU for males and 0-151 IU for females were reported. Higher values were observed during pregnancy but following parturition, a rapid decline in PRO values

occurred regardless of whether the patient was lactating or not. In a similar study, Bahn and Bates (12) attempted to bioassay PRO extracted from patients; however, they failed to obtain a positive response even with PRO samples extracted from urine obtained from patients with galactorrhea or from pregnant or lactating females.

Simkin et al. (314, 315) prepared acetone-acid extracts of PRO from blood samples obtained from normal males, cycling females, pregnant and lactating females, and children. The extracts were assayed in the pigeon and positive responses were only observed in extracts from cycling women and from pregnant and lactating females. Two PRO peaks were observed in normal cycling females, one in the first five days of the cycle and the second in the last 10 days of the cycle (314), with the greatest concentration of PRO, 0.04 IU/ml, found during the luteal phase of the menstrual cycle.

In 1954, Forbes et al. (95) described the clinical findings of a syndrome in which the patients had persistent galactorrhea and amenorrhea, with enlargement of the sella turcica. The findings were not associated with a recent pregnancy or with acromegaly. Pituitary tissue from several patients was examined and a chromophobe adenema was observed in each case. The investigators concluded that the tumor was secreting excessive amounts of PRO. A similar syndrome was described by Argonz and del Castillo (8). They also state that the clinical findings are due to hypersecretion of PRO, even though a normal sized sella turcica was observed in all of their patients.

The early (pre-1960's) morphological and clinical evidence favored the concept that a separate PRO was present in all primate pituitaries. However, after GH had been purified from human and monkey pituitaries and analyses of these two hormones began, the existence of a separate PRO hormone in primates was questioned.

In the first reports of the isolation of GH from primates by Li and Papkoff (206) and Raben (280) in the mid-1950's, no references were made with regard to the degree of contamination of these hormone preparations by other anterior lobe hormones, including PRO. However, in 1961, Lyons et al. (216) analyzed the biological activity of human GH prepared by Li and Raben and concluded the following regarding the two hormone preparations:

a) they promote growth of the epiphyseal cartilage of the H rat

b) are active in the local or systemic pigeon crop sac assay

c) cause hypertrophy of the corpora luteal cells

d) synergize with ovarian steroids to induce ductal and lobule alveolar growth of the mammary gland and

e) are lactogenic in H, ovarectomized and adrenalectomized rats.

At the same time Wilhelmi (352) reported that all of his purified preparations of human GH contained some bioassayable PRO. Conversely, his purified human PRO preparations had GH activity. Wilhelmi, therefore, concluded that these two hormones possessed similar biological properties.

Several reports were published to substantiate the findings of Lyons et al. (216) and Wilhelmi (352), and to promote the theory that the two activities reside on a single hormone molecule. Ferguson and Wallace (89) examined a few preparations of GH by zone electrophoresis on starch-gel and compared the results with those obtained with purified ovine PRO run under the same conditions. The electrophoretic patterns showed that four major components were present in both hormone preparations which had similar if not identical mobilities. PRO activity was present in all four components of the GH molecule with an overall pigeon crop sac activity of about 3 IU/mg. Chadwick et al. (41) isolated a GH preparation

using Li's procedure (206) and observed similar findings as Ferguson and Wallace (89) using starch-gel electrophoresis. They also reported that their GH caused lactation in pseudopregnant rabbits but was inactive in the crop sac assay. Forsyth, Folley and Chadwick (100) assayed 14 different preparations of human GH in the crop sac and pseudopregnant rabbit bioassays. All were active in the latter assay; however, only two were able to induce a response in the pigeon crop sac test. Based on their findings, they calculated the lactogenic activity of human GH to be from 15-22 IU/mg. Forsyth and Folley (99) in a later report observed similar findings with ten more human GH preparations. The discrepancy between the negative results obtained in the pigeon crop sac bioassay with primate GH's by Chadwick et al. (41) and Forsyth et al. (99, 100), and the slight but significant positive findings reported by Ferguson and Wallace (89) and Lyons et al. (216) is unexplainable.

Lyons et al. (215) demonstrated that human GH was active in promoting lactogenesis in both humans and monkeys. Patients who were unable to produce enough milk were treated daily with human GH to induce further lactogenesis. Throughout the experimental period the babies were weighed and the changes observed as compared to those babies whose mothers received a placebo were taken as an indication of the efficacy of the therapy. In the majority of cases, the babies whose mothers received GH had a greater increase in body weight than did the controls. In two women who ceased lactating, administration of GH subcutaneously or intraductally restored normal lactation.

Not only has purified human GH been shown to have PRO-like activities in man, ovine PRO has been reported to have induced metabolic effects in humans similar to those observed for GH. Bergenstal and Lipsett (26) and McGarry and Beck

(224) reported that ovine PRO causes nitrogen retention (26), an increase in excretion of calcium, reduced serum urea nitrogen and causes a decrease in carbohydrate tolerance (224). Ovine PRO did, however, fail to raise plasma free fatty acids in man which is a well documented activity of human GH (224).

Monkey GH has also been reported to have PRO-like activity. In the original report by Lyons et al. (216) in 1961, monkey GH from two different laboratories had the same PRO-like activities as those reported for human GH. Peckham et al. (274) observed minimal crop sac-stimulating effects from a single monkey GH preparation as well as from two human GH preparations isolated in their laboratory. They reported the activity to range from 0.89-1.09 IU/mg, which was about 1/16 as active as the purified ovine PRO standards. Forsyth (96) reported similar findings as Peckham et al. (274) although she observed a higher crop sac-stimulating effect with one monkey GH; 4 IU/mg. Forsyth also assayed monkey GH for its lactogenic activity following intraductal injection of the hormone into pseudopregnant rabbits and observed a potency of 15 IU/mg (96).

The lactogenic activity of both monkey and human GH has also been documented using in vitro techniques as well. Rivera et al. (297) selected GH preparations which had high growth promoting activity in the rat tibia assay, were extremely potent in inducing lactogenesis in pseudopregnant rabbits but had little crop sac-stimulating activity. These preparations stimulated histological changes in mammary tissue of mice grown in vitro, to the same degree or greater than did purified ovine PRO. The results showed that primate GH is lactogenic in both in vitro (297) as well as in vivo (100) systems even though the crop sac-stimulating activity is minimal (100). Rivera et al. (297) speculate that the latter activity may reside on a part of the molecule which is separate from that which induces lactation.

Some immunologic evidence that GH and PRO activities might be associated with some molecule in primates was reported by Hayashida in 1962 (140, 141, 147). Antibodies to human GH were able to neutralize the chondrogenic effect of GH on the tibial cartilage of H rats as well as the pigeon crop sac-stimulating activity of the hormone. Similar findings were reported by Damm et al. in 1964 (55). Both Irie and Barrett (165) and Hayashida (141) noted an immunochemical reaction of identity between two different "human PRO" preparations and human GH when using anti-human GH serum in their agar gel studies. No precipitin reaction was noted between the antiserum and purified ovine PRO by either group of investigators. Immunoelectrophoresis of purified human GH indicates that the hormone is quite homogeneous (140, 141); however, when examined by immunodiffusion on cellulose acetate several antigenic components can be detected (140). The investigators suggest that these components might represent a spectrum of antigenic components which are all related to the same hormone (147). They considered it unlikely that any of these components represented a separate PRO, although the possibility of the latter was not entirely ruled out.

Although the immunoelectrophoretic results showed that human GH is homogenous (140, 141), observations with polyacrylamide gel electrophoresis using primate PE's indicated that more than one GH component or band is present (243, 244) Nicholson (243, 244) observed that GH and PRO activities are associated with each band, based on immunodiffusion studies with antiserum to GH and results obtained using the crop sac bioassay. She further noted that the lactogenic activity is the same regardless of sex or stage at which the organism may be in its reproductive life. However, staining intensity of the bands did vary and may become more intense during pregnancy and lactation.

All of the aforementioned results led some investigators to believe that the human pituitary gland does not produce a separate lactogenic hormone and to, therefore, propose the theory that the PRO-like activity is an intrinsic part of the primate GH molecule (193). The latter concept is still well accepted; however, sufficient experimental evidence was published in the 1960's and early 1970's to prove that a separate PRO hormone is present in the primate pituitary. Tashjian (333) demonstrated that fractions of Wilhelmi's human GH preparation contained different proportions or ratios of biologically active PRO and GH. No correlation existed between the crop sac-stimulating activity of these fractions and the immunochemical behavior of the GH as measured by complement-fixation. Since the ratios of PRO:GH varied from 1-30:1 more, Wilhelmi (353) felt that such observations were inconsistent with the theory that the two activities resided solely on one hormone molecule.

Several investigators have compared the relative amounts of GH and PRO secreted by primate pituitary cultures. Pasteels et al. in 1963 (269) and Brauman et al. in 1964 (34) were the first to demonstrate a decrease in GH and an increase in PRO secretion into the culture medium in which two fetal human pituitaries were incubated for five weeks. The immunoassayable GH, as measured by complement fixation, fell progressively from day one to day 16 where it remained at a basal level until the end of the experiment, while the bioassayable PRO levels rose from day nine on. Channing et al. (43) and Nicoll et al. (253) recently reported that the absolute amount of PRO and GH released into the culture medium from organ cultures of adult human and monkey pituitaries decreased with time. However, the ratio of the amount of PRO:GH released increased throughout the experiment indicating that the two hormones were secreted independently. Nicoll et al. (253)

believe the differences in their results and those of Pasteels et al. (269) and Brauman et al. (34) are due to the type of medium used in each study. The latter two groups used a medium which induced cell proliferation while the medium employed by Nicoll et al. (240) did not. Nicoll et al. (253) substantiated their theory that the two hormones are secreted independently by treating the cultures with rat hypothalamic extracts and noting a rise in GH secretion without a change in PRO release. This concept is also supported by results obtained when blood from the cavernous sinus of male and female rhesus monkeys was collected and assayed for GH and PRO (248). Although the values for both hormones varied considerably from one sampling time to the next, the results strongly suggested that the mechanisms controlling the release of GH and PRO function independently.

The clinical evidence for a separate PRO has been well documented in the last ten years. Sectioning of the pituitary stalk has been used as a therapeutic treatment for patients with breast cancer (69). Plasma PRO values have been observed as high as 500 ng/ml (339) when measured in an in vitro bioassay system following stalk sectioning, while GH values were no higher than 4.0 ng/ml. Neither galactorrhea nor gynecomastia developed in these patients (339), although Ehni and Eckles (60) have observed lactation in their patients as early as one month following stalk sectioning.

Frantz and Kleinberg (102, 174) observed elevated PRO values in some patients with acromegaly whose GH values ranged from 13-180 ng/ml. The PRO levels were measured in an in vitro bioassay system employing mouse mammary tissue which is sensitive to both GH and PRO; however, the GH activity could be blocked with antiserum to human GH. After incubating the acromegalics' sera with anti-GH serum, the PRO values were still present and ranged from 0-16 ng/ml. These observations

are supported by Hwang et al. (163) who noted RIA PRO values of less than 20 ng/ml in all but one acromegalic. The GH values in the same patients ranged from 10 to more than 60 ng/ml (163). Forsyth and Edwards (98) have observed high levels of PRO in acromegalics using their in vitro PRO bioassay employing mammary tissue obtained from pseudopregnant rabbits. Similar findings have recently been reported by Sinha et al. (319) who observed an average PRO concentration of 40 ng/ml in five acromegalics studied.

Normal plasma GH levels have been observed in patients with galactorrhea resulting from several different etiologies, as well as normal responses of GH secretion following an oral glucose load (25, 328). Similar results were reported by Hwang et al. (163) and Friesen et al. (110) who noted GH values in all of their patients with galactorrhea to be less than 10 ng/ml. The PRO values in the same patients as measured by RIA (110, 163) ranged from 12-1800 ng/ml, which agrees with Jacobs et al. (166) who also recorded very similar RIA values in patients with Forbes-Albright syndrome. Peake et al. (276), Nasr et al. (239), Loewenstein et al. (210), Turkington (337), Frantz and Kleinberg (102, 174), Forsyth et al. (97, 101) all report elevated levels of bioassayable PRO in plasma and low levels of GH in patients with galactorrhea, steming from a variety of causes. Cultures of tumor pituitaries obtained from patients with Forbes-Albright syndrome produce and secrete very high levels of PRO and normal or abnormally low amounts of GH (117, 162, 239). Polyacrylamide gel electrophoresis of PE's prepared from these tumors reveals a slow moving band presumably PRO, which is absent in electrophoretic patterns of purified human GH but is present as a minor band in normal human PE's (239, 276).

Several different drugs have been shown to either elevate or reduce plasma PRO concentrations. Patients treated with derivatives of phenothiazine (174), reserpine, anti-depressants or α-methyldopamine (338) all showed elevated levels of bioassayable PRO. In some of these patients, GH values were not affected (174) Galactorrhea has been observed in patients treated with a variety of neuroleptic agents such as those mentioned above (6). Brom-ergocryptine (30, 54) and L-dopa (68) have been used successfully in arresting galactorrhea in patients with Forbes-Albright syndrome (68) or in appropriate lactation (30); however, in the patient receiving L-dopa (68) galactorrhea resumed after eight weeks despite continued drug therapy.

Bioassayable and RIA levels of PRO have been reported in normal males and females as well as during pregnancy and lactation. Earlier attempts to determine normal PRO values in extracts of urine or blood by the crop sac assay were conflicting and inconclusive (12, 50, 217, 229, 314, 315). Subsequent studies by Gati et al. (119) and Berle et al. (27) using extracts of plasma samples obtained during the menstrual cycle and assaying them in the crop sac bioassay failed to establish normal PRO levels in cycling females. The only successful attempt to assay human PRO in urine samples obtained from normal male and female subjects was recently reported by Sinha et al. (320). They measured PRO by RIA in unextracted urine samples and reported a normal daily excretion rate for males and females to be 5.2 µg and 3.1 µg respectively. Several investigators have attempted to measure PRO in plasmas from normal, pregnant and lactating females and in males using very sensitive in vitro bioassays (101, 102, 174, 210, 337). The results are all expressed in terms of an equivalent response obtained with an ovine PRO standard. PRO values in nearly all normal males and cycling females fell below

the minimum amount detectable in each assay system, while in postpartum females, values were recorded and ranged from 20-2000 ng/ml (101, 102, 174, 210, 337).

With the purification of human PRO and the development of the RIA for measuring primate PRO, a more accurate analysis of PRO secretion has been made. Hwang et al. (163), Friesen et al. (110, 115), and Jacobs et al. (166) have reported relatively low levels of PRO in normal males, cycling females and children. The mean value of PRO in these three groups is 5.0 ng/ml, 8.1 ng/ml, and 10.8 ng/ml, respectively (115). Sinha et al. (319), however, report PRO levels in normal males to be 13.0 ng/ml and in normal females 14.0 ng/ml which are significantly higher concentrations than observed by other investigators (110, 115, 163, 166). No changes in plasma PRO values are observed during the menstrual cycle. Plasma levels of PRO rise progressively during pregnancy from 25 ng/ml during the first trimester to 207 ng/ml at term (110, 340) and decrease rapidly to normal levels within a week postpartum regardless of whether the mother is lactating or not (163, 340). Nursing stimulates a sharp rise in plasma PRO concentrations within 30 minutes after the onset of suckling (110), peaking as high as 400 ng/ml. Beyond 80 days of lactation, however, this rapid rise is no longer observed following suckling.

GH values have been reported by a number of investigators in normal cycling females as well as during pregnancy and lactation. In general when PRO concentrations are changing, plasma GH levels remain unaltered supporting the assumption that these two protein hormones are separate in primates. Basal levels of GH in normal males and females as measured by RIA have been reported to be less than 5 ng/ml (105, 123). No changes were observed in basal GH values throughout the menstrual cycle; however, following ovulation and just prior to menstruation a rise in GH was observed in ambulatory patients (105). Yen et al. (358) reported low,

stable levels of GH in the follicular phase of the cycle, with a preovulatory peak followed by higher and very unstable values in the luteal phase of the cycle. All samples were drawn from ambulatory patients. Normal GH levels are observed throughout pregnancy (325, 327, 343, 357) as well as during the immediate post-partum period (25, 325, 327, 343, 357). Spellacy et al. (325) suggest that GH values during pregnancy are low due to the suppressive effect of HCS on the hypothalamouse which inhibits the secretion of GH releasing factor. In lactating females, plasma GH was either unchanged or slightly lower than normal (25, 326). Rimoin et al. (294, 295) have observed normal lactation in patients with isolated GH deficiencies. These dwarfs have a basal GH concentration of 1.5 ng/ml or less; however, they fail to respond with an increase in GH to a variety of stimuli. GH therefore, must not be a necessary factory for the initiation and/or maintenance of normal lactation.

Besides the persuasive clinical and experimental evidence, the histological findings have lent credence to the concept that PRO is a separate entity in the primate. Early morphological studies primarily by Dawson (59, 63) and Romeis (see Purves (278)) established that two acidophilic cell types are present in the primate pituitary. One acidophil stains with orange-G, is absent in dwarfs (158) and has been associated with GH secretion, while the other stains with azocarmine, increases in number during pregnancy and lactation (156),and is associated with PRO secretion. The latter corresponds to the pregnancy cell of Erdheim and Stumme (see Flodeus (92) and the "modified acidophile" of Dawson (59). Several investigators have used immunofluorescence with anti-GH serum and have identified the orangeophil as the somatotrophic cell in the primate pars distalis (22, 152, 156, 190). In normal pituitaries, 98.5% of the orangeophils fluoresced (22) while in pituitaries

from acromegalics, all of the tumor cells fluoresced with anti-GH serum (190). Fluorescence has also been observed in the orange-G cells of pituitaries obtained from pregnant and lactating humans (138, 156) and monkeys (152), but was absent in the pregnancy cells or carminophils of both primates when either anti-GH serum (138, 152, 156) or anti-HCS serum (138) was employed. It is thought by some investigators that the number of orange-G cells decreases during pregnancy and lactation in both humans (127) and monkeys (150) while in the normal non-pregnant non-lactating primate, the orangeophile is the predominant acidophil (127).

The number of stainable carminophils is negligible in humans except during pregnancy and lactation (127, 156) or in patients with pituitary tumors (271, 276) where the carminophils become hypertrophied and appear to outnumber the GH secreting orangeophils (127). In the rhesus monkey, carminophilic cells are present in the normal non-pregnant, non-lactating animal (59, 63, 151, 152, 271). A considerable number of carminophils can be observed in both the normal adult male and female rhesus monkey as well as during late pregnancy and lactation when they seem to outnumber all other cell types present in the anterior lobe (151, 152). These cells have been shown to contain a substance, presumably primate prolactin, by fluorescent antibody studies using anti-ovine PRO serum (151, 152, 271).

Electron microscopic studies of human pituitaries obtained from normal patients (94, 266, 347) and from patients with pituitary tumors (276, 303) reveal two cell types which are believed to be the somatotrophs and the lactotrophs, respectively. The former cell type has granules ranging from 350-500 mμ in diameter and seem to be the most common cell type observed (94, 303, 347). The lactotrophs are believed to be the cells with characteristic polymorphic secretory

granules which average about 500 mμ in diameter but may be as large as 1000 mμ especially in tumor cells (94, 266, 271, 303, 347).

The eventual isolation and purification of human and monkey PRO was first achieved by Friesen and co-workers (111-114, 117, 162, 164), followed later by the purification of human PRO by Lewis et al. (188) in the early 1970's (see above). Friesen's human PRO is the most potent and the purest of the two hormone preparations (164). The activity of his PRO preparation is 30.5 IU/mg as measured in an in vitro mouse mammary bioassay system, with only a 0.4% GH contamination on a weight-for-weight basis. A fair degree of homology exists between the primary structures of human PRO and ovine PRO (184) and between human GH and ovine PRO (32, 33). The overall homology between human and ovine PRO is 75% (184), while 60% of the amino acids are homologous between the latter two hormones (33). These observations probably account for the intrinsic PRO-activity observed in primate GH's and suggest that a fair degree of structural relatedness may be anticipated between human GH and PRO.

D. Summary and Conclusions

Prior to the purification of primate PRO by Friesen and colleagues et al. in the 1970's only indirect evidence had been published supporting the theory that PRO and GH were two separate hormone molecules in the primate pituitary. This was provided by a) clinical observations such as lactation in dwarfs, low plasma GH values in pregnancy, as well as during normal lactation and in patients with galactorrhea; b) by experimental evidence that GH and PRO are secreted independently in vitro and in vivo; and c) by the anatomical data showing the presence of two tinctorially different acidophilic cell types, the changes in the number of

carminophils during pregnancy and lactation, and lack of fluorescence within these cells using anti-GH serum. The biochemists failed to find and isolate a PRO which was devoid of GH activity, so along with the experimental endocrinologists they proceeded to demonstrate that purified and synthetic primate GH had lactogenic, mammogenic, luteotropic and pigeon crop sac-stimulating activity. The conclusion of their findings was the inference that GH was the only lactogenic hormone in primates in spite of the paucity of evidence in favor of their hypothesis.

This was in general the "state-of-affairs" at the inception of this research project. The objective was to determine if any evidence could be obtained using immunologic techniques which supported either the dualists' concept of the existence of separate GH and PRO molecules in primates or the unitarian idea of a single hormone with both GH and PRO activity. The approach to this problem was to first produce antisera to ovine PRO that would be capable of cross-reacting with PRO in primate pituitary extracts, as demonstrated by precipitin reactions in agar gel and by radioimmunoassay. Following this, the PRO antisera were to be used along with an antiserum to human GH, to obtain evidence for the separate identities of PRO and GH in extracts of pituitaries from immature and mature monkeys of both sexes, through employment of the above-mentioned immunochemical techniques as well as by the application of immunofluorescent and conventional light microscopic techniques to sections of pituitaries from such animals.

MATERIALS AND METHODS

A. Hormone Preparations

Purified human GH preparations were obtained through the courtesy of Drs. C.H. Li (#3477A, #3530F), M. Raben (#18) and A. Wilhelmi (#HS-612B, #HS-1394, #HS-624Bb-3N). The biological activity of only one human GH preparation was available, Wilhelmi #HS-1394 (2.0 USP units/mg). All of the other preparations were considered to be "highly purified" except Wilhelmi GH #HS-624Bb-3N which was a "clinical grade" hormone preparation. All three human GH preparations were used in agar gel diffusion studies and RIA; however, antiserum was raised to the Li (#3530F) and Raben (#18) GH preparations only. Absorption and neutralization of antisera were carried out using both the Li (#3477A) and the Wilhelmi (#HS-612B and #HS-624Bb-3N) human somatotropins, while the bioassay studies were conducted only with Wilhelmi's GH (#HS-1394). Two purified monkey GH preparations were provided by Dr. W. Peckham (#41 and #42) and used solely in the RIA. Several GH preparations from other mammalian species were generously provided as well. Highly purified bovine GH from Drs. Li (#3267C) and Wilhelmi (#B61-401B) were used for absorption of antisera and in the RIA. GH prepared from rat pituitaries (Ellis #47B (2.7 USP units/mg) and #4C (3.0 USP units/mg)), from porcine (Wilhelmi #P-495B (1.2 USP units/mg) and #P-496B-1 ("highly purified")) and from ovine hypophyses (NIH #NIH-GH-S9 (1.09 USP units/mg)) were employed exclusively in the RIA.

NIH kindly provided three preparations of ovine PRO (#NIH-P-S3, #NIH-P-S8, #NIH-P-S9) and one preparation of bovine PRO (#NIH-P-B2). The biological activity of the former preparations were 15.0, 28.38 and 30.3 IU/mg respectively,

while the bovine PRO was reported to have an activity of about 20.0 IU/mg. The ovine PRO was used for antisera production, absorption and neutralization of antisera, agar gel diffusion studies, RIA and bioassay. The bovine PRO along with a rat PRO preparation (Ellis #8C (25 IU/mg)) and an ovine PRO preparation from Dr. Li (#3118A (30 IU/mg)) were used exclusively in RIA studies.

B. Antisera Production

Three male Bonnet monkeys (Macaca radiata) weighing 2.4-2.75 kg, four male albino New Zealand rabbits ranging in weight from 2.5-3 kg, six male Sprague-Dawley rats approximately 500 gm in weight and 17 male guinea pigs of mixed breeds weighing 500-600 gm were initially immunized with either ovine PRO #P-S3 or #P-S8. The hormone was dissolved in sterile saline or water, emulsified in complete Freund's adjuvant and administered subcutaneously every two or three weeks for a period of 1.5-3.0 months. The last injection was frequently given in saline only. Each monkey initially received 6.5 mg of the hormone, the rabbits 2.3 mg, the rats 1.0 mg and the guinea pigs 1.5-2 mg of ovine PRO. All the animals were bled by cardiac puncture except the rabbits which were bled from the central artery of the external ear. The monkeys and rabbits were given subsequent boosts of 0.5 mg and 0.3 mg, respectively, of PRO at six week intervals for a period of four to six months and bled approximately one week following each boost. The rats and guinea pigs were usually sacrificed at the end of the initial immunization period.

Fourteen male guinea pigs of mixed breed weighing between 500-600 gm were immunized with Raben's (#18) human GH. Three additional guinea pigs were immunized with a highly purified preparation of Li's (#353OF) human GH. All of the guinea pigs received a total dose of 1-1.5 mg each of the respective human GH's

over a period of 2.5-3 months. The injections were given every two to three weeks in either complete Freund's adjuvant and sterile saline, or Freund's and sterile distilled water. All of the animals were bled by cardiac puncture and sacrificed at the end of the initial immunization period.

A number of male, New Zealand white rabbits weighing between 2.5-3 kg were immunized with either guinea pig gamma globulin (Pentex Inc. fraction II lot #10 or #410, or Immunology Inc., 7S fraction, log #1066) or human gamma globulin (Immunology Inc., 7S fraction, lot #766). The rabbits that received guinea pig gamma globulin were given a total dose of 5-20 mg of the globulin in Freund's adjuvant and sterile saline, or Freund's and sterile distilled water over a period of nine weeks at three week intervals. The animals were then bled from the central artery in the external ear and subsequently boosted every four to six weeks with 2-2.5 mg of gamma globulin. Blood samples were taken 12-16 days following each boost. The rabbits immunized with human gamma globulin were given initially 6 mg of globulin in Freund's adjuvant and sterile distilled water over a 12 week period in four separate injections. They were then bled and boosted every four to six weeks with 1 mg of human gamma globulin. Blood samples were obtained approximately two weeks following each boost.

All hormone specific antisera were absorbed before being used in any study to remove possible contaminating antibodies to serum proteins. Following a procedure similar to that previously published by Hayashida and Contopoulos (146), 20 µl of undiluted normal human serum were added to every 1 ml of anti-human GH, while 15-20 µl of undiluted sheep serum were added to every 1 ml of antiovine PRO serum. About 10 µl of a 1% solution of merthiolate (Thimerosal®) were also added to each 1

ml of absorbed serum to prevent bacterial growth. The sera were incubated for one hour at room temperature and stored overnight at 4°C prior to centrifugation. The supernatant was removed following centrifugation and stored at -20°C until used. Some of the anti-PRO serum was also absorbed with bovine GH (Li #3267C or Wilhelmi #B61-401B) to remove any possible antibodies produced to trace contaminants of GH present in the purified ovine PRO preparation. One hundred μg of the bovine GH were added to every 1 ml of serum which was previously absorbed with serum proteins. This was incubated first for one hour at room temperature and then overnight at 4°C. Following centrifugation for 20-30 minutes, the serum was decanted and the supernatant stored at -20°C.

In special studies, antisera to human GH and ovine PRO were neutralized or blocked with either purified human GH (Li #3477A, or Wilhelmi #HS-612B or #HS-624Bb-3N) or ovine PRO (#NIH-P-S8). Between 0.2 and 6 mg of either purified hormone were added to 1 ml of undiluted anti-GH or anti-PRO serum. The sera were incubated at room temperature for one hour before being placed at 4°C for 24-72 hours. They were then centrifuged and the decanted supernatant was stored at -20°C.

C. Animals

Pituitaries were obtained from normal juvenile (pre-pubertal) and adult (post-pubertal) male and female rhesus monkeys as well as from two pregnant and two lactating females. Additional glands were gathered from two adult female cynomolgus monkeys, and from four male and three female capuchin monkeys. These animals were purchased from the Primate Center in Davis, from private vendors on the East and West Coast, or donated by several investigators at the

Medical Center to whom I am grateful. Pituitaries were also obtained from one adult gorilla, one juvenile male orangutan and two presumably juvenile chimpanzees. These pituitaries were provided by Dr. T. Hayashida of the Department of Anatomy who obtained them from a variety of different sources.

D. Light Microscopy

The primate pituitaries were either fixed whole in 10% neutral buffered formalin, frozen and stored at -20°C, or hemisected in an anterior-posterior plane so that half of the pituitary was fixed in neutral buffered formalin and half was frozen and stored. The 10% neutral buffered formalin was prepared as follows:

a) 1000 ml 10% formalin neutralized over calcium carbonate crystals.

b) 4.0 gm sodium phosphate.

c) 6.5 gm anhydrous disodium phosphate.

Tissues stored at -20°C were used for PE's (see below) while pituitaries fixed in buffered formalin were embedded in paraffin and stained by Brookes' (36) trichrome technique. See Appendix for details of the methodology used for tissue processing and for information regarding the light microscopic staining procedure.

E. Fluorescent Antibody Technique

The indirect immunofluorescent technique for Weller and Coons (349) was applied to primate pituitaries primarily using anti-human GH or anti-ovine PRO serum prepared in the guinea pig. The conjugate, fractionated rabbit anti-guinea pig globulin labelled with fluorescein isothiocyanate, was purchased from Nutritional Biochemical Corp. (lot #3573, #3708, #7764, #9047). In addition, fluorescein-labelled anti-rat globulin (lot #5151), anti-monkey serum (lot #8851) and anti-rabbit globulin (lot #9963) were purchased for a few cursory studies using rat, monkey and rabbit anti-ovine PRO serum respectively. See Appendix

for details of the fluorescent antibody technique utilized in these studies.

The slides were examined with a Zeiss Standard GFL light microscope adapted for fluorescence microscopy using a high intensity mercury lamp, with a BG-38 red-suppressing filter, a BG-12 exciter filter and a #53 barrier filter. The identical area stained for light microscopy (see appendix for details) and photographed using Kodak Ektacolor Professional Type S color film was rephotographed using Kodak Hi Contrast Copy Film. Control experiments performed to assure specificity of fluorescence are summarized in Table 1.

F. Pituitary Extracts

Whole or hemisected frozen primate pituitaries were homogenized using a similar method as described by Hayashida (142). Either 20 or 40 mg of pituitary (wet weight) were homogenized in 1 ml of a 0.01 M phosphate-saline buffer pH 7.2 (0.01 M disodium phosphate, 0.01 M potassium phosphate, M/15 sodium chloride, and 0.01% merthiolate). 1% by volume of normal butyl alcohol was added as a preservative along with 50 K.I. units of Trasylol (FBA Pharmaceuticals, Inc.) as an enzymatic inhibitor. The extract was stored at $4^{o}C$ for 1-2 hours and then centrifuged. The supernatant was used as the PE and stored refrigerated at $4^{o}C$. Pituitaries from other mammalian and from submammalian species, obtained from commercial sources for immunoassays, were prepared in a similar fashion.

Four separate primate PE pools were prepared by homogenizing whole or hemisected pituitaries obtained from juvenile male, juvenile female, adult male and adult female rhesus monkeys. PE's from 5-7 individual monkeys from each of the four groups were combined to form their respective pools after being screened in a human GH RIA. The average body weight of the animals whose pituitaries were used in these pools was similar to the mean weight of the animals

TABLE I

FLUORESCENT ANTIBODY CONTROL STUDIES

	1st Reagent	2nd Reagent
1.	buffer	buffer
2.	buffer	fluorescein-labelled conjugate
3.	normal serum	fluorescein-labelled conjugate
4.	anti-hormone serum incubated with anti-gamma globulin serum	fluorescein-labelled conjugate
5.	anti-hormone serum incubated with original hormone antigen	fluorescein-labelled conjugate

whose pituitaries were examined histologically. In all of the pools except the juvenile male pool, at least 50% of the pituitaries used were hemisected glands in which the opposite portion of the gland was examined by light and fluorescence microscopy.

G. Agar Gel Diffusion

The double diffusion technique of Ouchterlony (257) was employed using small petri dishes containing 8-10 ml of an agar gel solution (1% Bacto agar (Difco Lab.) in normal saline, 0.5% sodium barbital and 0.01 merthiolate). The gel solution was poured into the dishes around Teflon-coated molds, cooled and then stored in a humidified chamber. All plates were allowed to develop at room temperature and usually photographed between 24-30 hours after the solutions were added to their respective wells.

H. Radioimmunoassay

A homolgous, double antibody RIA method was employed with guinea pig antiserum to human GH and to ovine PRO using a procedure similar to that previously reported by Schalch and Reichlen (302) for rat GH and by Arai et al. (7) for ovine PRO. The iodination was performed by Dr. Selna Kaplan, to whom I am very grateful for this courtesy, using a modification of the Hunter and Greenwood method (160) Five ug of hormone (either human GH (Wilhelmi #HS-612B or #HS-1394)) or ovine PRO (#NIH-P-S8) were iodinated with 2 millicuries of ^{131}I and the iodinated hormone was separated from unreacted hormone on a G-75 Sephadex column (0.9 x 20 cm). The column was equilibrated with 0.05 M barbital buffer pH 8.6 (0.05 M barbital, 0.05 M sodium barbital and 0.01% merthiolate) and coated with 5% bovine or human serum albumin. One ml eluates were collected in plastic tubes containing a drop of 25% bovine or human serum albumin and counted in a Nuclear Chicago Auto Gamma

Counter. The eluate with the highest amount of radioactivity (designated the "peak eluate") or the one immediately following the peak (designated the "after peak" eluate) was repurified on a second G-75 Sephadex column (0.9 x 13 cm). The peak and after-peak eluates from the repurification were then diluted with barbital buffer so that approximately 26,000 cpm/100 µl were present. These were stored at 4°C and used for two successive RIA's only.

The assay was carried out in disposable glass test tubes using 0.05 M barbital buffer containing 1% bovine or human serum albumin and 0.01 M EDTA (ethylenediaminete traacetic acid). The tubes were incubated at 4°C for about 90 hours prior to the addition of 25 µl of anti-gamma globulin serum and 0.5% normal guinea pig serum to each tube in order to precipitate all antigen-antibody complexes. After a second incubation at 4°C for 20-24 hours, the tubes were then centrifuged for 30 minutes and decanted. The precipitates were counted for one minute and the results were plotted on regular arithmetic graph paper or on logarithmic logistic probability paper.

The primate PRO RIA's were performed by Dr. Michel Aubert to whom I am deeply indebted. A heterologous RIA system was employed using anti-ovine PRO serum, provided by Dr. Hayashida's laboratory, and purified human PRO (obtained from Dr. U.J. Lewis #1-201-1 and #1-201-195-1) as both the standard and the tracer (^{125}I). The assay was carried out in a 0.01 M phosphate-saline buffer at pH 7.4 (0.01 M sodium phosphate, 0.01 M disodium phosphate and 0.09% saline) containing 1% bovine serum albumin and 0.01 M EDTA. The doublt antibody method was used with the two incubation times being the same as for the aforementioned homologous RIA's. Precipitates were counted for four minutes and the results plotted on logarithmic logistic probability paper.

I. Bioassay

An in vitro bioassay was employed according to the method of Loewenstein et al. (210) using 13-14 day primigravida female Charles River (CD-1) mice or 16 day primigravida female White-Swiss mice from Simonsens. Mammary tissue from the pectoral and abdominal glands were removed using sterile surgical instruments and then diced into 1 mm sections by a mechanical tissue chopper. The tissue was incubated for 48 hours in a Dubnoff metabolic shaker at 37° C using 2 ml of sterile culture medium 199. 10 μg/ml of insulin, 20 μg/ml of hydrocortisone hemisuccinate, 35 μg/ml of ampicillin (Omniperl®) and 1% normal human albumin were added to the medium to maintain the culture. Ovine PRO (#NIH-P-S8) was used as the standard and added to the culture flasks in concentrations ranging from 4-512 ng/ml. All results obtained from assaying human GH (Li #353OF and Wilhelmi #HS-1394), primate PE's and from human sera samples were expressed in terms of equivalents of ovine PRO. Following the incubation period, the tissue was homogenized in 200 μl of cold buffer pH 7.4 (0.04 M Tricine (Calbiochem)), 0.01 Mmmanganese chloride and 0.005 M mercaptoethanol) and then frozen.

The enzyme N-acetyllactosamine synthestase present in the homogenates catalyzes the following reaction:

UDP-galactose + N-acetylglucosamine (NAG) ⟶ UDP + N-acethyllactosamine (NAL). 20 μl of the homogenate were incubated for one hour at 37°C in the presence of 10 μl NAG, 45 μg UDP-galactose (dissolved in 0.02 M Tricine and 0.04 M manganese chloride) and 0.02 μ Ci of UDP-galactose ^{14}C. The ^{14}C became incorporated into the newly synthesized NAL and was separated from the unbound, free UDP-galactose ^{14}C by anion exchange chromatography. The radioactive NAL was then

counted in a Packard Tri-Carb Liquid Scintillation Spectrometer for five minutes and the results plotted on semilogarithmic paper.

J. Estrogen Studies

Eight intact juvenile female rhesus monkeys ranging in weight from 1.93-2.1 kg were selected for studies using estradiol benzoate (Progynon®). The animals were grouped so that two served as controls and received the injection vehicle (corn oil) only, three received 15 μg/day of the steroid and three received 150 μg/day of estrogen. The animals were injected daily intramuscularly for 10 days and sacrificed by exsanguination on the 11th day of the experiment. The monkeys were tranquilized with an intramuscular injection of 0.25-0.3 cc of Vetalar® (ketamine hydrochloride) before being handled. Blood samples were drawn by cardiac puncture at the beginning of the experiment, on the sixth day and on the last day of the experiment, just before the animals were sacrificed. The pituitary from each animal was removed and hemisected in an anterior-posterior plane. Half of the pituitary was frozen at -20 ^{o}C for the subsequent preparation of an extract and half was fixed in buffered formalin for examination by light and fluorescence microscopy. The blood samples were assayed by RIA to determine the concentration of GH and PRO. PE's from each of the eight animals were prepared and screened in a human GH RIA. They were then placed into three separate pools; a control PE pool, a low dose PE pool and a high dose PE pool. Only one PE was used in the former pool since one of the two control PE's was unresponsive in the human GH RIA. The amount of GH and PRO were then measured in these pools by RIA. Agar gel diffusion studies were also carried out on each of the three PE pools.

RESULTS

A. Agar Gel Diffusion Studies

1. Comparative Studies with Anti-ovine Prolactin Serum

Four different host animals, the guinea pig, rabbit, rat and monkey, were immunized with purified ovine PRO (#NIH-P-S3 or #NIH-P-S8), while only one host, the guinea pig, was immunized with purified human GH (Raben #18 or Li #353OF). The sera obtained from these animals were examined using the double-diffusion precipitin technique in agar gel as described by Ouchterlony (257). The antisera were screened to determine which host produced the most potent antiserum to ovine PRO, based on the strength of the precipitin reaction observed.

Fifty µl of antiserum from each of the four hosts immunized with ovine PRO were examined against 10 µg of the same ovine PRO preparation used for immunization. All of the antisera gave a precipitin line which indicates that ovine PRO was antigenic in all four species. The strength of these lines, however, varied from one host to another. The guinea pigs gave the strongest and most consistent precipitin reactions of the four hosts. All but one of the 14 guinea pigs gave a good to an excellent precipitin response against purified ovine PRO. The host which gave the next strongest precipitin reaction was the monkey. Three monkeys were immunized with ovine PRO; however, only one gave a good precipitin reaction after 11 weeks of immunization. The other two monkeys gave only a fair response after being immunized for the same period of time. All three monkeys were boosted at 4 to 6 week intervals and bled within a week following each boost. The titer of antiserum decreased in each of the three monkeys and the strength of the precipitin reaction was noticeably weaker following each successive boost. One monkey failed to give a precipitin reaction except after the initial immunization period despite

repeated boosts with the antigen. The six rats and four rabbits immunized with ovine PRO gave the poorest precipitin reactions. The antiserum obtained from the rat produced a slightly stronger precipitin line than that obtained from the rabbit. In general, these reactions were either fair or relatively weak. The rabbits were boosted and bled on a similar schedule as the monkeys. The boosts induced a slight increase in the antibody titer in two of the four rabbits, based on the strength of the precipitin reaction. In the other two animals, the titer seemed to decrease following each boost, much like that observed in the monkeys. The intensity of the precipitin reaction from the rabbits or the rats was never equal to that seen from either the guinea pigs or the "best" monkey.

Good to excellent precipitin reactions were observed when 50 µl of sera obtained from guinea pigs immunized with human GH were examined against 10 µg of the original antigen in agar gel. The antibody responses were quite consistent, with all the guinea pigs giving relatively strong precipitin reactions after a 12 week immunization period. These results along with those obtained with antisera obtained from guinea pigs immunized with ovine PRO indicate that the guinea pig is an excellent host for immunization with ovine PRO or human GH. However, during the immunization period, with either human GH or ovine PRO, several guinea pigs died of unknown causes. These deaths occurred after the animals were injected at least three times with the hormone preparation. It is possible that they succumbed from anaphylactic shock since human GH (149) and ovine PRO (140) have been previously demonstrated to be an effective antigen for sensitization of guinea pigs.

Precipitin reactions in agar gel were also carried out to determine the extent of cross-reactivity between anti-ovine PRO serum and pituitary extracts (PE's) from several vertebrate species. Precipitin reactions were observed between monkey,

guinea pig, rabbit and rat antiserum to ovine PRO and PE's from the ox, giraffe, sheep, seal and pig. The PE's of the first three mammals gave reactions of qualitative identity with each other and with purified ovine PRO. The seal and pig PE's however, only gave reactions of partial identity with ovine PRO or the giraffe PE. Rabbit, rat and guinea pig antisera were reacted in agar against a monkey PE; however, only the latter two antisera cross-reacted. The precipitin line formed gave a reaction of partial identity with ovine PRO. Only the monkey and rat antisera cross-reacted and gave a precipitin reaction with horse PE. The precipitin line from the horse PE gave a reaction of identity with the line from the pig PE and a reaction of partial identity with a seal PE. A precipitin reaction was observed between the monkey and guinea pig antisera and a turtle PE; however, only the former antiserum reacted with PE's from the chicken, a urodele amphibian (Necturus) and the rat. No cross-reaction was present between the monkey antiserum and PE's from the opossum, duck, toad, salmon, or sturgeon. These comparative studies using anti-ovine PRO serum in agar gel against selected vertebrate PE's are summarized in Table II.

2. Studies with Primate Pituitary Extracts

Since a precipitin line formed between a rhesus monkey PE and a guinea pig anti-ovine PRO serum, agar gel diffusion studies were conducted to determine the qualitative relationship between ovine PRO and the PRO in primate PE's. Similar studies using antiserum to human GH were performed to show the relationship between human GH and the GH in primate PE's. In addition, two separate agar plates, were set up to determine whether any cross-reactivity was present between anti-ovine PRO serum and purified human GH or between anti-human GH serum and purified ovine PRO.

TABLE II

REACTION OF VERTEBRATE PITUITARY EXTRACTS IN AGAR GEL AGAINST ANTISERA TO OVINE PROLACTIN

Host of Anti-ovine PRO Serum	Species of Pituitary Extract[1]															
	Monkey	Rat	Seal	Horse	Pig	Giraffe	Ox	Sheep	Opossum	Chicken	Duck	Turtle	Necturus	Toad	Salmon	Sturgeon
Guinea Pig	+	-	+	±	+	+	+	+	-	-		+				
Rabbit	-	-	±	-	+	+	+	+	-	-		-				
Rat	±	-	+	+	+	+	+	+	-	-		-				
Monkey		+	+	+	+	+	+	+	-	+	-	+	±	-	-	-

[1]Amount of tissue homogenate used in each agar study varied from 0.25-2mg. In most cases the higher level of 1-2 mg were used.

+ = Precipitin reaction was clearly visible.

± = Precipitin reaction was questionable.

- = No precipitin reaction.

blank = Precipitin reaction was not tested.

a. Antiserum to Ovine Prolactin

In Figure 1, 150 µl of guinea pig #8 antiserum to ovine PRO previously absorbed to remove any contaminating antibodies formed to serum proteins or GH were placed in the center well and allowed to react with 1-2 mg of several PE's located in the peripheral wells. A precipitin reaction formed between the antiserum and 7.5 µg of ovine PRO (#NIH-P-S8), as expected. In addition, a reaction was noted from the adult female rhesus monkey PE and from the lactating (23 days post-partum) rhesus PE. These lines were relatively weak but were apparent within 28 hours as shown in Figure 1. The line from the lactating female PE appeared to be giving a reaction of partial identity with the ovine PRO line, as indicated by a spur where the two lines join. The precipitin reactions formed from these two female rhesus PE's is presumed to be a reaction between the PRO within the PE's and the antiserum. Although not illustrated by Figure 1, a reaction of partial identity has also been observed between ovine PRO and PE's from a few other adult female monkeys. This precipitin reaction, however, is quite variable in its intensity and in some instances is bearly perceptible even after 72 hours. No precipitin line formed with the adult male, juvenile male or juvenile female PE (Fig. 1). In other agar gel studies, a very weak reaction has been observed only once or twice from adult male, juvenile male and juvenile female PE's. This reaction is generally not apparent until 50-72 hours after the agar plate has been set up.

Figure 2 illustrates the relationship between the PRO in PE's from two pregnant and one lactating female rhesus monkey, and highly purified ovine PRO. A volume of 150 µl of the same absorbed anti-ovine PRO serum as used in Figure 1 was placed in the center well and allowed to react with 10 µg of ovine PRO

(#NIH-P-S8) and 1 mg of the primate PE's which were located in the peripheral wells. The precipitin reaction observed from highly purified ovine PRO after 24 hours was composed of three separate lines. This observation has been frequently observed and its overall significance is unclear. The guinea pig may have produced different antibody populations to several components of the ovine PRO molecule which react and form precipitin lines with the hormone in slightly different ways. Similar observations have been reported by Emmart et al. (77) for ovine PRO and by Sinha et al. for mouse, rat (316) and human PRO (319). A single, broad and fairly intense line was present from each of the two pregnant female PE's and from the lactating PE as shown in Figure 2. These precipitin reactions are again presumed to represent a reaction between the antiserum and PRO in each of the primate PE's. Each line formed from the PE's gave a reaction of partial identity with the ovine PRO line, as indicated by the formation of a spur where the lines meet.

Three different concentrations of purified human GH (Wilhelmi #HS-1394) were examined by agar diffusion against 150 µl of the same absorbed guinea pig anti-ovine PRO serum used in the previous two agar gel studies. The results, illustrated in Figure 3, show a good single precipitin reaction with 7.5 µg of ovine PRO (#NIH-P-S8) after 48 hours. Again, more than one component is present in the ovine PRO line. No detectable reaction was present with 50, 100 or 200 µg of human GH even after 72 hours. Therefore, any precipitin lines observed between the anti-ovine PRO serum and primate PE's are not due to cross-reaction between the antiserum and the GH in the PE's.

b. Antiserum to Human Growth Hormone

Two agar gel diffusion studies were performed using guinea pig #5 or guinea pig #30 antiserum to human GH which had been absorbed for possible contaminating antibodies to serum proteins. In one study, shown in Figure 4, the relationship between purified human GH and the GH within each of the four rhesus PE pools, juvenile male, juvenile female, adult male and adult female PE pool were examined. Absorbed guinea pig #30 anti-human GH serum was placed in the center well, in a volume of 150 µl and reacted with 0.4-0.75 mg of each of the primate PE pools and 10 ug of human GH (Wilhelmi #HS-1394), which were placed in the peripheral wells. Single precipitin lines formed with each of the four PE pools which gave a reaction of qualitative identity with each other and with a line from the human GH preparation. The substance in the monkey PE's which is reacting with the antiserum is presumed to be GH which is immunochemically identical (qualitatively) to highly purified human GH. Purified monkey GH has previously been shown to be immunochemically identical to human GH, based on studies by Hayashida and Li (149) using anti-human GH serum in agar gel.

Three different preparations of human GH (Li #3477A, Wilhelmi #HS-1394 and Raben #18) and three concentrations of ovine PRO (#NIH-P-S8) were examined against the guinea pig #5 anti-human GH. In the center well, 150 µl of absorbed guinea pig anti-human GH serum was placed and reacted against 5 µg of each of the three human GH preparations, and against 50, 100 and 200 µg of ovine PRO. A single precipitin line formed from each of the three human GH's. The intensity of the line from Wilhelmi's GH was much stronger than that observed from either Li's or Raben's GH preparations. The line from the Li GH seemed to be slightly

more intense and longer than the line from the Raben GH. No precipitin line formed from any of the three concentrations of ovine PRO, even after 48 hours. Similar findings were reported by Hayashida in 1962 (141) using rabbit antiserum to human GH. These results demonstrate that the precipitin lines observed between the anti-human GH serum and the primate PE pools were not due to any cross-reactivity with the PRO within the PE pools.

B. Radioimmunoassay

1. Comparative Studies Using A Homologous Ovine Prolactin Radioimmunoassay

A homologous, double antibody radioimmunoassay was set up to study the amount of cross-reactivity between the antiserum and PE's from a few selected vertebrate species. The assays were performed using guinea pig #8 pig anti-ovine PRO serum, which was previously absorbed for possible contaminating antibodies to serum proteins and to GH, at a final concentration of 1:200,000-1:500,000/ml of reaction mixture. The mean antibody concentration employed was 1:340,000. Highly purified ovine PRO (#NIH-P-S8) was used as both the standard and the ^{131}I-labelled tracer. The PE's were prepared so that approximately 0.063 mg of PE was considered to be equivalent to 1 µg of the ovine PRO standard, following a similar procedure previously described by Hayashida (142). The combined results of several RIA's are represented in Figure 6. The maximum amount of ^{131}I-labelled ovine PRO that bound to the antiserum in the absence of any unlabelled hormone (termed total binding) averaged 57% with a range of 39-88%. The total amount of tracer which "reacted" nonspecifically with the buffer and/or adhered to the glassware in the absence of the antibody or unlabelled hormone, termed the nonspecific, averaged 5% with a range of 2-8%. It is assumed that any inhibition of the binding between the antiserum and the tracer by dilutions of either the

standard or of PE's represents a competitive binding reaction between PRO in the PE and the antiserum. This assumption was verified by preincubating 1 ml of guinea pig anti-ovine PRO serum with 5 mg of purified ovine PRO (NIH-P-S8). When this was used in the RIA at a final dilution of 1:500,000, the total binding was reduced to 6%, with the control being 52%. The standard curve was completely abolished indicating that all of the available binding sites on the antibody molecule were saturated following the 72 hour preincubation with ovine PRO. Thus, the formation of the standard curve represents an inhibition of the binding of the tracer to the antibody by increasing amounts of unlabelled ovine PRO.

Figure 6 is an arithmetic plot showing the extent of cross-reactivity between a few vertebrate PE's and guinea pig #8 anti-ovine PRO serum. The B/T ratio, (representing the amount of tracer bound to the antibody (B) as a function of the total amount of tracer available for binding (T)), is shown on the ordinate and the concentration of unlabelled ovine PRO is shown in ng on the abscissa. The amount of PE equivalent to a given concentration of ovine PRO is represented at the top of the graph. The assay system is quite sensitive and can detect as little as 0.125 ng of ovine PRO standard. The range of sensitivity is from 0.125 to 1 ng before the curve begins to flatten out and become less sensitive. The curve from an ovine PE closely approximated the standard curve and represented the greatest degree of reactivity of any of the vertebrate PE's examined. A significant amount of cross-reactivity was also observed between the giraffe PE and the anti-ovine PRO serum; however, considerably less cross-reactivity was shown by PE's obtained from the rabbit and the seal. The seal PE always inhibited the tracer to a greater extent than did the rabbit PE. The curves from the first two mammalian species, the

sheep and the giraffe, were nearly identical to the standard curve, suggesting immunologic identity between ovine PRO and PRO in these two PE's. The slope of the curves from the rabbit and seal PE's, however, was markedly different from the standard curve indicating immunologic relatedness but not identity between the PRO in these two PE's and ovine PRO. These RIA results corroborate the findings seen in agar gel studies. No cross-reactivity was observed with the rat or the guinea pig homogenates or with the two avian PE's, the duck and the chicken.

The curves obtained with a second NIH ovine PRO (#NIH-P-S9) and an ovine PRO preparation donated by Dr. C.H. Li (#3118A) were identical to the standard curve formed by ovine PRO (#NIH-P-S8). The slope of the bovine PRO curve was nearly parallel to the standard curve; however, the degree of cross-reactivity was less than expected since there is only a two amino acid difference in the primary structure of these two hormones (307). A significant amount of cross-reactivity about 1%, was noted with ovine GH (#NIH-GH-9). The slope of the ovine GH line was essentially identical to that of the standard ovine PRO curve, suggesting that a substance was present in the ovine GH which was immunologically identical to ovine PRO. This substance is probably ovine PRO since NIH reports a PRO contamination equal to 0.66 IU/mg present in the ovine GH preparation. Although not shown in Figure 6, a significant amount of cross-reaction was also observed with bovine GH (Wilhelmi #B61-401B) which may also be due to a contamination of the bovine GH preparation with PRO. Some cross-reactivity was present between the anti-PRO serum and purified rat PRO (Ellis #8Q, even though rat PE failed to show any inhibition of the tracer. The significance of this is unknown; however, it is possible that the hormone was "altered" during the purification procedure so that antigenic determinants were exposed, which in the native hormone were not available for binding.

2. Studies with Primate Pituitary Extracts

a. Homologous Ovine Prolactin Radioimmunoassay

An attempt was made to assay PRO values in several primate PE's employing the same homologous ovine PRO RIA system as used in the above comparative immunochemical studies. The results are shown in Figure 7, with the legends being the same as in Figure 6. The amount of cross-reactivity was the same from juvenile male and female rhesus monkey PE's. This was much less than observed with PE's from adult males and females or from lactating and pregnant female PE's. The adult female rhesus monkeys cross-reacted to about the same degree as the pregnant female rhesus monkeys; however, the greatest amount of cross-reactivity was noted with the lactating rhesus females. The curves from each of the groups of primate PE's were bi- or multiphasic. There was some degree of inhibition of the tracer between 0.75 ng and 4 ng equivalents where the curves flatten out, as if each had reached its maximum degree of binding. However, at higher concentrations of the PE, further inhibition of the tracer was observed. This could represent two populations of antibodies which have different binding affinities for the PRO in the primate PE's.

The curves in Figure 7 represent a mean or average of the results obtained from individual PE's within each of the six groups illustrated in this graph. These curves are all nearly identical to each other, thereby indicating that a common substance, presumably PRO, is being measured in both the juvenile and adults, as well as in the pregnant and lactating females. These lines, however, have a different slope than the standard curve, indicating partial immunologic relatedness between ovine and primate PRO. Since the curves were not parallel and the degree

of cross-reactivity from the primate PE's was so low, it was not possible to use this assay system to quantitate absolute concentrations of PRO in the rhesus monkey PE's. In addition, the results obtained from individual PE's were quite variable and frequently did not readily conform to any predictable pattern. Therefore, a mean of the responses obtained, as shown in Figure 7, had to be taken as an example of the degree of cross-reactivity observed between each of the six groups of primate PE's and the anti-ovine PRO serum.

Figure 8 illustrates the extent of cross-reaction shown by five different human GH preparations (Li #3477A; Raben #18; Wilhelmi #HS-612B, #HS-1394, #HS-624Bb-3N) and two monkey GH preparations (Peckham #41 and #42) using the same homologous ovine PRO RIA system as used in the previous studies. The legends for Figure 8 are the same as in Figure 6. One of the two monkey GH preparations, #42, cross-reacted with the anti-ovine PRO serum. The amount of inhibition of the tracer was only 2% when 2000 ng of monkey GH #42 was incubated with the antiserum, which is almost negligible. This suggests that any cross-reactivity observed in RIA between the rhesus PE's and the anti-PRO serum is probably not due to GH within the PE's.

A greater degree of binding is observed with each of the five human GH preparations. The curves from two Wilhelmi GH's (#HS-1394 and #HS-612B) and from the Raben GH (#18) are paralleled to each other but have different slopes than the standard curve. The lines from two other human somatotropins, Wilhelmi #HS-624Bb-3N ("clinical grade") and Li #3477A, have slopes which appear to be similar if not identical to the standard ovine PRO curve. The maximum percent of cross-reaction in each of the five human GH preparations as measured by RIA is a) 0.001% Wilhelmi #HS-612B and #HS-1394; b) 0.002%, Raben #18; c) 0.003%, Wilhelmi

#HS-624Bb-3N ("clinical grade"); and d) 0.01% Li #3477A.

The cross-reaction between human GH (Li #3477A) and antiserum to ovine PRO was not abolished when 1 ml of the antibody was preincubated with either 1 or 6 mg of human GH. The total binding, however, was reduced by 17-23% following preincubation of the antibody with human GH. The cross-reaction from the Li GH was only eliminated when the antiserum was preincubated with ovine PRO.

b. Heterologous Human Prolactin Radioimmunoassay

Since the homologous ovine PRO RIA system was unsuitable for measuring absolute value of PRO in primate PE's, another RIA system was employed for this purpose. Dr. Michel Aubert set up a heterologous RIA using rabbit #246 antiserum to ovine PRO provided by Dr. Hayashida's laboratory, ^{125}I-labelled human PRO (Lewis #201-1 or #201-195-1) and human PRO (Lewis #201-1) as the standard. Several different anti-ovine PRO sera were first screened using ^{131}I-labelled porcine PRO and a human pregnancy serum sample as the standard to determine which antiserum would be most suitable for the assay. A rabbit anti-ovine PRO serum was chosen from these preliminary assays and used at a final concentration of 1:32,000/ml of reaction mixture in most RIA's. The average total binding was 26%, while the mean nonspecific binding was only 1%. This assay system was employed to measure the amount of PRO in each of the four rhesus PE pools: juvenile male, juvenile female, adult male and adult female PE pool.

Figure 9 shows a typical standard curve and the curves obtained from each of the four rhesus PE pool. The results are plotted by logarithmic logistic probability transformation. The B/Bo ratio (representing the amount of tracer bound to the antibody in the presence of unlabelled PRO (B)as a function of the amount of tracer

bound in the absence of unlabelled PRO (Bo)) is plotted on the ordinate and the concentration of unlabelled PRO is shown on the abscissa. The amount of PE equivalent to a given amount of human PRO is shown at the top of the graph. The amounts are less than those shown at the top of Figures 6 and 7 because the four PE pools were prepared at half the concentration of the PE's used in the homologous ovine PRO RIA studies. The assay is more sensitive than the homologous ovine RIA (Fig. 6), with the minimum sensitivity being less than 0.05 ng and a range of sensitivity up to 2 ng of human PRO. There is a negligible amount of cross-reaction observed with two human GH preparations at concentrations greater than 1000 ng, 0.4% with Wilhelmi GH (#HS-1394) and 0.2% with Raben (#18). No cross-reactivity was seen with Li GH (#3530F) at concentrations up to 10,000 ng. Ovine PRO (#NIH-P-S8) cross-reacts to a greater extent than the human PRO standard, as indicated by the very precipitous ovine PRO curve. This is probably due to the greater binding affinity of the antiserum for ovine PRO than for human PRO, since the antiserum was prepared against ovine PRO.

The curves from the four rhesus PE pools were always parallel to each other but frequently were not parallel to the standard human PRO curve. This is probably due to the difference in binding affinities of the anti-ovine PRO serum for human and monkey PRO. In one homologous human PRO RIA, in which an antiserum to human PRO provided by Dr. Henry Friesen was used (final concentration of 1:60,000) along with the aforementioned human PRO preparation as the standard and the tracer, all four primate PE's were parallel to the standard curve. The parallelism indicates that the substance being measured in the primate PE pools in both the homologous and heterologous RIA's is primate PRO which is immunochemically identical to purified human PRO.

The absolute concentrations of PRO in each of the four pools is shown in Table III. The amount of PRO, in μg/mg pituitary wet weight, is nearly the same in the two juvenile PE pools, 1.01 μg/mg in the juvenile male pool and 0.91 μg/mg in the juvenile female pool. Approximately twice as much PRO is present in adult male pituitaries, 2.17 μg/mg, with the greatest amount being present in the adult female, 4.96 μg/mg.

c. Homologous Human Growth Hormone Radioimmunoassay

A homologous, double antibody human GH RIA was set up to examine the degree of cross-reactivity between various mammalian GH's and to quantitate the amount of GH in each of the four rhesus PE pools. Guinea pig #5 antiserum to human GH was used after being absorbed for possible contaminating antibodies to serum proteins, at a final concentration which averaged 1:400,000/ml of reaction mixture. The range of antibody concentration employed was 1:300,000-1:750,000. Highly purified human GH (Wilhelmi #HS-1394) served as both the standard and the ^{131}I-labelled tracer.

Figure 10 is an arithmetic plot showing the immunochemical relationship of several human GH preparations as well as the degree of cross-reactivity from several purified mammalian GH's. This graph is a composite of results obtained from a number of RIA's with the B/T ratio plotted on the ordinate and the concentration of the unlabelled hormone on the abscissa. The mean total binding was 53%, with a range of 30-75% and the average nonspecific binding was 5%, with a range of 1-9%. The standard curve shows that a significant amount of inhibition of the tracer occurs at 0.1 ng, with a range of sensitivity up to 2 ng before it becomes less sensitive. To primate GH preparations, Peckham's monkey GH (#41) and Li's human GH (#3477A) had curves with identical slopes to the standard human GH

TABLE III

CONCENTRATION OF PITUITARY GROWTH HORMONE AND PROLACTIN IN THE RHESUS MONKEY

Animal Group	GH (μg/mg Pituitary Wet Weight)[1]	PRO (μg/mg Pituitary Wet Weight)[1]	Body Weight (kg)[1]	Rating of the Average No. of PRO Cells	Number of Pituitaries Examined Histologically
Juvenile Male	20.42 ± 0.42[2]	1.01 ± 0.05[3]	3.06 ± 0.18[4]	0	5
Juvenile Female	25.67 ± 0.33[2]	0.91 ± 0.07[3]	2.90 ± 0.15[4]	0	9
Adult Male	33.75 ± 1.30	2.17 ± 0.10	7.82 ± 0.54	0.97 +	8
Adult Female	92.00 ± 6.36	4.96 ± 0.14	4.78 ± 0.23	1.35 +	12

[1] mean ± SE

[2] $p < 0.005$ in Students' t-test (juvenile male vs. juvenile female)

[3, 4] no significant difference in Students' t-test (juvenile male vs. juvenile female)

(Wilhelmi #HS-1394) curve. The other primate GH's monkey GH (Peckham #42) and human GH (Wilhelmi #HS-612B and #HS-624Bb-3N, and Raben #18) cross-reacted to a slightly lesser degree. The curves from these latter hormones were essentially parallel to the standard curve indicating immunologic identity between all seven primate GH's. No cross-reaction was observed with rat GH (Ellis #4C and #47B), bovine GH (Li #3267C or Wilhelmi #B61-501B), porcine GH (Wilhelmi #P-495B or #P-496B-1) ovine GH (#NIH-GH-S9) or ovine PRO (#NIH-P-S8 or #NIH-P-S9) at a concentration up to 2000 ng. At higher concentrations, a small amount of cross-reactivity was present with ovine PRO (#NIH-P-S8) with a maximum degree of cross reaction being about 0.001%. Thus, any cross-reaction seen with primate PE's and anti-human GH serum is probably not due to binding, between the PRO in the PE's and the antiserum. Although not shown in Figure 10, individual rhesus monkey PE's were examined in this assay system and screened before being placed in their respective PE pools. All were parallel to the standard curve, showing immunologic identity between purified human GH and the GH within the PE's.

To assure that the standard curve represents an inhibition of the tracer by increasing amounts of unlabelled hormone, 0.2, 0.6 and 1.6 mg of human GH (Li #3477A) were incubated with 1 ml of antiserum for 72 hours. When these were used in the RIA at a final concentration of 1:250,000 the total binding was reduced to 8% with the control binding being 67%. The standard curve was abolished as well as the curve from an adult female rhesus monkey. These results indicate that the standard curve represents an inhibition of binding by increasing amounts of unlabelled hormone and that the cross-reaction observed with primate PE's represents the binding of GH within the PE to the anti-GH serum.

The homologous human GH RIA was also employed to measure the amount of GH present in each of the four rhesus monkey PE pools. The results are shown in Figure II and are plotted by logarithmic logistic probability transformation. The B/Bo ratio is shown on the ordinate, the concentration of human GH standard (Wilhelmi #HS-1394) on the abscissa and the PE equivalents placed at the top of the graph. The curves from all four rhesus PE pools were always parallel to the standard curve. Two of the four PE pools, the adult male and adult female pool, had to be diluted so that a more accurate estimation of their GH content could be made. The juvenile male PE pool had significantly less ($P < .005$) GH than did the juvenile female PE pool, 20.42 µg/mg, pituitary wet weight and 25.67 µg/mg, respectively. Less GH was also present in the adult male PE pool, 33.75 µg/mg, than was observed in the adult female PE pool, 92.0 µg/mg.

C. Light and Fluorescence Microscopy

Serial sections from whole or hemisected pituitaries obtained from juvenile and adult male and female rhesus monkeys and from pregnant and lactating females were stained by Brookes' trichrome technique (36). This staining procedure tinctorially differentiates the acidophils into orangeophils and carminophils. Every 10th-15th section was examined to determine the regional distribution of these two acidophilic cell types and to demonstrate the relative difference in numbers of carminophils within the aforementioned group of animals. Pituitaries obtained from two adult female cynomolgus monkeys, four male and three female adult capuchin monkeys, one adult male gorilla, one juvenile male orangutan and two presumably juvenile chimpanzees were also examined. The number of rhesus monkeys studied histologically is shown in Table III along with the mean body

weights of all the monkeys whose pituitaries were examined both histologically and used in the PE pools. The average body weight of the animals whose pituitaries were used in the PE pools was similar to the mean body weight of the monkeys whose pituitaries were examined histologically.

I. Immunofluorescence of Primate Pituitaries

Figures 12a, c and 13a, c illustrate the two tinctorially different acidophilic cell types. The orangeophils are the finely granulated cells which are about the same size as the small carminophils. The latter cells shown in Figures 12c and 13c, average 8.21 $\mu \pm 0.07$ ($\pm$ SE) in diameter in normal juvenile and adult rhesus monkeys as well as in pregnant and lactating females. The nucleus is slightly eccentrically located in the cytoplasm. These smaller carminophils are generally not found in discrete groups or clusters and are frequently observed towards the periphery of the anterior lobe. The granules are coarser than those present in the orangeophils; however, the density of granulation is less than in the larger carminophils. The larger cells, shown mainly in Figures 12a and 13a are about 13.74 $\mu \pm 0.14$ in diameter in normal rhesus monkeys, while in pregnant and lactating females they are slightly larger 14.43 $\mu \pm 0.11$. These cells tend to be found in groups or clusters of cells as shown in Figures 12a and 13a. A characteristic feature of the large carminophils is a negative image of the Golgi apparatus located in the supranuclear region of the cell. Due to the high density of granulation, especially during pregnancy and lactation, the nucleus becomes polarized and is observed towards the periphery of the large carminophils. In Figures 12a, c and 13a, c, intermediate sized carminophils can also be observed along with the small and large sized cells . The smaller carminophils are generally more

abundant in adult rhesus monkey pituitaries, as well as during pregnancy and lactation. Intermediate and large sized cells were found in pituitaries from all rhesus monkeys regardless of age or stage of the reproductive life of the animal, although their relative numbers or concentrations were the highest in pituitaries of pregnant and lactating females.

The indirect fluorescent antibody technique (349) was employed to determine the histophysiology of the two acidophils. Guinea pig antiserum to human GH and to ovine PRO were used, along with rabbit anti-guinea pig globulin labelled with fluorescein isothiocyanate. The identical areas stained and photographed for light microscopy were used for immunofluorescence. See Appendix for details of the light microscopic staining procedure and the protocol used in the fluorescent antibody technique. Figures 12a, b and 13a, b show the results of the studies. Only the orangeophils fluoresced with guinea pig #5 anti-human serum (Fig. 13a, b). Several guinea pig antisera prepared against human GH gave positive fluorescence, indicating that the results were not an unusual finding of only one antiserum. These results support the observations of several investigators (22, 152, 156, 190, 268, 271) and provide evidence that the orangeophils are the cells in the rhesus pituitary which synthesize and store GH. The orangeophils will hereafter be referred to as GH cells or somatotrophs.

Figure 12a, b shows the results using guinea pig #8 anti-ovine PRO serum. Only the carminophils fluoresced with the anti-ovine PRO serum indicating that these cells were responsible for the storage and presumably the secretion of PRO. Therefore, they will be referred to as PRO cells or lactotrophs. These findings were first reported by Herbert and Hayashida in 1970 (152) and later by Pasteels et al. (268, 271). Antisera from several guinea pigs showed positive fluorescence

as well as rabbit, rat and monkey anti-ovine PRO serum. Fluorescence was quite bright with the rat antiserum but was only fair or rather dull with either the rabbit or monkey antiserum. Neither of these three antisera produced fluorescence which was as intense as that observed with the guinea pig antisera. The monkey and rabbit antisera failed to produce a precipitin reaction in agar gel diffusion studies against rhesus monkey PE's but did show positive fluorescence in the lactotrophs of the rhesus. This suggests that the latter technique is considerably more sensitive than the agar gel diffusion technique. Recently Nayak et al. (240) and Moger and Geschwind (234) have suggested that the fluorescent antibody technique is qualitatively the most sensitive of all the immunologic procedures, including agar gel, RIA and hemagglutination.

Fluorescence was observed in cells which were barely stainable by light microscopy and in some which appeared to be devoid of granules. The intensity of fluorescence in these cells was usually less than in cells with a greater density of granulation. Porteous et al. (277) have observed in human fetal pituitaries the appearance of positive fluorescence in cells which could not be stained by light microscopic stains. These observations as well as those made on the rhesus pituitaries again demonstrate that the immunofluorescent technique is much more sensitive than light microscopic stains. Fewer granules are, therefore, needed for positive staining with the former method than with conventional histologic dyes.

Using anti-human GH and anti-ovine PRO serum, respectively, fluorescence was present in the orangeophils and carminophils of pituitaries obtained from all primate species, including the rhesus, cynomolgus and capuchin monkeys and the three species of apes. It was also observed in pituitaries taken from two fetal

rhesus monkeys, one male and one female, which were about 148 days old (fetal age) and in one 23 day-old neonatal male rhesus monkey.

a. Control Studies

Several control experiments were performed to assure specificity of fluorescence. These are summarized in Table I and illustrated in Figure 14 a-e. Figure 14a represents the degree of autofluorescence present in pituitaries fixed with neutral buffered formalin. A dull, homogeneous reddish-brown color fluorescence was present in the anterior, intermediate and posterior lobes of the rhesus monkey pituitaries. No specific, positive fluorescence was observed. Some nonspecific flourescence was present in the other four controls, Figures 14 b-e, all of which were exposed to the fluorescein-labelled conjugate. This nonspecific fluorescence was dull, yellow-green in color and was present in most tissue components. It was frequently brighter in red blood cells and in some of the connective tissue fibers. Nonspecific staining appears to be a nonimmunologic phenomenon caused by electrical attractions between tissues and serum proteins (126). Some non-specific fluorescence can be reduced or eliminated by diluting both the first and second antisera and/or by incubating the tissue with normal serum (species used is identical to the host species in which the second antibody was prepared) before treating the tissue with either antiserum. The control studies 2-5 (Table I) were carried out to demonstrate:

a) that the fluorescein conjugate alone was not inducing any positive fluorescence (control #2),

b) that nonspecific components in guinea pig serum were not responsible for specific fluorescence (control #3),

c) the specificity of the reaction between the first and second antibodies (control #4), and

d) the fluorescence observed when using either anti-human GH or anti-ovine PRO serum was due to a specific reaction between GH or PRO, respectively and the cytoplasmic components of the acidophils (control #5).

Special control studies were conducted to determine whether preincubation of anti-human GH serum with ovine PRO (3 mg/ml undiluted antiserum) or of anti-ovine PRO serum with human GH (3-3.2 mg/ml undiluted serum) would cause a decrease in the intensity of fluorescence observed with the same, untreated antisera. No change in the intensity of fluorescence was detected in either of these two special studies when compared to the degree of fluorescence present from the control, untreated sera. Therefore, no cross-reaction occurred between the anti-GH serum and ovine PRO or between the anti-PRO serum and human GH. Thus, positive fluorescence observed in any of the immunofluorescent studies demonstrates the specific localization of either GH or PRO in its respective cell type.

2. Light Microscopy of Primate Pituitaries

a. Normal Juvenile and Adult Rhesus Monkeys

Pituitaries were stained and examined to demonstrate the regional distribution of the GH and the PRO cells and to determine the relative number of PRO cells present in the pars distalis of pituitaries obtained from rhesus, cynomolgus and capuchin monkeys, and from the gorilla, orangutan and chimpanzee. Between 15 and 30 slides from each monkey or ape were examined. The slides observed represented essentially all areas of the pars distalis. The overall number of PRO cells was determined and scored accordingly. An arbitrary scoring system of 0-4 + was devised to rate the number of PRO cells. Each succeeding increment

represents a doubling or a 100% increase in the number of PRO cells present over the preceding one. Any pituitaries falling between two ratings were scored as 1.5, 2.5, etc. Figure 15 a-e illustrate a representative example of each of the five categories in the rating system. Three sagittal sections taken from a juvenile male and a juvenile female rhesus monkey are shown in Figures 16 and 17 a-c, respectively. The orientation of these micrographs and all others in which sagittal sections of primate pituitaries are shown is with the superior pole facing upwards and the anterior region of the pituitary facing towards the left. The micrographs were taken from three separate areas of the pituitary. Figures 16a and 17a represent areas near the midline or about 25% of the distance away from the midline, towards the lateral pole of the pars distalis. Figures 16b and 17b are areas from 40-60% of the distance away from the midline and Figures 16c and 17c are from 75-90% of the distance away from the midline, very close to the lateral pole of the anterior lobe. A similar pattern is observed in both juvenile male and female pituitaries. At higher magnifications it can be observed that near the midline, a few PRO cells are present in the medial and anterior regions of the gland. These cells are almost entirely clusters of large PRO cells, which seem to be slightly more prevalent in juvenile males. The GH cells appear to be the predominant cell type in the anterior lobe. They are mainly found posteriorly, adjacent to the intermediate and posterior lobes and at the superior and inferior poles. Therefore, in juvenile rhesus monkeys of both sexes a negligible number of PRO cells are present. These are mostly found anteriorly and medially, between the midline and about 60% of the distance from the midline. A rating of 0 was given to the relative

number of PRO cells present in the juveniles as shown in Table III. The GH cells are found nearly everywhere, but predominate in the posterior and lateral regions as well as at the superior and inferior poles. Two female rhesus monkeys which had reached puberty 2-3 months before being sacrificed were also examined. More PRO cells were present in one of the two glands than was observed in the majority of the prepubertal juvenile rhesus monkeys. The histology of the pituitary obtained from the second pubertal female, however, resembled that of a typical juvenile rhesus. The number of PRO present in these two females was rated .25 + and 0 respectively. From such a small sampling, it is not possible to accurately assess the histologic changes occurring around the time of puberty.

Figure 18 a-c illustrates three sagittal sections taken from an adult male rhesus monkey. The orientation of these micrographs is the same as in Figures 16 and 17. The three micrographs represent areas from approximately 25-30% (Fig. 18a), 50% (Fig. 18b) and 75-80% (Fig. 18c) of the distance away from the midline and towards the lateral pole of the anterior lobe. More PRO cells were apparent in all of the adult male pituitaries than were present in juveniles of either sex. These were mostly observed to be located anteriorly, antero-inferiorly and medially. The clusters of large PRO cells were more numerous than the small, randonly distributed carminophils, which were located towards the periphery of the gland. Greater intermixing of the GH and PRO cells occurred where the small carminophils were located than where the larger carminophils were present. The greatest number of PRO cells were located between the midline and about 60-70% of the

distance away from it. The GH cells in the adult male predominated in similar regions as observed in the juveniles, posteriorly, laterally and at the superior and inferior poles. The number of PRO cells in eight adult males examined histologically averaged 0.93 +, as shown in Table III. Several but not all of the adult males which were quite heavy and presumably older than some of the other animals had significantly more PRO cells than the average adult male rhesus monkey..

Three sagittal sections from an adult female pituitary with the greatest number of PRO cells observed among all of the normal rhesus pituitaries are illustrated in Figure 19 a-c. The levels of these sections are approximately the same as those shown in Figure 18 a-c. The orientation of these micrographs is the same as Figure 16 except the anterior region of the pituitary is facing towards the right. More PRO cells are present in the adult female pituitary than in the adult male or in the juvenile male or female pituitary. These PRO cells are mostly clusters of large cells which predominate in the anterior, antero-inferior and medial regions of the pars distalis from the midline to about 75-90% of the distance from it. The smaller PRO cells are again found towards the periphery of the anterior lobe. Intermixing of the two acidophils occurs posteriorly, postero-inferiorly and towards the lateral poleswhere the number of GH cells increases. The adult female had about 50% more PRO cells than the adult males. The average rating of PRO cells observed in 12 adult rhesus females is 1.35 + and is shown in Table III. Since the stage of the menstrual cycle was not known for a majority of the adult females, no correlation could be made between the number of PRO cells and the phase of the cycle. Two adult female cynomolgus monkey pituitaries were also examined histologically. Both had nearly as many PRO cells as were present in the adult rhesus female with the largest number of lactotrophs (Fig. 19a-c). These cells were located in regions

of the anterior lobe of the cynomolgus monkey which were similar to those of the rhesus monkey pituitary.

Table III and Figure 20 summarize the histologic and RIA data from normal juvenile and adult rhesus monkeys. The body weights (kg) shown in Table III are the weights of animals whose pituitaries were examined both histologically and those used in the PE pools. Whole or hemisected pituitaries from five juvenile males, seven juvenile females, five adult males and seven adult females were used to prepare each of the four primate PE pools. In all but one of the PE pools, at least 50% of the pituitaries were hemisected glands in which the opposite half was studied histologically. The concentration of GH and PRO represent values obtained in RIA studies carried out on each of the four pools. The ratings indicate the average of the relative number of PRO cells present in the juvenile and adult rhesus monkey pituitaries which were examined by light and fluorescence microscopy. The mean body weight of the juvenile males and females closely approximated each other with no statistically significant difference between the two. The concentration of PRO present in the pituitary of juvenile males and females was also quite similar, and again no significant difference existed between the two groups. Slightly more PRO cells were present in juvenile male pituitaries than was observed in juvenile female pituitaries; however, the concentration of GH was higher in the female than in the male juvenile rhesus monkey PE ($p < .005$).

The mean body weight of the adult females 4.78 kg which was about 70% lower than the mean weight of the adult males. Nearly three times as much GH was present in the adult females PE pools as compared to the adult males PE pools, while a difference of only two fold existed in the concentration of PRO contained within the same adult PE pools. The number of PRO cells was also greater in adult female

pituitaries than in the adult males; however, this difference was only about 50%.

Figure 20 summarizes the regional distribution of GH and PRO cells in normal juvenile and adult male and female rhesus monkey pituitaries. The areas in which each type predominates are shown in both horizontal and sagittal sections taken from five different levels of the anterior lobe. The areas shown by the diagonal and broken lines are those in which the PRO and GH cells, respectively, predominate. Those areas where both diagonal and broken lines are present depict the regions where intermixing of the two cell types occurs. The gray areas are those occupied by the intermediate and posterior lobes. At no time was either cell type excluded from an area in which the other cell type predominated. Intermixing of the two acidophilic cells occurred in most areas of the anterior lobe; however, it was most frequently observed in the more lateral and inferior regions. The PRO cells predominated in the anterior, antero-inferior and median regions while the GH cells predominated posteriorly, laterally and at the superior and inferior poles of the pars distalis. A preliminary report of the regional distribution of the two acidophils in normal primates was made at the Anatomists' meeting in 1972 (151).

b. Pregnant and Lactating Rhesus Monkeys

Figures 21 a-c and 22 a-c are photomicrographs taken from three different areas of the pars distalis of a pregnant (Fig. 21) and a lactating (Fig. 22) rhesus female. Two pregnant rhesus female monkeys were obtained which were presumably in their third trimester of pregnancy, about 148 days gestational age. Two entirely different histologic patterns were obtained from these animals, with regard to the number of PRO cells observed in the respective pituitaries. The number of PRO cells present in the pituitary from one of the pregnant females is only slightly greater than the number present in the adult female illustrated in Figure 19. In

the second pregnant female (Fig. 21 a-c), the number of PRO cells exceeded all other cell types combined. It is presumed that the first monkey was not as far advanced in her pregnancy as the second and that an incorrect gestational age was given. The body weight, pituitary weight and fetal weight were all significantly greater in the second pregnant female than in the first. In addition, the histology of the second pregnant rhesus more closely resembled that observed in lactating females (Fig. 22 a-c), suggesting that she was closer to term than the first animal.

The PRO cells present in the one pregnant female (Fig. 21) and in the two lactating rhesus females (23 days postpartum (Fig. 22) and six weeks postpartum)) seemed to occupy anywhere from 60-80% of the total area of the anterior lobe of these animals. The regional distribution of GH and PRO cells seen in normal rhesus monkeys was completely obliterated. The number of stainable GH cells were less than observed in normal adults and the areas normally occupied by the somatotrophs was frequently filled with PRO cells. A majority of the PRO cells in both the pregnant and lactating females were clusters of the large variety, with the greatest number of smaller PRO cells predominating in areas near the lateral pole of the gland. Approximately the same number of lactotrophs were present in the two lactating females; however, more stainable carminophils were observed in the one pregnant female than were present in either lactating female pituitary. This latter observation is probably due to the higher rate of secretion rather than storage of PRO occurring in lactating females than in pregnant females.

c. Fetal and Neonatal Rhesus Monkeys

The pituitaries obtained from the fetuses of both pregnant females, one male and one female, and from the 23 day neonatal male taken from a lactating mother, were examined histologically. GH cells were tinctorially differentiated in both

fetal pituitaries; however, it was questionable whether there were any carminophils present in either pituitary. The cells present in one fetal pituitary seemed more organized, larger and more densely granulated than the other. The animal from which this pituitary was obtained was the fetus of the pregnant female with the greatest number of PRO cells. When examined by immunofluorescence with anti-GH serum, the GH cells were brighter in the pituitary from this fetus as well. The intensity of fluorescence in either fetal pituitary, however, was less than observed in juvenile or adult rhesus monkeys which is due to the relative sparsity of secretory granules present in the fetal somatotrophs as compared to the somatotrophs of more mature monkeys. A few cells fluoresced in the two fetal pituitaries, with anti-ovine PRO serum; however, these same cells did not stain with the carmoisine dye in the light microscopic stain. Apparently the number of granules present in the cytoplasm was too small to be stained histologically but could be detected by immunofluorescence. The histology of the 23 day-old neonatal pituitary was similar to that observed in juvenile rhesus monkeys. A few clusters of large PRO cells were confined to the posterior, lateral, superior and inferior regions.

d. Pituitaries from Other Species of Primates

1. Capuchin Monkeys

Pituitaries were obtained from four adult males and three adult female capuchin monkeys. Essentially the same results were found in both the males and females as illustrated in Figure 23 a-c. These three micrographs, obtained from an adult male capuchin monkey, are horizontal sections taken at the level of the superior pole, (Fig. 23a), about halfway between the superior and inferior poles (Fig. 23b) and very close to the inferior pole (Fig. 23c) The

orientation is with the anterior region facing the bottom and the lateral areas facing towards the right and the left sides of the micrograph. GH cells are easily recognized and predominate in the same regions as noted above in the rhesus monkeys. The difference between rhesus and capuchin monkeys is the relative paucity of PRO cells observed in the adult capuchins. Fewer PRO cells were noted in these animals than were present in the majority of the juvenile rhesus monkeys of either sex. This unusual finding may be a characteristic feature of adult capuchin monkeys or the age of these animals was inaccurately supplied.

2. Apes

Figure 24 a-c represent sections from pituitaries obtained from adult male gorilla (Fig. 24a), the juvenile male orangutan (Fig. 24b) and one of the two juvenile female chimpanzees (Fig. 24c). The orientation of the gorilla pituitary was unknown and, therefore, the plane of section was impossible to discern. The orangutan pituitary was sectioned sagitally and the two chimpanzee pituitaries were cut horizontally. Very few PRO cells were observed in any of the three apes by either light or fluorescence microscopy. On a relative basis, however, more were present in the adult gorilla pituitary than in either the juvenile orangutan or the two juvenile chimpanzee pituitaries. The few lactotrophs present were confined to the anterior regions of the pars distalis. GH cells were abundant in all three species and where it was possible to determine, these seemed to be confined to similar regions of the pituitary as the GH cells in the rhesus monkeys.

D. Bioassay

An *in vitro* mouse mammary bioassay was set up to measure PRO levels in each of the four rhesus PE pools following the procedure published by Loewenstein *et al*. (210). Mammary tissue from two strains of mice were used in the assay, the

CD-1 Charles River and the White-Swiss strain from Simonsens. Ovine PRO (#NIH-P-S8) served as the standard. All values obtained from the four PE pools as well as from two preparations of human GH were expressed in terms of ovine PRO equivalents. Figure 25 is a semilogarithmic plot illustrating the standard curves obtained from both species of mice. These are a composite of results gained from several bioassays. The standard error of each point is shown by the bars, the counts per minute (CPM) per ml of culture medium are shown on the ordinate and the concentration of ovine PRO in ng/ml of culture medium are shown on the abscissa. The White-Swiss strain was more sensitive and responded to smaller amounts of ovine PRO than the CD-1 strain. Both animals gave biphasic responses to the standard. The possible significance of this may be explained on the basis of the presence of two receptors in the mammary tissue which are capable of binding PRO. One receptor may be sensitive to low concentrations of PRO while the other may be sensitive only to very high concentrations of the hormone. A significant amount of intra- and interassay variation was present in all assays which was probably due to differences in responses obtained from individual animals. About 80% of the response to the ovine PRO standard was abolished when anti-ovine PRO serum was added to the culture flasks containing purified ovine PRO. The amount of antiserum added to each flask depended on the concentration of PRO being assayed. The proportions of antiserum to PRO were about 0.4 ul of antibody per 10 ng of ovine PRO assayed. About 5-10% of the response was lost when an equivalent amount of anti-human GH was added to each 10 ng of ovine PRO assayed. Normal guinea pig serum by itself was found to be slightly active, so whenever antiserum was added to the culture flasks, an equal amount of normal serum was added to the flasks containing the standard.

Two purified human GH preparations, Li #353OF and Wilhelmi #HS-1394, were both active in this assay system. No detectable PRO contamination was observed in the Li #353OF human GH preparation, while a PRO contamination of 0.04% was present in the Wilhelmi #1394 preparation, based on results obtained in the heterologous human PRO RIA. Other investigators, including Loewenstein et al. (210) have observed positive responses with purified human GH preparations assayed in in vitro bioassay systems (101, 102, 174, 297, 337). The responses observed from the two human GH's varied between 6% and 27% of that obtained when using an equal amount of purified ovine PRO. The human GH curves were never parallel to the standard curve. When 0.4 ul of anti-human GH was added to each 10 ng of GH assayed, the response was completely abolished. No change occurred when equivalent amounts of anti-ovine PRO was added to the flasks containing human GH.

A significant amount of variation in response was observed when each of the four rhesus PE pools were assayed in this system, making it very difficult to obtain accurate values for any of the PE pools. A comparison of results obtained in each of the PE pools by bioassay and by RIA are shown in Table IV. Only those PRO values measured by bioassay in the adult female PE pool agree with the results obtained by RIA. All of the rest are from 73-148% higher than the RIA values. When the PE pools were preincubated with anti-ovine PRO serum 0.3-0.5 ml antiserum/mg PE pool) and then added to the assay, no change in response occurred. However, when the same amount of anti-human GH serum was preincubated with the PE pool, the entire response was obliterated. These results suggest that the responses seen in the control PE pools, to which no anti-serum was added, was due entirely to GH present in the PE pools and not PRO.

TABLE IV

CONCENTRATION OF PROLACTIN IN RHESUS MONKEYS AS MEASURED BY BIOASSAY AND RADIOIMMUNOASSAY

Animal Group	Bioassay Results[1]	Radioimmunoassay[1] Results	Ratio of Bioassay: Radioimmunoassa Results
Juvenile Male	2.51 ± 1.23[2]	1.01 ± 0.05[2]	2.48: 1
Juvenile Female	1.77 ± 0.45[3]	0.91 ± 0.07[3]	1.94: 1
Adult Male	3.76 ± 0.90[4]	2.17 ± 0.10[4]	1.73: 1
Adult Female	4.74 ± 0.84	4.96 ± 0.14	0.95: 1

[1] mean $\pm$ SE. PRO values are in ug/mg pituitary wet weight.

[2,3] $p < .005$ in Students' t-test (bioassay vs. radioimmunoassay results)

[4] $p = 0.05$ in Students' t-test (bioassay vs. radioimmunoassay results)

These findings were observed with both strains of mice, including the CD-1 strain used by Loewenstein et al. (210) in their original assay procedure. In addition similar findings were observed when a plasma sample from a patient with galactorrhea was assayed. The values were nearly abolished when anti-GH serum was added to the culture flasks containing the plasma, while relatively little change occurred when anti-PRO was added.

E. Estrogen Studies

Eight intact juvenile female rhesus monkeys were used to determine the effect of estradiol benzoate (EB) on the synthesis and secretion of pituitary PRO. The animals were grouped so that two served as controls and received the corn oil vehicle, three received 15 μg/day of EB and three received 150 μg/day for 10 consecutive days. On the 11th day the animals were sacrificed. Blood samples were drawn at three time intervals and assayed by RIA for their GH and PRO content. The pituitaries obtained from these animals were hemisected, with half being fixed for histologic examination and half were used to make PE's for agar gel diffusion studies and RIA.

1. Agar Gel Diffusion Studies

Agar gel studies were carried out to determine the relationship between purified ovine PRO and presumed PRO in the PE's, as illustrated in Figure 26. A volume of 150 μl of absorbed guinea pig #8 anti-ovine PRO serum was placed in the center well and reacted against 10 ug of ovine PRO (#NIH-P-S8) and 2.6 mg of the PE pools (juvenile female PE pool, low dose PE pool, high dose PE pool). The ovine PRO gave a very strong precipitin reaction as compared to the relatively weak reaction observed from each of the three PE pools. The ovine PRO line was composed of several components, a phenomenon which was frequently observed as noted above.

The line from each of the PE pools formed a reaction of partial identity with the ovine PRO line, as indicated by the formation of a spur where the lines join. The PE lines are presumably due to PRO within each of the PE pools which is immunochemically related to but not identical to purified ovine PRO. These results parallel those obtained with normal rhesus PE's as shown in Figure 1. A second line which was shorter and broader than the first was visible with the low dose PE pool only. In figures 26 and 27 the normal juvenile female PE pool was substituted for the control PE pool because the latter was exhausted in other immunologic studies. Agar gel studies not illustrated here were previously carried out using the control PE pool and the results were identical to those obtained with the juvenile female PE pool as illustrated in Figures 26 and 27.

An agar gel study using absorbed guinea pig #30 anti-human GH serum was set up to determine the relationship between human GH and the presumed GH in the PE's. This is shown in Figure 27. A volume of 150 µl of the antiersum was placed in the center well, while 15 µg of the purified human GH (Wilhelmi #HS-1394) and 0.75-1.5 mg of each of the three PE pools (juvenile female, low dose, and high dose PE pool) were placed in the peripheral wells. A good single precipitin line was present from the three PE pools and from the purified human GH. All of the lines fused with each other forming reactions of qualitative identity. These findings suggest that the GH within the PE pools is immunologically identical to purified human GH. Similar results are shown in Figure 4 with normal rhesus PE pools.

2. Radioimmunoassay of Pituitary and Serum Concentrations of Growth Hormone and Prolactin

RIA studies were carried out on the control, low dose and high dose PE pools using the same assay systems for PRO and GH as previously described and illustrated

in Figures 9 and 11, respectively. Figure 28 shows the curves obtained with the three PE pools assayed in the heterologous human PRO RIA (Fig. 9). The results are plotted by logarithmic logistic probability transformation with the B/Bo ratio shown on the ordinate, the concentration of human PRO standard on the abscissa and the amount of PE equivalent to a given concentration of standard at the top of the graph. The PE lines were always parallel to each other but frequently were not parallel to the standard human PRO curve. In a homologous human PRO RIA, the curves from the PE pools were parallel to the standard curve indicating immunologic identity between human and monkey PRO. The absolute values of PRO (μg/mg pituitary wet weight) in each of the three pools is shown in Table V. The control PE pool had the least amount of PRO, 0.54 μg/mg, which is about half the concentration observed in the normal juvenile female rhesus PE pool. The greatest concentration of PRO was present in the low dose PE pool 7.11 μg/mg. It appears as if the animals treated with the higher dose of EB (150 μg/day) became refractory to the steroid treatment since the amount of PRO was about 60% less in this group than in the low dose PE pool.

The three PE pools were also assayed for their GH content using a homologous human GH RIA, with results shown by Figure 29. The assay system is the same as previously described and illustrated in Figure 11. The legends in Figure 29 are also the same as in Figure 11. The lines from the three PE pools were always parallel to the standard human GH curve, suggesting immunochemical identity between human GH and GH in the primate PE pools. The concentration of GH (μg/mg pituitary weight) in each of the PE pools is also shown in Table V. The GH content of the control PE was 22.4 μg/mg which was nearly identical to the amount of GH present in normal juvenile rhesus females. Significantly less GH was present

TABLE V

CONCENTRATION OF PITUITARY GROWTH HORMONE AND PROLACTIN IN ESTRADIOL BENZOATE TREATED JUVENILE RHESUS MONKEYS

Animal Group	GH (μg/mg Pituitary Wet Weight)[2]	PRO (μg/mg Pituitary Wet Weight)[2]	Body Weight (kg)[2,3]	Pituitary Weight (mg)[2]	Rating of the Average No. of PRO Cells
Control n = 2	22.4 ± 2.79[4]	0.54 ± 0.10	1.93 ± 0.00	30.75 ± 0.75[5]	0
Low Dose n = 3	17.50 ± 0.46[4]	7.11 ± 0.39	2.03 ± 0.05	36.73 ± 1.46	1.67 +
High Dose n = 3	6.00 ± 0.56	4.25 ± 0.18	2.01 ± 0.07	33.40 ± 0.60[5]	1.25 +

[1] n is the number of animals in each group.

[2] mean ± SE.

[3] body weight at the beginning of the experiment.

[4, 5] p = 0.05 in Student's t-test (control vs. low dose; control vs. high dose)

in both the low and high dose PE pools, with the latter having the lowest concentration of GH, 6 μg/mg. Thus, the synthesis of pituitary GH appeared to be inhibited in those animals treated with EB.

Also illustrated in Table V are the mean body weights (kg) and the mean pituitary weights (mg) of each animal used in this experiment. No significant difference was present in the initial body weights of the three groups, or in the pituitary weight of the two groups that received the steroid treatment. There was, however, a significant difference ($p=0.05$) in the mean pituitary weight of the animals which received the steroid as compared to the mean pituitary weight of the controls. No statistically significant difference existed in the pituitary weights of the two groups treated with EB.

Blood samples were drawn from each of the eight animals before the experiment began, on the 6th day and on the last day of the experiment. These were assayed in the heterologous human PRO RIA and in the homologous human GH RIA in order to quantitate the concentration of PRO and GH, respectively, in each blood sample. The results are expressed as the mean serum PRO or GH (ng/ml) value for each of the three groups and are summarized on Table VI. The PRO values obtained from each of the three groups before the experiment began were all approximately equal, with no significant difference between the three groups. Similarly, no change was observed in the concentration of the PRO in the control animals throughout the entire experimental period. There was an increase in PRO concentration in both groups of animals treated with EB, up to the 6th day of the experiment, however, no significant increase in PRO levels occurred in either group from the 6th day until the end of the experiment. Thus, administration of EB was effective in stimulating the

TABLE VI

CONCENTRATION OF SERUM GROWTH HORMONE AND PROLACTIN IN ESTRADIOL BENZOATE TREATED JUVENILE RHESUS MONKEYS

Animal Group	Beginning of the Experiment (1st day)		Middle of the Experiment 6th day)		End of the Experiment (11th day)	
	GH (ng/ml serum)[1]	PRO (ng/ml serum)[1]	GH[1]	PRO[1]	GH[1]	PRO[1]
Control	7.81 ± 3.08[2]	7.20 ± 0.80[3]	9.15 ± 5.40	7.20 ± 0.70	29.50 ± 18.50[2]	9.30 ± 0.95[3]
Low Dose	54.42 ± 17.61	8.27 ± 0.23	90.37 ± 20.01	16.67 ± 3.21[4]	20.07 ± 8.30	13.07 ± 1.56[4]
High Dose	30.36 ± 6.19	6.47 ± 0.94	24.00 ± 6.39	13.82 ± 2.25[5]	70.40 ± 18.24	17.47 ± 2.38[5]

[1] mean ± SE. GH and PRO values are ng/ml serum.

[2-5] no significant difference in Students' t-test (control GH: 1st day vs. 11th day; control PRO: 1st day vs. 11th day; low dose PRO: 6th day vs. 11th day; high dose PRO: 6th day vs. 11th day).

secretion of the PRO over a short period of time; however, prolonged treatment with the steroid does not appear to enhance further increases in serum PRO levels. EB administration was also effective in stimulating the synthesis of pituitary PRO as reflected by the increase of PRO values in the pituitaries of the animals given the steroid (Table V).

The serum GH levels were very erratic, as indicated by the relatively high standard errors for each of the values. In general, the monkeys treated with EB had higher serum GH values than the controls. No significant difference was present in the GH values of the control animals throughout the experiment. There were marked flucturations in serum GH concentrations in both groups treated with EB during the 11 day experimental period, with neither of the two groups showing a consistent pattern of GH values.

The stress of capturing and tranquilizing the monkeys may have had some effect on the secretion of both GH and PRO. Stress induced by drugs has been shown by several investigators to be a stimulant of GH and PRO in humans (104, 124). This may have affected GH secretion more than PRO and may account for the fluctuations in GH values observed during the experimental period.

3. Light and Fluorescence Microscopy

Figures 30-32 a-c, are photomicrographs of sagittal sections of a representative member of each of the three groups of monkeys. The micrographs were taken from three separate levels of the pituitary which correspond to the same levels shown in Figure 18 a-c. The control animals (Fig. 30 a-c) had very few PRO cells present in their pituitaries, which was similar to that observed in normal male and female juvenile rhesus monkeys (Fig. 16-17 a-c). These cells were found almost entirely

in the more anterior regions of the pars distalis. The GH cells were quite abundant in number and were present in the same regions as in normal rhesus monkeys (Fig. 20). A marked and significant increase was observed in the number of PRO cells present in the pituitaries of the animals treated with the low dose (Fig. 31 a-c) and the high dose (Fig. 32 a-c) of EB. The PRO cells were mostly the larger variety and were frequently found in small clusters in the medial and anterior regions of the pars distalis. Very often the PRO cells were observed to be randomly arranged in the most posterior regions, intermixing with the GH cells. The number of stainable GH cells appeared to be less in both groups of animals treated with the steroid as compared to the controls, which is in agreement with the RIA results.

Sagittal sections from approximately the same area of the pituitary of a control, a low dose and a high dose treated animal are shown in Figure 33 a-c, respectively. Each section was stained by the indirect fluorescent antibody technique using anti-ovine PRO serum. Very few PRO cells fluoresced in the pituitaries from the controls, as compared to either group which received the EB. Slightly more PRO cells fluoresced in the animals treated with the lower dose of EB. These histologic findings were reported at the IV Internal Congress of Endocrinology (153). It was reported at that time that the number of PRO cells were "slightly greater" in the animals given the higher dose of EB. More critical examination of these pituitaries in the light of the RIA results, show that the greatest number of PRO cells are present in the pituitaries of the animals given the lower dose of EB.

The relative number of PRO cells observed in the pituitaries of the animals treated with the steroid was determined employing the same rating system used to score the number of PRO cells in normal rhesus pituitaries. The average rating of the number of PRO cells present in the pituitaries of the monkeys treated with the low dose of the EB was 1.67 +, as shown in Table V. Fewer PRO cells, 1.25 +, were

observed in the animals receiving the higher dose of EB. When the RIA and histologic findings are compared between the two groups treated with the steroid, there is 67% more PRO and 34% more lactotrophs in the pituitaries of the animals treated with the lower dose of EB. The hormone treatment was effective in stimulating both the synthesis (Table V) and the secretion (Table VI) of PRO in these juvenile rhesus females, even though it appears as if the group which received the high dose of EB may have become refractory to the steroid.

DISCUSSION

A. Hormone Immunization

With the hope of obtaining an antiserum that would be capable of detecting primate PRO in rhesus monkey PE's, ovine PRO was utilized for the immunization of several different animal species. The hormone was antigenic in the guinea pig, rat, rabbit, and monkey, as indicated by the precipitin reactions observed in agar gel. There was a significant amount of intraspecies and interspecies variation in antibody response present from three of the four hosts immunized with ovine PRO. These differences were reflected in the character of the precipitin reaction observed from each animal injected with ovine PRO. The most consistent precipitin reactions were obtained from the guinea pig anti-ovine PRO serum. Hayashida and Contopoulos (146) have also observed interspecies variations in antibody response between three hosts immunized with rat GH. In their study, no detectable antibody response was elicited in any of the several rabbits immunized with rat GH on the basis of agar gel diffusion tests; however, a fair response was observed in the guinea pigs and a good to excellent response was noted for the majority of monkeys. A determining factor regarding which species of animal will produce the most potent antiserum seems to be related in part to the phylogenetic distance between the host animal and the species from which the antigen was purified. The greater the distance, the more unrelated or "foreign" the antigen appears to the host and the better the antibody response.

Interspecies variations were also noted in the intensity of fluorescence observed when antisera from each of the four hosts were used to detect the cellular source of PRO in the primate pituitary by immunofluorescence. The brightest and most

intense fluorescence was observed with guinea pig antisera, while the least intense reaction was obtained with rabbit and monkey antisera. The low degree of fluorescence was unexpected with the later antiserum since in agar gel studies a strong precipitin reaction was observed. It is not possible, however, to obtain a direct correlation between the strength of the precipitin reaction observed in agar gel and the intensity of fluorescence. An uncontrolled factor, the fluorescein-labelled conjugate, is present in the latter technique. There is a considerable amount of variation in the quality of the commercially available conjugates. In addition, there seems to be a marked difference in the affinities of some sera for the fluorescein isothiocyanate. This factor alone can act to either enhance the fluorescence obtained with a rather weak antiserum or to "repress" a very potent antiserum, thereby giving an incorrect view of the antibody titer. Even though it is less sensitive than immunofluorescence (234, 240), the agar gel diffusion technique has fewer uncontrolled factors and in general is a more reliable method for the determination of antibody titer.

B. Comparative Immunochemistry of Growth Hormone and Prolactin

The comparative studies using anti-ovine PRO serum with selected vertebrate PE's in agar gel and in the RIA indicate that no apparent phylogenetic relationship exists among the vertebrate PRO's. The quality of the precipitin reactions observed in agar gel appeared to be mainly dependent on the species of the host producing the particular antiserum. Most of the mammals examined gave precipitin reactions when using antisera from each of the four hosts. The most extensive cross-reactivity was observed with the monkey antiserum. Apparently the primates, which are phylogenetically quite distantly removed from the sheep, produced antibodies more effectively to sites on the ovine PRO molecule which are related among

the mammals, than did the other three hosts.

The immunochemical relatedness between mammalian PRO's in agar gel and the RIA suggests that a certain amount of species specificity is present within this class of vertebrates. The PRO's from the ox, giraffe and sheep, appear to be very closely related if not identical immunochemically. The precipitin lines observed from these animals gave reactions of qualitative identity with each other. In addition, the slope of curves from the ox, giraffe and sheep PE's or purified PRO's were all very similar to each other in the RIA studies, indicating again the immunochemical relatedness of these mammalian PRO's. Prolactin in PE's from the seal and rabbit, and from the horse and pig, respectively, appear to be identical to each other but differ significantly from other mammalian PRO's tested in this study. One of the mammals, the opossum, failed to cross-react with any of the antisera in agar gel. Precipitin reactions were also observed, with PE's from a few submammalian species, mainly with monkey anti-PRO serum. No cross-reaction occurred between duck or chicken PE's and guinea pig antiserum in the RIA.

Sinha et al. (319) report some cross-reactivity between purified submammalian PRO's and rabbit anti-human PRO serum in agar gel. Precipitin lines were observed with PRO's from the human, ox, sheep, pig, mouse, whale, turtle and frog. No reaction was observed with rat or shark PRO. The apparent lack of phylogenetic relationship among the vertebrate PRO's have been studied quite extensively by Nicoll and Bryant (38, 249) using a variety of heterologous RIA systems with rabbit and human anti-ovine PRO sera, and rabbit anti-human PRO serum. The slopes of the inhibition curves varied so extensively that no evolutionary order could be assigned to the vertebrate PRO's. They observed more extensive cross-reactions

with PE's of several primitive vertebrate species than with a few more highly evolved animals.

It is readily apparent from the observations reported in this dissertation, as well as those by Sinha et al. (319) and Nicoll and Bryant (38, 249) that fewer shared antigenic determinants are present among the vertebrate PRO's than are present among the vertebrate GH's (142, 144). The PRO molecule appears more species specific than the vertebrate GH's, which could be related to the vast evolutionary changes undergone by the former hormone. More biological activities have been assigned to PRO than to any other pituitary hormone (28, 247). Changes in the primary structure of the hormone must have occurred throughout evolution so that each of these functions could be expressed. The chemistry of the PRO has probably become more complex with time so that fewer structural similarities, including antigenic determinants, are shared between species. It does appear, however, as if the structural aspects associated with the biological activity of the vertebrate PRO's have evolved at a slower rate than those responsible for the immunologic activity. PRO of the more highly evolved species have retained their very primitive biological activities (28, 247), while at the same time appear to have lost many of their shared antigenic determinants. Therefore, the concept of a molecular core with regard to the vertebrate PRO's is more difficult to conceive than for vertebrate GH's. It seems, however, as if the vertebrate GH molecule has evolved quite slowly. This is probably related to the fact that this hormone has at least one major biological activity in the vertebrates, that being to promote somatic growth. Few evolutionary changes must have occurred so that this activity

could be retained in all vertebrate GH's. In addition minimal alterations in the immunochemical activity of GH must have taken place, accounting for the relatively large number of shared antigenic determinants which seem to be present in the vertebrate GH's. (142, 144).

Agar gel and RIA studies illustrate the presence (to some degree) of partial immunochemical relatedness between primate PRO and ovine PRO. The precipitin lines from the primate PE's form reactions of partial identity with ovine PRO suggesting that a substance presumably PRO is present in the PE's which is similar to ovine PRO. In addition the slopes of the curves from the primate PE's and ovine PRO are different in both the homologous and the heterologous RIA's indicating further the partial immunochemical relatedness between these two hormones. This relatedness between ovine and primate PRO has been used to study the cellular source of human (270, 271) and monkey PRO (151, 152, 270, 271) as well as in the purification of PRO from cultures of human (106, 114, 117, 162, 315) and monkey pituitaries (112). It has also been important in the development of heterologous RIA systems used to measure plasma levels of PRO in humans (11, 166, 255). It is not surprising that immunochemical relatedness has been demonstrated between primate and ovine PRO since these two hormones are very similar in primary structure. The overall homology reported between the primary structure of human and ovine PRO is about 75% (184). Both have the amino acid cystine at the C-terminus (184).

A significant amount of immunochemical species specificity exists among the mammalian GH's. Studies using agar gel and RIA with antibodies to rat GH indicate the presence of immunologic identity among the nonprimate mammalian GH's (142).

However, it appears as if the primate GH's are immunochemically different from all other mammalian GH's. In the present investigation, the results from the agar gel and RIA studies using guinea pig anti-human GH serum support this concept. Immunologic identity was observed among several human and monkey GH preparations as well as from the GH within the primate PE's. No immunochemical relatedness was found with a few purified mammalian GH's, such as ox, sheep, or pig GH. Similar findings have been reported by Hayashida and Li (149). They observed precipitin reactions in agar gel from purified human and monkey GH's using rabbit anti-human GH serum, but not with ox, sheep, or whale GH. The only immunologic technique which is sensitive enough to show immunochemical relatedness between primate and nonprimate mammalian GH's appears to be the fluorescent antibody technique. In this study, fluorescence was observed in the GH secreting cells of the monkey and the dogfish shark, using either guinea pig anti-human or monkey anti-rat GH serum. Similar observations were also noted in horse and sheep pituitaries with guinea pig anti-human GH serum. Nayak et al. (240) have localized fluorescence in the somatotrophs of the ox, rat and pig using rabbit anti-human GH serum but failed to fine fluorescence in human pituitaries with rabbit anti-ovine or anti-bovine GH serum.

Species specificity is also observed with regards to the biological activity of mammalian GH's. Several investigators have shown that purified primate GH's are biologically active in the rat (121, 206, 207); however, nonprimate GH's such as sheep, ox and porcine GH, fail to stimulate somatic growth in primates (121, 175). The more highly evolved GH's, therefore, have retained the ability to elicit

a biological response in lower animals; however, the converse is untrue. It is possible that there may be a significant difference between the binding receptors in the non-primate mammals as compared to those present in the primates which accounts for the lack of growth promoting activity of nonprimate GH in primates.

Primate GH and ovine PRO are unrelated immunochemically, even though there are significant similarities between these molecules in their amino acid compositions and in their biological activities. Bewley and Li (33) report a structural homology of 60% between these two hormones. Human and monkey GH have been shown to possess biological activities similar to those of ovine PRO (41, 89, 96, 99, 100, 164, 216, 274) and have been used to induce lactation in female human subjects (114). Ovine PRO has been reported to elicit similar metabolic effects as GH when given to human subjects (26, 224). The lactogenic activity of primate GH may be an intrinsic part of the hormone molecule as suggested by Li (193) or it may be phenomenon induced by inadvertent biochemical manipulation of the molecule during its purification. It is not known whether native primate GH has any effect on lactation in vivo. The amino acids responsible for this activity may be so positioned in the molecule that they cannot exert their biological activity in vivo. During the purification of the hormone, these residues may have been exposed and the molecule "opened up" allowing the amino acids to express their PRO-like activity. What is being observed experimentally may be an artifact induced from the biochemical insult of primate GH. If this is not true and if native GH does possess intrinsic lactogenic activity, then one might expect lactation to occur more frequently in acromegalics (85) and plasma GH levels to be higher during pregnancy and

lactation as well as in patients with galactorrhea than they really are (25, 163, 325, 326, 327, 357). It is possible that the receptors in the epithelium of human and monkey mammary tissue are unresponsive in vivo to that portion of the GH molecule responsible for inducing lactation while at the same time are sensitive only to the residues capable of general somatic growth of the mammary gland. The only substantial and direct evidence which demonstrates that native human GH possesses intrinsic lactogenic activity are the findings made by Frantz and Kleinberg (102, 174) in their in vitro mouse mammary assay system. When unextracted human blood samples were preincubated with rabbit anti-human GH serum, there was a significant decrease in the response observed as compared to those samples which were not pretreated with the antiserum. It is assumed that the antiserum removed all of the GH within the serum, and therefore, effectively neutralized the lactogenic response which was being induced by GH.

C. Radioimmunoassay

The homolgous ovine PRO and human GH RIA systems used in this investigation as well as the heterologous human PRO RIA system were all quite sensitive and very reliable assay techniques. There appeared to be a negligible amount of cross-reactivity between anti-ovine PRO serum and purified primate GH, and between anti-human GH serum and purified ovine PRO. The maximum percentage of cross-reactivity between anti-PRO and primate GH was 0.01%, while the maximum cross-reactivity between anti-GH serum and ovine PRO was 0.001%. Even though these cross-reactions were only minimal, there seemed to be some significance to them. In the homolgous ovine PRO RIA, five different human GH preparations and

one of the two monkey GH preparations cross-reacted. The curves from the monkey GH #42 and from three human GH preparations, Wilhelmi #HS-612B, #HS-1394 and Raben #18, were all parallel to each other (Fig. 8) and had a similar slope to the curves from the primate PE's (Fig. 7). All these lines, however, had slopes which were different from the standard ovine PRO curve. On the other hand, the curves from Li GH #3477A and Wilhelmi GH #HS-624Bb-3N were nearly identical to the standard ovine PRO indicating that the material which is being measured in these two GH preparations must be very closely related immunochemically to ovine PRO. It is not clear as to why certain primate GH preparations show parallelism to ovine PRO while others do not. The nature of the material present in highly purified human GH preparations responsible for the cross-reactions observed with anti-ovine PRO serum is unknown. It may be due to the portions of human GH molecule responsible for the intrinsic PRO-like activity of primate GH or to a contamination of the hormones by primate PRO. Also it is not clear why the cross-reaction observed with human GH remained after its preincubation with anti-ovine PRO serum. Although it appears unlikely, the possibility of a third factor present in these hormone preparations besides GH itself or PRO cannot be ruled out. The results of these observations raise the possibility, therefore, that either GH itself or a factor other than PRO may be influencing the cross-reactions noted between the primate PE's and anti-ovine PRO serum.

D. Bioassay of Primate Pituitary Extracts

An *in vitro* mouse mammary bioassay system was set up to measure PRO values in the rhesus PE pools following a procedure similar to that reported by Loewenstein *et al.* (210). Similar results were obtained using either the CD-1 or the White-Swiss

strain of mouse. Ovine PRO as well as two human GH preparations were active in the assay, confirming the findings previously reported by several investigators (101, 102, 174, 210, 337). These responses were quite specific since each could be nearly abolished when the homologous antiserum was added. The bioassay curves obtained with ovine PRO and human GH were not parallel, which suggests the possibility that each hormone may be stimulating a different set of receptors on the plasma membrane of the mammary epithelial cells.

The primate PE pools and a plasma sample obtained from a patient with galactorrhea were also active in the bioassay. The responses from both the PE pools and the plasma sample were abolished by the addition of anti-human GH serum to the incubation flasks but were not altered significantly when anti-ovine PRO was added to the flasks. These observations were noted with both strains of mice and suggest the possibility that only GH in the PE's and the plasma sample is being measured in the assay. Rivera et al. (296, 297) have demonstrated that some strains of mice are more sensitive to purified GH's than others. This seems to be an inherent factor present in the mammary epithelium of certain strains of mice. The results obtained in this assay, however, cannot be explained solely on the basis of the difference in responsiveness of the two strains of mice, since of the strains used was identical to that employed by the investigators who established the technique (210). The GH concentration was too low in the plasma sample (1.5 ng/ml) to account for all the bioactivity measured when this sample was assayed. Therefore, it appears as if the assay was detecting and measuring PRO in the PE pools and the plasma sample. The concentration of PRO in the homogenates as measured by the bioassay was no

more than two and a half times higher than the values obtained using the RIA. The differences in bioassayable and immunoassayable PRO values are significant but are also quite similar to those differences reported by Frantz et al. (104)and Friesen et al. (115).

It is not clear as to why the anti-ovine PRO failed to inhibit or abolish the responses obtained with the PE' pools and the plasma sample. The same antibody used in this study apparently binds to some part on the primate PRO molecule because positive fluorescence in the PRO-containing carminophils can be obtained when the antiserum is employed in the fluorescent antibody technique. Possibly the anti-ovine PRO serum is capable of combining with the immunologic sites located on the surface of the monkey PRO molecule but is incapable of binding to or neutralizing the residues responsible for the lactogenic activity of primate PRO. This implies that the amino acids responsible for the immunologic and biological activities are quite distant from each other on the surface of the hormone. Frantz et al. (103) have been successful in abolishing the biological activity of PRO in human plasma samples with rabbit anti-ovine PRO serum in their in vitro bioassay system. Loewenstein et al. (210) observed only a partial loss of PRO activity when a plasma sample from a lactating human female was incubated with guinea pig anti-ovine PRO and then used in bioassay.

Antibodies to human GH, which were effective in abolishing the mouse mammary responses observed with the PE pools and the plasma sample, apparently some how reacted with and neutralized the biological activity of primate PRO. The same antiserum has been used in agar gel studies with ovine PRO and in immunofluorescent studies carried out on sections of primate pituitary tissue. No precipitin

line forms in agar gel with ovine PRO nor is any fluorescence observed in the lactotrophs of the rhesus monkey with anti-human GH serum. It appears as if the antiserum fails to combine with the immuno-reactive sites on the primate PRO molecule but interacts in some way to block those molecular sites which are responsible for the biological activity of the hormone. Again this suggests that these two sites are separated from each other on the surface of the PRO molecule. This concept may also explain the cause for the 10% decrease in response when ovine PRO was incubated in the presence of anti-human GH serum. Loewenstein et al. (210) have observed an 85% decrease in the response due to a plasma sample obtained from a lactating human female, which was cultured with anti-human GH serum. They attribute this decrease to a contamination of PRO which was present in the GH preparation used for immunization, resulting in the presence of anti-PRO antibodies in the anti-GH serum.

E. Light and Fluorescence Microscopy of Primate Pituitaries

The orangeophilic and carminophilic cells observed in the pituitary of the rhesus, capuchin, and cynomolgus monkeys as well as in the pars distalis of the three species of apes studied were identified by immunofluorescence as the somatotroph and the lactotroph respectively. These findings demonstrate unequivically that GH and PRO are two distinct hormones in the primate pituitary gland and that these hormones are synthesized and secreted by separate cell types. All of the acidophils present in the primate pituitary gland appear to be either GH or PRO secreting cells. Two sizes of PRO cells are generally observed in the primate pituitary. The small PRO cell is more frequently found in the postpubertal adult

animal while the large PRO cell is present in all pituitaries regardless of age or sex. The small PRO cell may be an immature form of the larger cell type. They do not contain as many secretory granules as the larger carminophils. The intensity of fluorescence is always quite weak in the small PRO cells, indicating that a small amount of hormone is present within these cells. Since they are found principally in adult and pregnant or lactating pituitaries which are actively synthesizing and presumably secreting PRO, they may represent newly differentiated cells which are formed due to the physiologic need of the animal.

The number of PRO cells observed in the rhesus monkey varied depending on the age, or sex of the animal. Very few PRO cells were identified in the two fetal pituitaries examined. These cells were only recognized by immunofluorescence using antibodies to ovine PRO. These observations do not corroborate with the RIA data reported by Friesen et al. (110). They report serum PRO values in human fetuses which are essentially identical to those present in the maternal serum. In addition, ten times more PRO is present in the amniotic fluid than in the serum of the fetus. Histologic studies conducted on human fetal pituitaries collected throughout gestation show that nearly all of the acidophils fluoresce with anti-human GH serum (277). This suggests that the fetal pituitary contains very few PRO cells and is storing only a small amount of hormone. Goluboff and Ezrin (127) observed very few stainable carminophils in human fetal pituitaries. The elevated serum PRO values (110) cannot be from de novo synthesis of the hormone by the fetal pituitary alone. The PRO being measured in fetal serum may be maternal PRO which has cross the placenta. The PRO values in fetal and maternal sera are the same (110) which suggests that an equilibrium has been established between the

levels of PRO in the fetus and the mother. At parturition, the equilibrium is disturbed, and the plasma PRO values begin to decrease in the neonate until 4-6 weeks of age when they are within normal range (110). If de novo synthesis were responsible for the elevated PRO levels in the fetus, then no change would be expected to occur after parturition. Examination of the 23 day-old neonatal rhesus monkey pituitary indicated that very little PRO was being synthesized at this early stage of life. The elevated PRO levels in the amniotic fluid reported by Friesen et al. (110) may be due to an accumulation of PRO which is being excreted by the fetus. Friesen et al. (115) administered ^{125}I-labelled PRO to a 95 day pregnant monkey and found that a small amount of hormone crossed the placenta. They also reported lower PRO values in the fetal serum than in the maternal serum in four pregnant rhesus monkeys studied. This suggests that the kinetics of PRO transport are different in the rhesus monkey than in humans and that maternal PRO may pass more freely through the human placenta than through the monkey placenta. They observe high PRO values in the amniotic fluid of rhesus monkeys which they speculate may arise from the chorion (115).

Very few PRO cells were present in the juvenile, nonbreeding rhesus monkeys, the adult capuchin monkeys, and in the three species of apes: the adult gorilla, the juvenile orangutan and the two juvenile chimpanzees. The concentration of pituitary PRO was very low in the normal juvenile rhesus monkeys studied: 0.91 µg/mg pituitary wet weight in the females and 1.01 µg/mg in males, which suggests that PRO is relatively nonfunctional in the juvenile, prepubertal rhesus monkey. Pasteels et al. (271) recently observed similar findings in infant rhesus monkeys as

those reported in this study for juvenile rhesus monkeys. In humans, very few carminophils were present in normal nonpregnant, nonlactating pituitaries regardless of age or sex (127).

The number of PRO cells as well as the concentration of pituitary PRO, as measured by RIA, were significantly higher in all of the adult male and female rhesus monkeys examined compared to the findings in the prepubertal or juvenile monkeys. The adult females contained a higher concentration of pituitary PRO and possessed more stainable PRO cells than the adult males. The two adult female cynomolgus pituitaries examined also contained a large number of PRO cells. Pasteels et al. (271) report that the number of PRO cells present in adult male and female rhesus monkey pituitaries are essentially the same. They measured the concentration of PRO in homogenates of juvenile and adult monkey pituitaries by densitometry performed on polyacrylamide gel electrophoretic columns. Juvenile rhesus monkeys and adult males were observed to have the same concentrations of pituitary PRO, while slightly higher values were noted in adult females (271). The PRO values obtained by Pasteels et al. (271) for adult male and female rhesus monkeys, as measured by densitometry, appear to be similar to values obtained in the present investigation in the adult male and female rhesus PE pools measured by RIA. However, Pasteels et al. (271) report much higher PRO values in infant monkeys than those observed in the juvenile male and female PE pools examined in the present study.

F. Effect of Estradiol Benzoate on Primate Prolactin

PRO cells are abundant in all adult rhesus monkey pituitaries, but appear quite scarce in nonpregnant, nonlactating human pituitaries according to Goluboff and Ezrin (177). Apparently the lactotrophs present in adult rhesus monkey pituitaries are more responsive to the rise in circulating levels of the sex steroids following puberty than in humans. These steroid hormones are probably acting both at the level of the hypothalamus and the pituitary to stimulate PRO synthesis and secretion from the adult rhesus monkey pituitary. Numerous reports have been published showing the influence of estrogens on the histology of the pituitary PRO cells and on the plasma and pituitary levels of PRO in several mammalian species 16, 45, 63, 120, 227, 230, 236, 251, 288). Estrogen implants placed in either the pars distalis (120) or the hypothalamus of the rat (236) cause hypertrophy of the PRO secreting cells (120) and an increase in the concentration of PRO in the plasma and the pituitary (236). Progesterone administration causes a very small rise in pituitary and plasma PRO levels in the rat (45). When progesterone is implanted in the pituitary, no change occurs in the histology of the PRO cells (14); however, a decrease in the size and granular content of PRO cells was noted when this steroid was given subcutaneously to rats (15). In the present investigation intact juvenile female rhesus monkeys were treated with estradiol benzoate (EB) to determine the effect of this steroid on the morphology of PRO cells and on the levels of pituitary and serum PRO. The steroid treatment increased the number of the PRO cells as well as causing a significant rise in pituitary and serum levels of PRO. The number of PRO cells and the pituitary concentrations of the hormone

noted in the EB treated juvenile females was nearly equal to or greater than that observed in normal adult rhesus females. This strongly suggests that the increase in circulating levels of estrogenic steroids, which presumably occurs after puberty in adult cycling female rhesus monkeys, are responsible at least in part for the histologic changes and the increase in hormone content observed in post-pubertal females. Since normal serum concentrations of PRO were not established for adult females, it is not known whether the PRO levels observed in the EB treated animals are similar to those present in cycling adult females.

Two other investigators have observed histologic changes in monkeys following the administration of sex steroids. Dawson observed an increase in the number of carminophilic cells following choronic treatment of ovarectomized rhesus females with estrogen alone or a combination of estrogen and progesterone (63). A similar observation was noted by Girod (122) after injection of reserpine or estrogen into presumably adult male and female rhesus monkeys. Nicoll et al. (248) stimulated th release of bioassayable PRO in cavernous sinus blood of male rhesus monkeys after injection of a combination of delestrogen and perphenazine. A few studies have reported the effects of steroid administration on PRO levels in human subjects. No change occurs in plasma PRO following acute (170) or chronic (191) administration of progesterone. Conflicting reports have been reported regarding the effect of estrogens or PRO in humans (104, 170, 191). Kastin et al (170) report no change in PRO levels in males given daily injections of 20 mg of estrogens for three days, while Frantz et al. (104) observe a small but significant rise in PRO values in their patients treated with 15 or 50 mg/day of estrogens over a seven-day period. An increase in plasma PRO values has also been observed in patients given estrogenic steroids for prostatic cancer (104). L'Hermite et al (191)

administered estrogens orally and intravenously to several groups of patients. They noted considerable variation in response which they attribute to the differences in physiologic status of their patients, in the dosage of steroids given, and in the route of administration of the hormone.

Testosterone may be stimulating PRO synthesis and secretion in the post-pubertal adult male rhesus monkey. This would account for the increase in the number of lactotrophs and the concentration of pituitary PRO recorded in the adult males. Dawson (61) treated an ovariectomized female rhesus monkey with both estrogens and testosterone and observed hyperplasia of the carminophils. Testosterone proprionate administration has been shown to increase the content of pituitary PRO in lactating rats (230) and the plasma levels of PRO in castrated male or female rats (169). In either of these studies, the effect of testosterone was not as pronounced as it was following estrogen administration, but nevertheless it was significant. A greater rise in plasma PRO was observed when estrogen and testosterone were given together than when either hormone was administered alone (169). This suggests that the increase in the number of caminophils observed by Dawson (61) was due to the combined effects of both estrogen and testosterone and not solely due to estrogen. MacLeod et al. (219) incubated the pituitaries of male rats in the presence of ^{3}H-labelled leucine following administration of testosterone to these animals. The labelled amino acid became incorporated into newly synthesized PRO indicating a stimulatory effect of the steroid on PRO production.

G. Role of Prolactin in Adult Rhesus Monkeys

Since the number of PRO cells and the concentration of pituitary PRO increases

significantly in male and female rhesus monkeys following puberty, the question arises as to the possible role of PRO in the normal adult rhesus monkey. In females, PRO may be involved in the growth and development of the mammary gland. Lobulo-alveolar growth is principally regulated by estrogen, progesterone and PRO (214) and has been observed in normal, nonpregnant, nonlactating adult primates (93). PRO may also be involved in cholesterol metabolism and steroid production in the ovary. This action may be a tonic one since PRO levels appear to be essentially unchanged during menstrual cycle of humans (110, 163) and are presumed to be unchanged in cycling monkeys as well. Armstrong and co-workers (9, 10) have shown that PRO increases cholesterol synthesis, and the concentration of cholesterol esters and progesterone in the corpus luteum of the rat. PRO has little effect on cholesterol metabolism or steroid production in the interstial tissue elements of the rat ovary. Behrman et al. (23) have reported an increase in the levels of the two enzymes involved in the synthesis and hydrolysis of cholesterol esters in rat corpora luteal tissue following administration of PRO to $\bar{H}$ rats. PRO effectively restored the concentration of these enzymes to normal while luteinizing hormone (LH) progesterone or estrogen had no effect.

PRO may be involved in maintaining the normal physiology of the testes and sex accessory glands of the adult male rhesus monkey. Bartke and collaborators (18, 20, 133, 134, 135, 235) have conducted a series of studies on male mice and rats to determine the effect of PRO on cholesterol metabolism. Administration of PRO to intact or $\bar{H}$ adult male mice increases the concentration of total cholesterol and cholesterol esters in the testes of these animals (18, 20). Similar observations

were made on hereditary dwarf mice bearing hypophyseal transplants (18). Injection of PRO into hereditary dwarf mice has restored the activity of two enzymes involved in the biosynthesis of testosterone (135, 235). In rats, there appears to be a synergistic action of PRO and LH in the regulation of testicular enzymatic activity (135), testosterone biosynthesis (133) and on plasma levels of testosterone (134). PRO and LH have also been shown to stimulate spermatogenesis in mice (19, 21) and rats (355). This latter effect may be an indirect action of PRO and LH which isprimarily mediated by testosterone. Several investigators have shown that PRO alone or in combination with the testosterone can cause an increase in the weight and secretory activity of the prostate (28, 346) and the seminal vesicles (19, 24) of mice or rats. PRO seems to enhance or potentiate the effect of testosterone on the sex accessory glands (183). Thus, PRO may play a very important role in steroid hormone biosynthesis and sperm production in male rhesus monkeys, as well as in maintaining normal physiologic activities of the prostrate and the seminal vesicles.

H. Prolactin During Pregnancy and Lactation

The numbers of PRO cells observed in pituitaries obtained from late pregnant and lactating monkeys were much greater than the number present in normal adult female pituitaries. The lactotroph appears to be the predominant cell type in the pars distalis during late pregnancy and lactation. These changes are probably due in part to an increase in circulating levels of estrogens which presumably occurs during pregnancy as well as to the effect of suckling by the newborn which

has been shown to be a potent stimulus of PRO secretion (104, 163) in the lactating female. Serum PRO levels were not measured in the pregnant and lactating monkeys in the study, so it is not known whether concomitant rises in circulating levels occurred in these animals. Goluboff and Ezrin (127) and Pasteels et al. (270, 271) have also observed a hyperplasia of the lactotrophs in pregnant and lactating humans and monkeys. Friesen et al. (115) report no change in the serum levels of PRO during pregnancy in rhesus monkeys. They conclude that the lack of elevated PRO levels are due to the apparent low concentration of plasma estrogens present during pregnancy. This is based on the observation that lower urinary levels of estrogens are observed in pregnant monkeys than in pregnant humans. However, Hodgen et al. (159) have assayed plasma estradiol levels throughout pregnancy in rhesus monkeys. They report a slow rise in steroid levels from about day 40 to day 100, when the estrogen levels reach their peak and remain high until parturition (about day 160). The maximum levels recorded were about 5-6 fold higher than those values observed during the early stages of pregnancy. Urinary concentrations of estrogen were quite low during the latter stages of pregnancy (159), as previously reported by Friesen et al. (115). Friesen et al. (115) have conducted a few preliminary studies measuring PRO levels in lactating rhesus monkeys. The results show inconsistent changes in PRO levels following suckling. In some animals a decrease in plasma PRO values was recorded, while in others a significant increase was observed. The serum PRO values reported by Friesen et al. (115) in pregnant and lactating female rhesus monkeys are not consonant with the

histologic findings. These discrepancies may be explained on the basis that elevated plasma estrogens observed during pregnancy are affecting synthesis and not secretion of PRO. It is not known, however, why suckling does not promote a more consistent rise in plasma PRO values in the lactating rhesus monkey.

I. Growth Hormone in Primate Pituitaries

The concentration of GH was measured in the PE's of juvenile and adult male and female rhesus monkeys and in PE's and the serum of the estrogen-treated animals. The GH values were the highest in the adult rhesus monkey PE's, with the greatest concentration being noted in the adult females. Pasteels et al. (271) report that pituitary GH values are quite similar in male and female adult rhesus monkeys and in lactating females. They observed the highest concentration of GH in juvenile rhesus monkeys as measured by densitometry of polyacrylamide gel electrophoretic columns. The number of GH cells observed in juvenile, adult and lactating monkeys appear to be in good agreement with the values of pituitary GH found in these animals (271). Goluboff and Ezrin (127), however, have reported a decrease in the number of GH cells present during pregnancy and lactating in humans. We observed similar findings in the pregnant and lactating rhesus monkey pituitaries when examined by light and fluorescence microscopy.

The decrease in the number of GH cells during late pregnancy and lactation in humans and rhesus monkeys may be due to an inhibitory effect of estrogens on the somatotrophs. In the pituitaries obtained from the intact juvenile female monkeys treated with EB, there appeared to be a decrease in the number of stainable GH cells. Concomitant with this was a significant decrease in the levels of pituitary

GH in both groups of animals treated with EB, and a small increase in the serum levels of GH. Pantić and Genbačev (259) noted a decrease in the number of secretory granules present in the somatotrophs of male rats treated with estrogen. They also observed a decrease in the staining density of the GH band following polyacrylamide gel electrophoresis of pituitary homogenates prepared from the steroid treated males. Implantation of estrogen into the pars distalis of the rat causes a reduction in the size and possibly the number of GH cells (120). Frantz and Rabkin (105) measured basal and ambulatory plasma levels of GH in estrogen-treated human male subjects and observed a rise in plasma GH values only in the ambulatory state.

From the results observed in the juvenile monkeys treated with estrogens as well as those reported by other investigators, it appears as if estrogenic steroids have an inhibitory effect on the synthesis of GH in the pituitary. This is reflected by a decrease in the number of GH cells present as well as a drop in the pituitary levels of GH. The rise in serum GH observed in our steroid-treated monkeys and in the patients studied by Frantz and Rabkin (105) may be directly attributed to the stress of the steroid therapy, including any psychological and physiological stresses acting on the animal or patient during the course of the experiment. Stress has been shown to be an effective agent in causing increases in plasma GH levels (124).

J. Regional Distribution of the Acidophils in the Primate Pituitary

A regional distribution pattern of the GH and PRO cells was observed in the juvenile and adult rhesus monkey pituitaries which followed an expected evolutionary pattern. The GH cells predominated posteriorly, laterally and at the superior and

inferior poles of the pars distalis, while the PRO cells were mainly observed anteriorly, antero-inferiorly and medially. Neither cell type was restricted from the region in which the other predominated. Other investigators have observed a similar distribution of the orangeophils in man (278) and the rhesus monkey (56) as that reported in this study for the rhesus monkey. The distribution of GH cells in the capuchin monkeys as well as the orangutan and chimpanzee are also similar to that observed in the rhesus monkeys. Pasteels et al. (271) report finding PRO cells throughout the entire pars distalis of the adult rhesus monkeys while Daniel et al. (56) note that the cells which they assume to be the lactotrophs are present mainly in the antero-median regions of the rhesus monkey pituitary.

Some but not all of the regions in which the PRO cells predominated correlate with the zona tuberalis described in rhesus monkeys by Dawson in 1948 (63). This area is related to the pars tuberalis and merges with the latter in the superior regions of the rhesus monkey pituitary. The zona tuberalis is located in the anterior and antero-medial regions of the pars distalis. It receives the highest concentration of portal blood and is also the area in which the greatest change in cell population occurs (63). This suggests that the cells in the zona tuberalis are being influenced more by the hypothalamic hormones than the cells in other areas outside the zona tuberalis which are less vascularized. The zona tuberalis is also the area where Dawson observed the highest concentration of carminophils in the monkey, while the orangeophils are located more laterally and away from the zona tuberalis (63).

The fact that the PRO cells are located more anteriorly or rostrally than the GH

secreting cells in primates is a characteristic feature of the acidophils in most vertebrate species beginning with the elasmobranchs (150). In most submammalian vertebrates, the GH and PRO cells are restricted to separate regions of the pars distalis, which may be related to the development of the pituitary. In the submammalian vertebrates Rathkes pouch is constricted so that the pars distalis becomes divided into lobes, lobules or regions (155). Thus, the two acidophils become separated and are restricted to a given lobe or region of the pars distalis.

Only one acidophilic cell is observed in the cyclostomes or that cyclostome PRO is not immunochemically related to mammalian PRO (1). Two acidophils can be tinctorially differentiated in the elasmobranch (150) and teleost pituitaries (17, 31, 75, 78, 306). The carminophils, which have been shown by immunofluorescence in the teleosts to contain PRO (75, 78, 223, 226), are restricted to the rostral pars distalit while the orangeophils, which have been shown to contain GH in elasmobranchs (150) and teleosts (226) are found only in the proximal pars distalis just caudal to the rostral pars distalis. In the lungfish the carminophils are found in all regions of the pars distalis, while the orangeophils are located posteriorly (17, 172). These cells are presumed to secrete PRO and GH respectively (172, 336). A considerable amount of confusion exists regarding the histophysiology of the acidophils in the amphibians which has been brought about by the use of several different staining techniques and of nomenclature which was based on the tinctorial characteristics of the cells instead of their functional role (17). Two acidophilic cells have been differentiated in the anurans (64, 256, 342) and urodele (4, 49, 171, 344) amphibians, each of which may stain red or orange depending on the technique used.

It has been proposed that the more anteriorly located carminophils secrete GH and the more posteriorly located orangeophils secrete PRO (341). The only immunofluorescence study conducted on the amphibian pituitary indicates that in the newt, PRO is secreted by the acidophils which are located in the dorso-central region of the pars distalis. Two acidophils, an orangeophil and a carminophil, are present in the reptilian pituitoary. In most species, these cells are localized in the caudal and rostral regions, respectively, of the pars distalis (136, 209), although the opposite distribution has been reported in a few species of reptiles (88). Experimental evidence suggests that the carminophils secrete PRO (209); however, there have been no immunofluorescence studies to confirm these observations. Rahn and Painter (281, 282) have described a characteristic pattern of the acidophils which was noted in 18 different species of birds. They observed the orangeophils in the cephalic lobe and the carminophils in the caudal lobe of the pars distalis. Experimental data suggest that the cells located in the cephalic lobe secrete PRO while those found in the caudal lobe secrete GH (91, 272). McKeown (225) has localized PRO in the acidophils of the cephalic lobe of the pars distalis of the pigeon employing the fluorescent antibody technique with anti-ovine PRO serum. Only a few studies have been carried out on the regional distribution of the acidophils in mammals. In the rat, guinea pig (345) and gold hamster (53) there appears to be no regional distribution of the acidophils. Both cell types are located in all regions of the pars distalis. The orangeophils predominate in the lateral regions of the pituitary of the ox (324) and cat (62), while in the dog they are mainly observed laterally and antero-laterally (279). The carminophils in the pars distalis of the cat are found

towards the periphery of the gland (108), in the dog they are mainly located in the centro-median regions (279) and in the rabbit (2) they are found in the lateral and postero-central areas of the pituitary (2). In squirrels, the cells which are presumed to secrete GH are located postero-laterally and posteriorly while the presumed PRO-secreting cells are located anteriorly and laterally (67).

K. Speculations Concerning the Relations Between Growth Hormone and Prolactin Cells

There is some weak, indirect evidence to suggest that GH secreting cells can be transformed into functional lactotrophs, or vice versa, depending on the physiologic need of the organism. There seemed to be fewer GH cells in the pituitaries of the EB treated animals. The concentration of GH was lower in the steroid treated animals, while the concentration of pituitary PRO and the number of PRO cells increased. Fewer GH cells were also present in the pituitaries of the pregnant and lactating rhesus monkeys. The PRO cells in these same animals were so numerous that they appeared to outnumber all other cell types combined, thereby obliterating any regional distribution of cell types. The PRO cells in the pregnant and lactating pituitaries were present in large numbers in all regions of the pars distalis including those areas in which the GH cells were the predominant cell type in the nonpregnant, nonlactating rhesus monkey. Cell division alone probably cannot account for the marked hyperplasia of PRO cells during late pregnancy and lactation. Some of the lactotrophs could have arisen from chromophobes; however, it does not seem possible that cell differentiation could account for all the PRO cells observed. Dawson (61) observed a decrease in the number of

chromophobes and orangeophils in an ovariectomized female monkey treated with estrogen and testosterone, while the number of carminophils increased significantly. Friedgood and Dawson have observed fluctuations in the number of carminophils and orangeophils during the estrus cycle and throughout pregnancy and lactation in the pituitary of the rabbit (107) and cat (108). On the basis of the staining reactions of the two acidophils in the rabbit and cat pituitaries, they postulated that some of the carminophils may have evolved from pre-existing orangeophils. Transitional cell types which stain with both stains were not observed (107); however, they believe this may be due to limitations in the staining technique. Goluboff and Ezrin (127) report the presence of "mixed cells" which stain with both orange-G and carmoisine in pituitaries of pregnant and lactating humans. During pregnancy and lactation the number of GH cells decreases and the number of PRO cells increases in the human pituitary (127). Plasma GH levels are relatively low during pregnancy (325-327, 343, 357), lactation (25, 325-327, 343, 357), and patients with galactorrhea (25, 110, 163, 328), while plasma PRO levels are elevated (110, 163). The reverse is observed in some patients with acromegaly (163). MacLeod et al. (220) report that a reciprocal relationship exists between some factors which are known to effect GH and PRO secretion. They note that GH synthesis is decreased in rat pituitaries when estrogens, reserpine and perphenazine are given to the animal. PRO levels are increased by all three agents. Cyclic AMP elicits the opposite effect; it increases GH and decreases PRO synthesis. All of the aforementioned histologic, clinical and experimental findings suggest that a balance exists between the number of acidophils and the concentration of GH and PRO present

in the pituitary of mammals. It can be speculated that if some factor disturbs this balance and an increased demand for the secretion of one hormone is created by the animals, then the concentration of the second hormone decreases. Thus, if the demand is prolonged, i.e., pregnancy, the cell type producing the second hormone de-differentiates and re-differentiates into one which is synthesizing and secreting the first hormone. The most direct evidence to support this concept comes from the investigations of Tashjian et al. (332) who carried out studies on clonal strains of rat pituitary tumor cells in vitro. The clones, which were presumably derived from a single cell, are capable of synthesizing and secreting both GH and PRO. Even though these cells are tumor cells, these results strongly support the concept that a single acidophilic cell may be capable of secreting either hormone if given the appropriate stimulus.

There is some chemical evidence indicating that GH and PRO may have evolved from a common ancestral molecule. This concept is supported by several investigators (31, 32, 242, 250) based on polyacrylamide gel electrophoretic studies (250) and the analysis of the primary structure of several mammalian hormones (31, 32, 242, 315). Both GH and PRO activity have been found to be associated with the same band following polyacrylamide gel electrophoresis of PE's from some vertebrate species (250) as well as in some purified mammalian GH and PRO preparations (216, 250, 274). Bewley et al (31-33) and Niall et al. (242) have shown that the primary amino acid sequences of ovine GH, ovine PRO and human GH are structurally quite similar. A homology of 60% exists between the total amino acid residues in the latter two hormones (33). Niall et al. (242) speculate that the

primordial protein which may have given rise to the vertebrate GH's and PRO's must have been a very small peptide which closely resembled PRO. It is possible that this primitive, ancestral molecule may be present in the cyclostome pituitary. Only one acidophilc cell type has been described in the cyclostomes (90, 91). The hormone in this cell does not cross-react with anti-ovine PRO serum (1) which suggests that it is either a GH or a PRO which is not immunochemically related to ovine PRO. There is some experimental evidence suggesting that a hormone with PRO-like activity is present in cyclostomes (299). Since PRO has been reported to promote somatic growth in some vertebrates (28, 247), it is possible that the hormone present in the acidophils of cyclostomes is PRO which has growth-promoting activity. This hormone may then be the ancestral molecule from which all vertebrate GH's and PRO's have descended. Thus, through evolution two hormones GH and PRO have evolved which have notable similarities in their molecular structure and their biological activities. They are secreted by two separate cell types, which are both categorized as acidophils; however, these tinctorially different cell types may have the ability to alter their physiology and secrete either GH or PRO depending on the needs and demands of the animal. If one speculates further, the orangeophil and the carminophil may actually represent different functional states of a single cell type.

SUMMARY

The principle objective of this dissertation was to demonstrate whether or not growth hormone (GH) and prolactin (PRO) are present in the primate pituitary gland as two separate and distinct hormone molecules. Morphological, immunochemical and biological assays and techniques were employed during the course of this investigation. The main findings and results of this study can be summarized as follows:

1. Highly purified ovine PRO is antigenic in guinea pigs, rats, rabbits, and monkeys. Considerable intra- and interspecies variation in antibody response can be observed among these host animals.
2. The immunochemical relatedness of the vertebrate PRO's did not show correlation with the phylogenetic classification of the respective vertebrate species.
3. A significant amount of immunochemical species specificity exists among the mammalian PRO's, and between nonprimate and primate GH's.
4. Highly purified ovine PRO is partially related immunochemically to the monkey PRO contained within primate pituitary extracts (PE's), while highly purified human GH is immunochemically identical to the monkey GH contained within the primate PE's. No immunochemical relatedness was demonstrable between ovine PRO and human GH.
5. Two tinctorially different acidophilic cell types, an orangeophil and a carminophil, can be differentiated in the pituitary gland of the rhesus, cynomolgus and capuchin monkeys, as well as in the gorilla, chimpanzee and the orangutan. These two cell types appear to comprise all of the acidophilic cells present in the primate pituitary.

6. The acidophils predominate in separate regions of the primate pituitary. The orangeophils are principly located posteriorly, laterally, and at the superior and inferior poles of the pars distalis, while the carminophils are observed anteriorly, antero-inferiorly and medially. This topographical distribution of the acidophilic cells follows a typical evolutionary pattern.
7. In all species of primates studied, the orangeophils are identified as the somatotrophs and the carminophils as the lactotrophs.
8. The number of lactotrophs and the concentration of PRO in the pituitary of juvenile rhesus monkeys of either sex are negligible. Marked increases in both the number of PRO cells and the concentration of PRO occurs in the postpubertal adult male and female rhesus monkey, which suggests that the presence of PRO in adult rhesus monkeys may be related to the circulating levels of sex steroids.
9. The lowest concentration of pituitary GH is present in male and female juvenile rhesus monkeys, while the highest levels are observed in the adult females.
10. It is estimated that the lactotrophs seem to occupy 60-80% of the total area of the pars distalis during late pregnancy and lactation in the rhesus monkey. The number of somatotrophs in these same animals appears to be less than in normal adult female rhesus monkeys.
11. Administration of estradiol benzoate to intact juvenile female rhesus monkeys stimulates the synthesis and secretion of pituitary PRO and inhibits the synthesis of pituitary GH. The concentration of PRO and the number of lactotrophs observed in this animal was equal to or greater than that observed in normal adult females.

12. Primate PRO present within rhesus monkey PE's as well as highly purified ovine PRO and purified human GH are capable of stimulating mouse mammary tissue in vitro .

APPENDIX

A. Processing of Pituitaries for Histologic Examination

1. Tissues fixed for 4-6 hrs. in 10% neutral buffered formalin.
2. Dehydrated in alcohol
   a) overnight in 70%.
   b) 30 minutes in 80%.
   c) 30 minutes in each of two changes of 90%.
   d) 1-2 hrs. in each of three changes of absolute alcohol.
3. Alcohol cleared with two 30-60 min. changes of cedar wood oil and stored overnight in a third change of cedar wood oil.
4. Infiltrated under vacuum with three changes of paraffin (45 min. each).
5. Embedded in paraffin wax blocks.
6. Sectioned at 5-6 μ.

B. Light Microscopic Staining Procedure - Modified from L.D. Brookes (36).

1. Sections de-paraffinized with two changes of xylene and hydrated through decreasing concentrations of alcohol to water.
2. 30 min. in 1% Carmoisine L (E. Gurr, Ltd.) in 1% acetic acid.
3. Rinsed in distilled water.
4. 1-2 min. in 95% alcohol.
5. 5 min. in 0.5% orange-G (Allied Chemical Corp.) in 2% phosphotungstic acid in 95% alcohol (agitated 2-3 times).
6. Rinsed in 2% phosphotungstic acid in 95% alcohol followed by a rinse through four changes of distilled water.

7. 4 min. in 1% Carmoisine L in 1% acetic acid.
8. Rinsed in distilled water.
9. 10 min. in 0.5% Wool Green S (E. Gurr, Ltd.) in 0.5% acetic acid.
10. Rinsed in distilled water.
11. 2 min. in 1% acetic acid.
12. 1-2 min. in each of three changes of absolute alcohol.
13. 5 min. in each of two changes of xylol before mounting.

C. Fluorescent Antibody Technique

1. Sections stained using the aforementioned light microscopic staining procedure and photographed.
2. Coverslip removed with xylol and slides rinsed in two changes of xylol.
3. Rinsed in absolute, 95% and 80% alcohol.
4. 1 hr. in 2% HCL-70% alcohol to remove the light microscopic stain.
5. 5 min. in 70% alcohol.
6. Rinsed in distilled water.
7. 15 min. in 1000 ml of a 0.02 M phosphate-buffered saline solution pH 7.4 (800 ml m/15 disodium phosphate, 200 ml M/15 potassium phosphate, 2400 ml normal saline) using a magnetic stirrer (repeated twice).
8. Slide placed in moise chamber with a drop (15-20 µl) of anti-hormone serum placed directly over the tissue section for 1 hr. at room temperature. Serum was generally diluted to a pre-determined concentration with saline (usually between 1:10 and 1:20).
9. 20 min. in 1000 ml of 0;02 M phosphate-buffered saline solution pH 7.2 using a magnetic stirrer.

10. Slide placed in moist chamber with drop of anti-gamma globulin serum labelled with fluorescein isothiocyanate placed directly over the tissue section for 1 hr. at room temperature (conjugate was filtered through a Millipore swinny adapter and diluted to a pre-determined concentration with saline (usually between 1:5 and 1:10).
11. 20 min. in 1000 ml of 0.02 M phosphate-buffered saline solution pH 7.4 using a magnetic stirrer.
12. Mounted using a glycerine: phosphate-buffered saline mounting medium, ratio 9:1, and covered with a #1½ coverslip (Corning Co.).

Figure 1. Agar gel study with guinea pig #8 antiserum to ovine PRO showing the immunochemical relatedness of ovine PRO and the PRO in several rhesus monkey PE's. The center well contains 150 µl of the antiserum, while the peripheral wells contain 7.5 µg of ovine PRO (SPRO), and 2 mg of each primate PE except the lactating female PE which represents 1 mg. A precipitin reaction is present only from the adult female and the lactating female PE's. The latter is giving a reaction of partial identity with ovine PRO. Photographed at 28 hrs.

Figure 2. Agar gel study showing precipitin reactions observed between guinea pig #8 anti-ovine PRO serum and PE's from pregnant and lactating rhesus females. A volume of 150 µl of the antiserum is located in the center well. The peripheral wells contain 10 µg of ovine PRO (SPRO) and 1 mg of either the pregnant or lactating PE. A precipitin line is present from all three primate PE's which is giving a reaction of partial identity with ovine PRO. Photographed at 24 hrs.

Figure 3. Agar gel study showing the lack of cross-reaction observed between guinea pig #8 anti-ovine PRO serum and purified human GH. The center well contains 150 µl of the antiserum, while the peripheral wells contain 7.5 µg of ovine PRO (SPRO) and three concentrations of human GH (HGH), 50 µg, 100 µg, and 200 µg. No precipitin reaction is present from any of the three concentrations of human GH. Photographed at 48 hrs.


JUVENILE ♂
ADULT ♀
LACTATING ♀
ADULT ♂
SPRO

1


PREGNANT ♀
SPRO
SPRO
LACTATING ♀
SPRO
PREGNANT ♀

2


HGH 100μg
HGH 200μg
HGH 50μg
SPRO 7.5μg

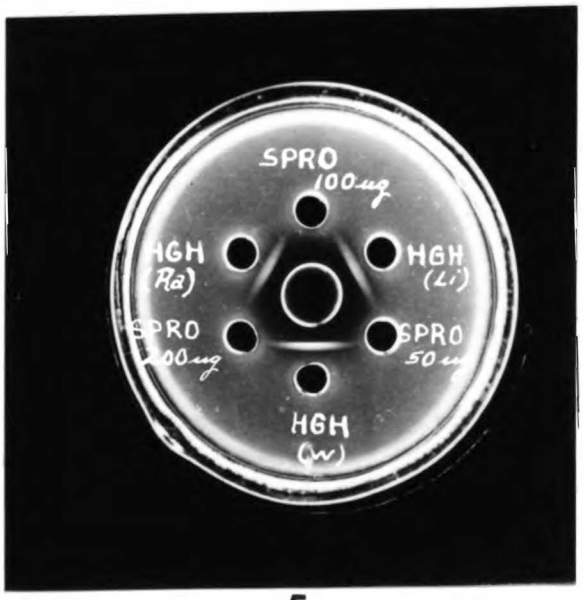
3

Figure 4. Agar gel study showing the immunochemical relatedness of human GH and GH in each of the four rhesus monkey PE pools. The center well contains 150 µl of guinea pig #30 antiserum to human GH. The peripheral wells contain 10 µg of human GH (HGH) and 0.75 mg of the primate PE pools except the adult femalt PE pool which represents 0.40 mg. All of the PE pools are showing precipitin reactions. The lines from the PE pools are giving a reaction of qualitative identity with each other and with purified human GH. Photographed at 28 hrs.

Figure 5. Agar gel study showing the lack of cross-reaction observed between guinea pig #5 anti-human GH serum and purified ovine PRO. A volume of 150 µl of antiserum is located in the center well, while 50 µg, 100 µg, and 200 µg of ovine PRO (SPRO) are present in the peripheral wells. Also located in the peripheral wells are three preparations of human GH (HGH), Wilhelmi #HS-1394 (W), Li #3477A (Li), and Raben #18 (Ra), each representing 5 µg of the hormone. No precipitin reaction is present from any of the three concentrations of ovine PRO. A precipitin line is observed from each of the human GH preparations. Photographed at 48 hrs.


ADULT ♂
JUVENILE ♀
ADULT ♀
JUVENILE ♂
HGH

4


SPRO 100 ug
HGH (Ra)
HGH (Li)
SPRO 200 ug
SPRO 50 ug
HGH (Gw)

5

Figure 6. Composite of several radioimmunoassay studies showing the degree of cross-reactivity of purified mammalian pituitary hormones and selected vertebrate PE's in a homologous ovine PRO assay system.

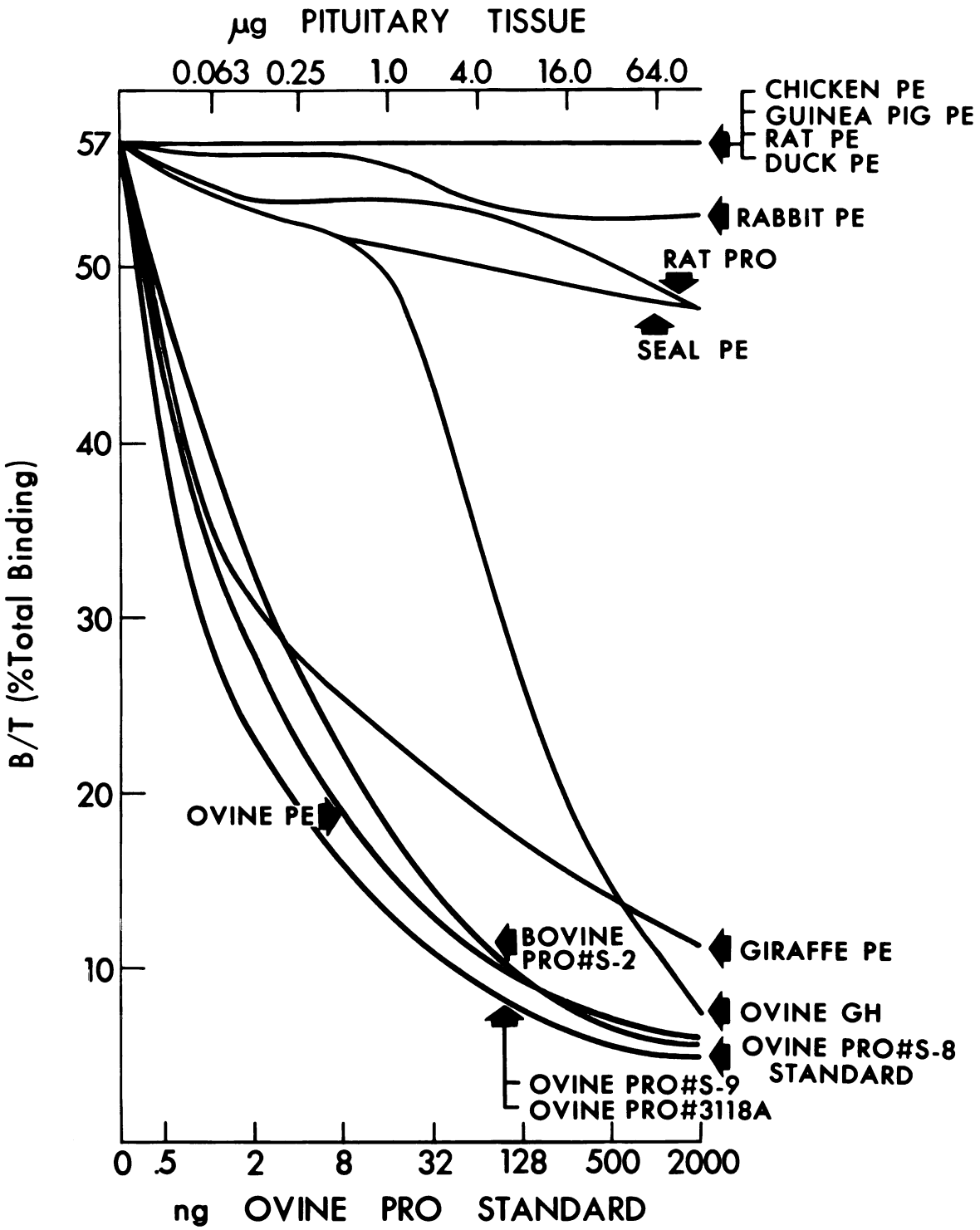

µg PITUITARY TISSUE
0.063 0.25 1.0 4.0 16.0 64.0
CHICKEN PE
GUINEA PIG PE
RAT PE
DUCK PE
RABBIT PE
RAT PRO
SEAL PE
OVINE PE
BOVINE PRO#S-2
GIRAFFE PE
OVINE GH
OVINE PRO#S-8 STANDARD
OVINE PRO#S-9
OVINE PRO#3118A
B/T (%Total Binding)
57
50
40
30
20
10
0 .5 2 8 32 128 500 2000
ng OVINE PRO STANDARD

Figure 7. Radioimmunoassay studies showing the immunochemical relatedness of PRO in several rhesus monkey PE's to each other and to purified ovine PRO in a homologous ovine PRO assay system. The curves from the primate PE's represent the mean response obtained from individual animals comprising each of the six groups.

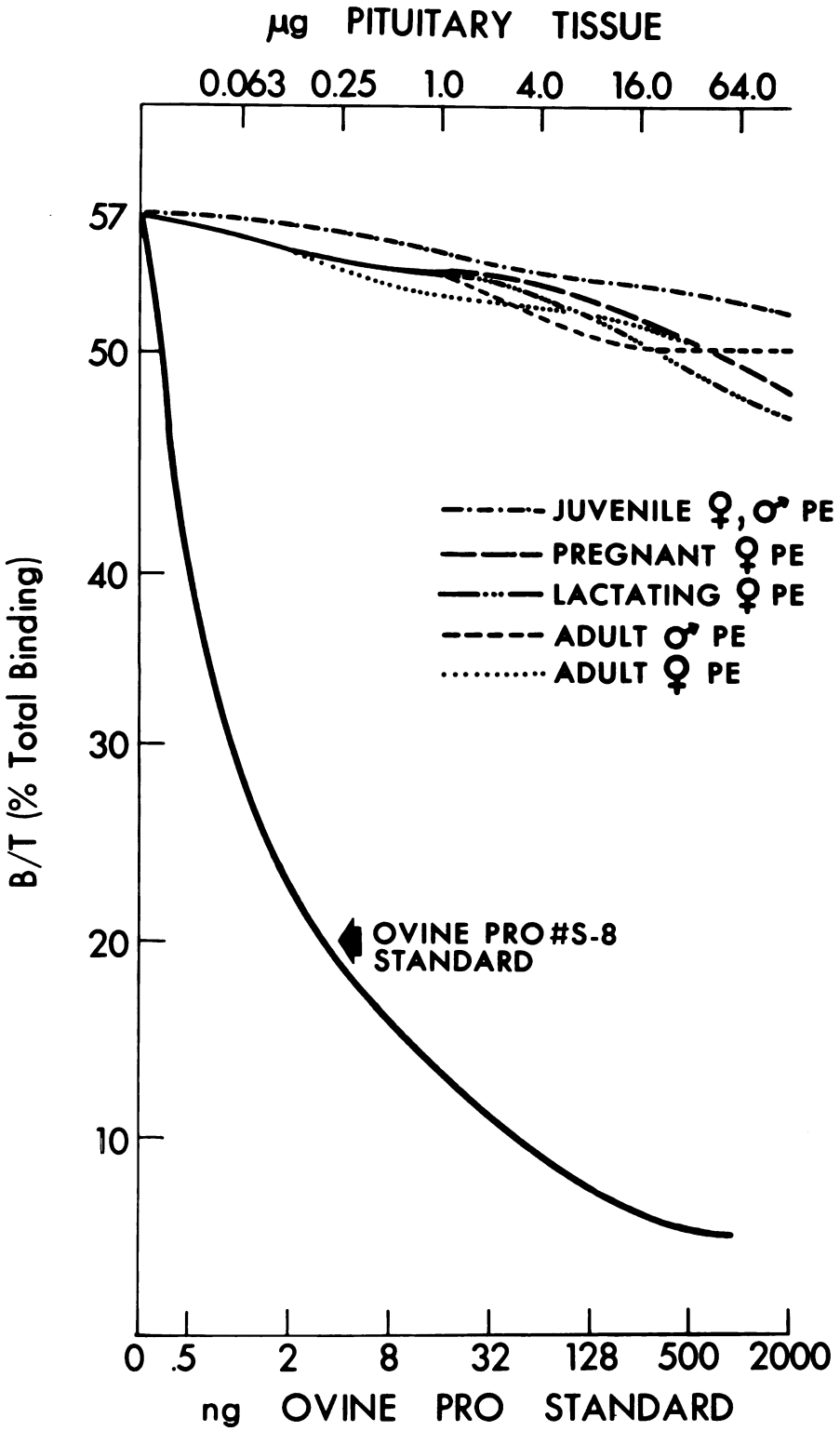

µg PITUITARY TISSUE
0.063 0.25 1.0 4.0 16.0 64.0
57
50
40
30
20
10
B/T (% Total Binding)
JUVENILE ♀, ♂ PE
PREGNANT ♀ PE
LACTATING ♀ PE
ADULT ♂ PE
ADULT ♀ PE
OVINE PRO #S-8 STANDARD
0 .5 2 8 32 128 500 2000
ng OVINE PRO STANDARD

Figure 8. Radioimmunoassay studies showing the immunochemical relatedness of several purified human and monkey GH preparations to each other and to purified ovine PRO in a homologous ovine PRO assay system.

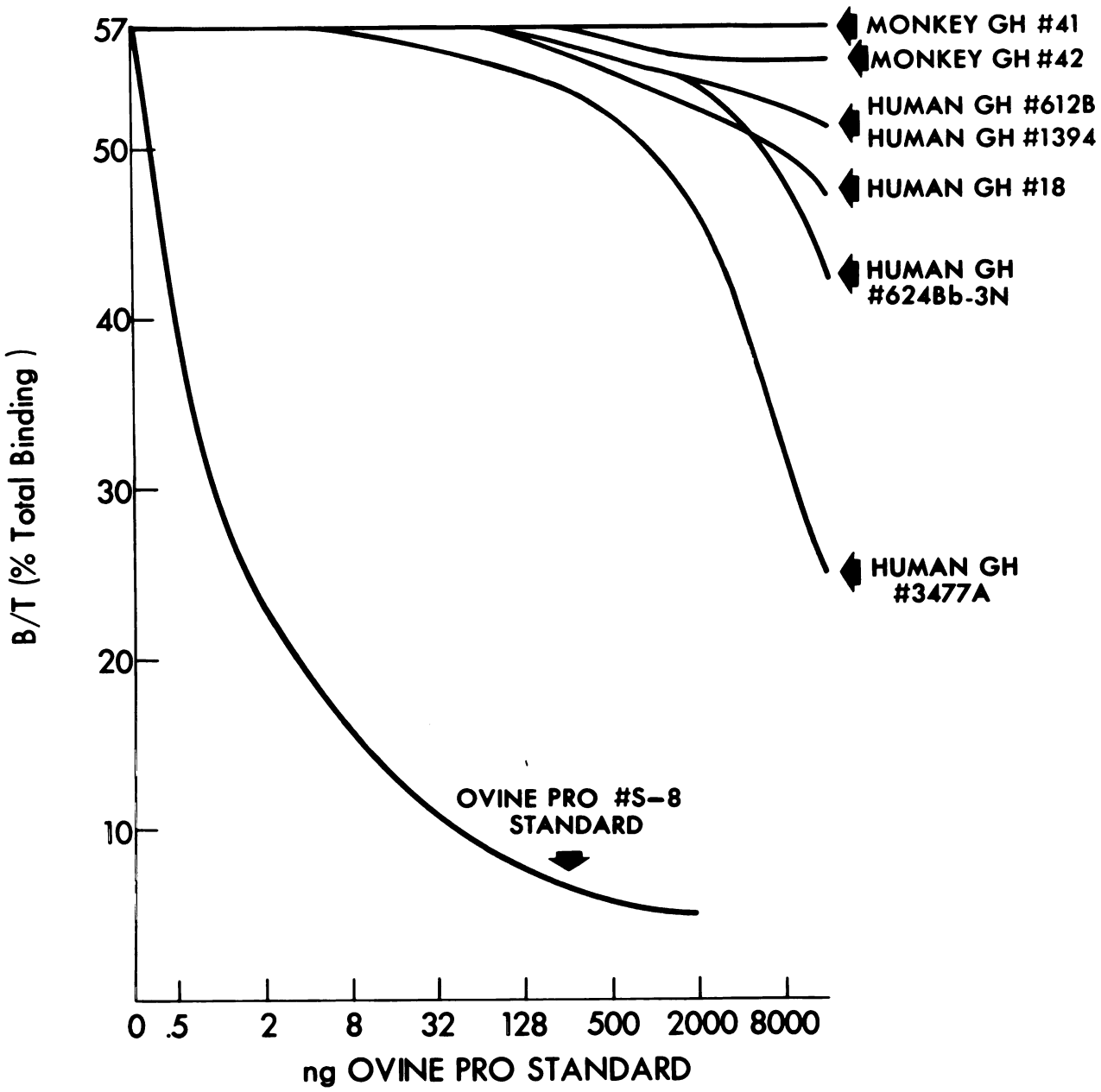

MONKEY GH #41
MONKEY GH #42
HUMAN GH #612B
HUMAN GH #1394
HUMAN GH #18
HUMAN GH #624Bb-3N
HUMAN GH #3477A
OVINE PRO #S-8 STANDARD
B/T (% Total Binding)
57
50
40
30
20
10
0 .5 2 8 32 128 500 2000 8000
ng OVINE PRO STANDARD

Figure 9. Radioimmunoassay studies showing the extent of cross-reaction of the four rhesus monkey PE pools, several purified human GH preparations and purified ovine PRO in a heterologous human PRO assay system. The results are shown by logarithmic logistic probability transformation.

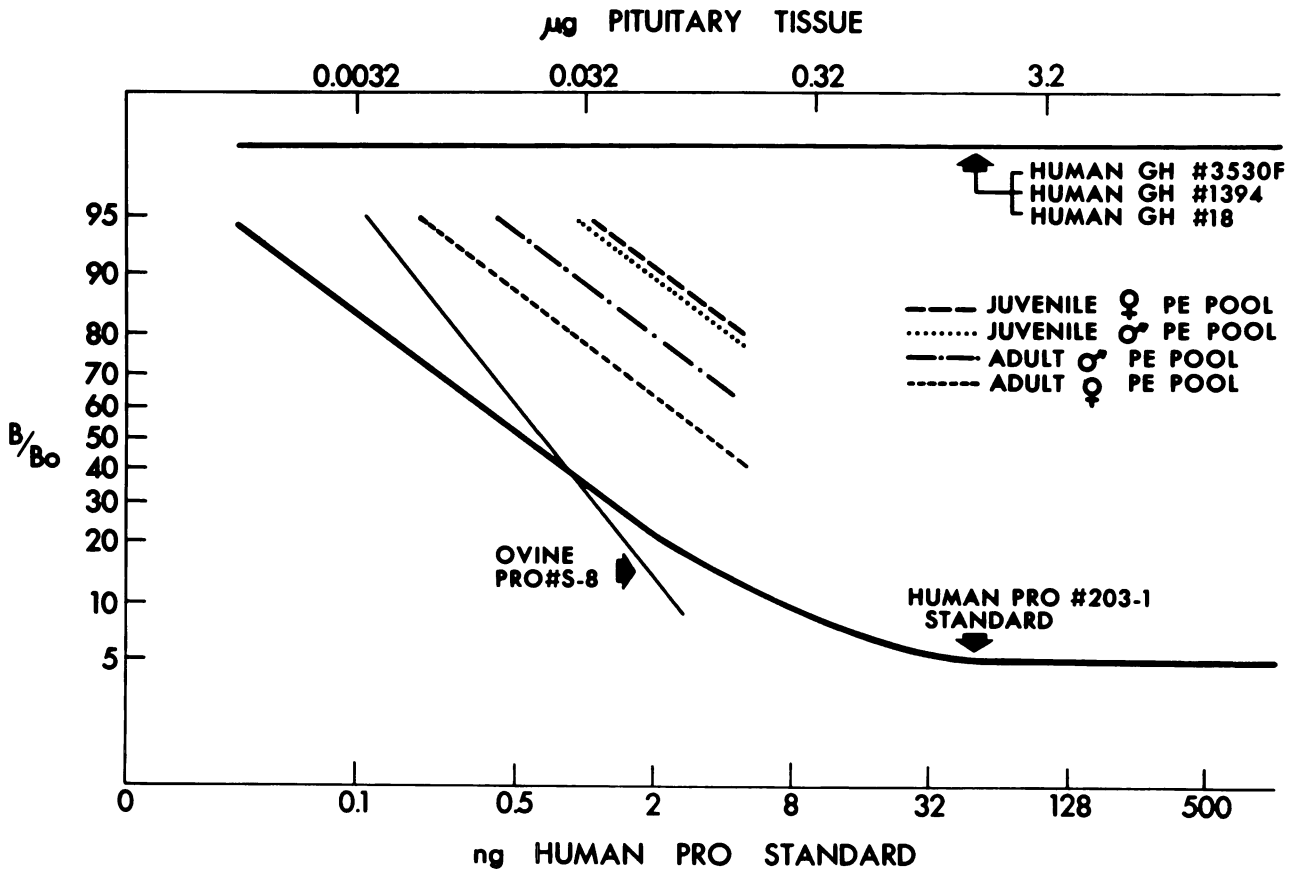

µg PITUITARY TISSUE
0.0032
0.032
0.32
3.2
HUMAN GH #3530F
HUMAN GH #1394
HUMAN GH #18
JUVENILE ♀ PE POOL
JUVENILE ♂ PE POOL
ADULT ♂ PE POOL
ADULT ♀ PE POOL
B/Bo
95
90
80
70
60
50
40
30
20
10
5
OVINE PRO#S-8
HUMAN PRO #203-1 STANDARD
0
0.1
0.5
2
8
32
128
500
ng HUMAN PRO STANDARD

Figure 10. Radioimmunoassay studies showing the immunochemical relatedness of several purified human and monkey GH preparations to each other and to selected GH and PRO preparations from a few subprimate mammalian species in a homologous human GH assay system.

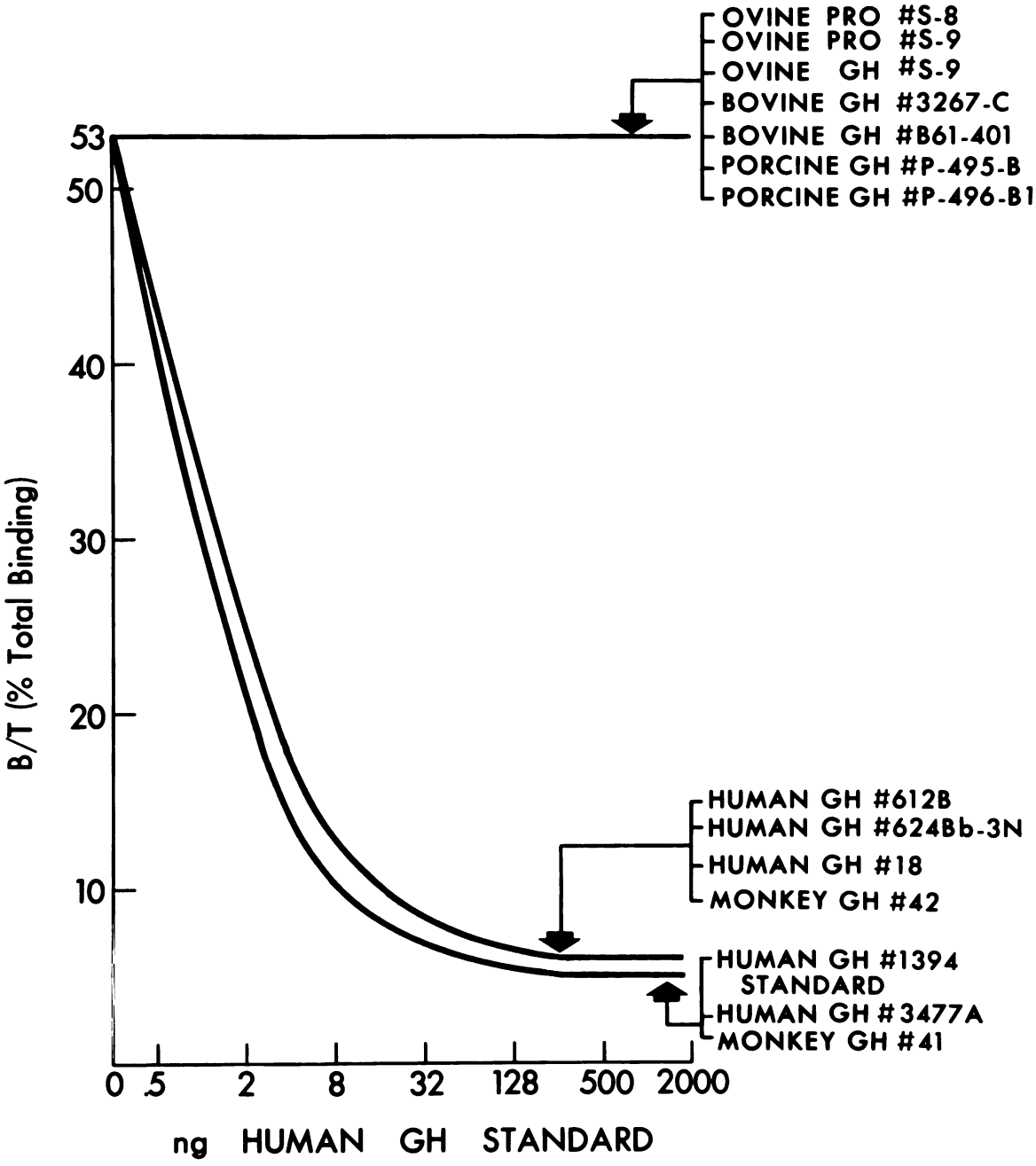

OVINE PRO #S-8
OVINE PRO #S-9
OVINE GH #S-9
BOVINE GH #3267-C
BOVINE GH #B61-401
PORCINE GH #P-495-B
PORCINE GH #P-496-B1
53
50
40
30
20
10
B/T (% Total Binding)
HUMAN GH #612B
HUMAN GH #624Bb-3N
HUMAN GH #18
MONKEY GH #42
HUMAN GH #1394
STANDARD
HUMAN GH #3477A
MONKEY GH #41
0 .5 2 8 32 128 500 2000
ng HUMAN GH STANDARD

Figure 11. Ratioimmunoassay studies showing the extent of cross-reactivity of PRO in the four rhesus monkey PE pools in a homologous human GH assay system. The results are shown by logarithmic logistic probability transformation.

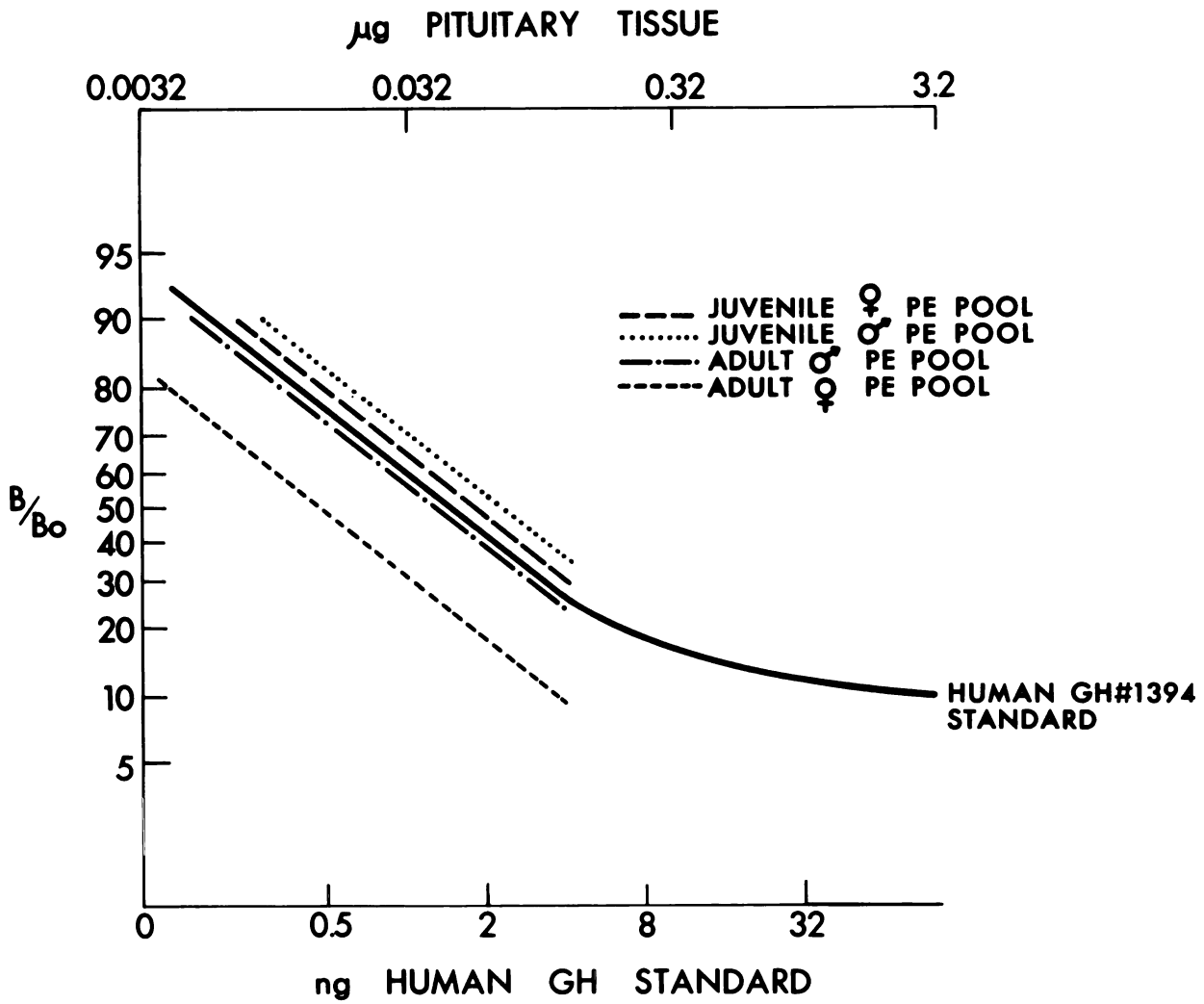
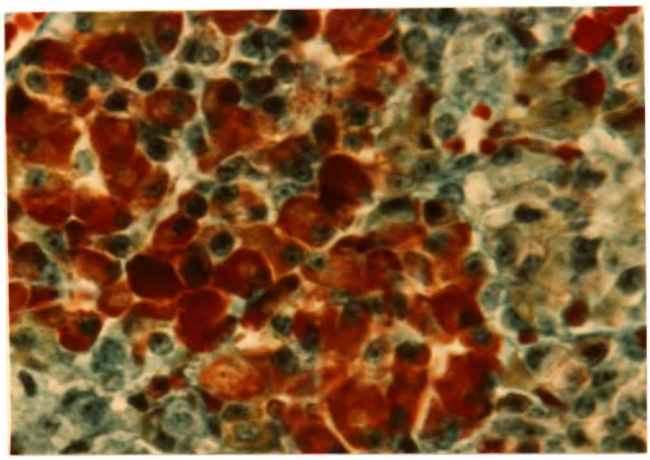
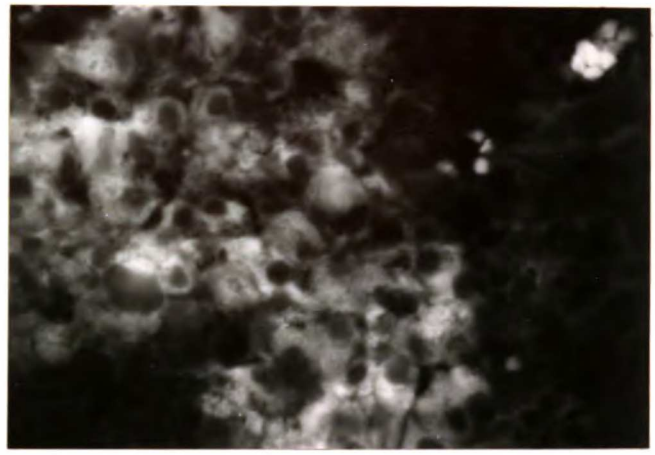

µg PITUITARY TISSUE
0.0032
0.032
0.32
3.2
95
90
80
70
60
50
40
30
20
10
5
B/Bo
JUVENILE ♀ PE POOL
JUVENILE ♂ PE POOL
ADULT ♂ PE POOL
ADULT ♀ PE POOL
HUMAN GH#1394 STANDARD
0
0.5
2
8
32
ng HUMAN GH STANDARD

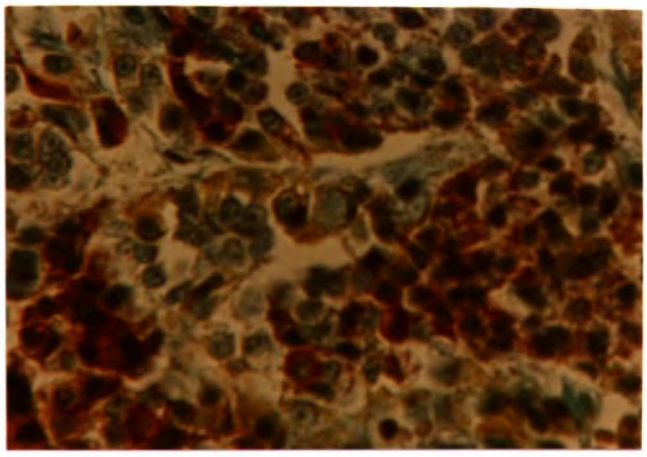
Figure 12. A and C are light photomicrographs showing the orangeophils and the large and small sized carminophils in a section from an adult female and an adult male rhesus monkey pituitary, respectively. B is a fluorescence micrograph of the identical areas as shown in A following immunofluorescent staining with anti-ovine PRO serum. Only the carminophils are shown to fluoresce identifying them as the PRO secreting cells. Magnification 650X.



A

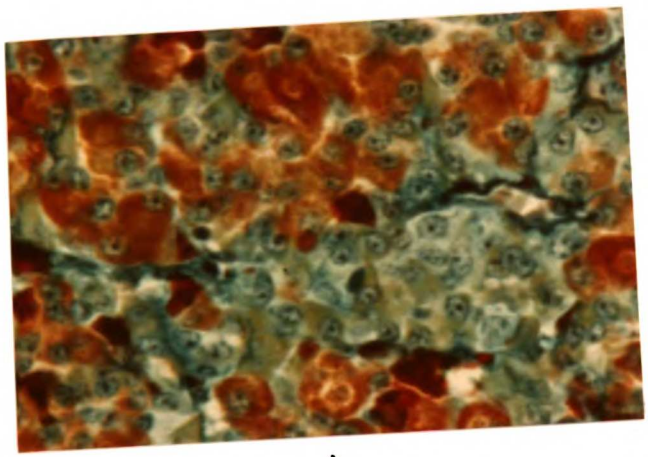


B

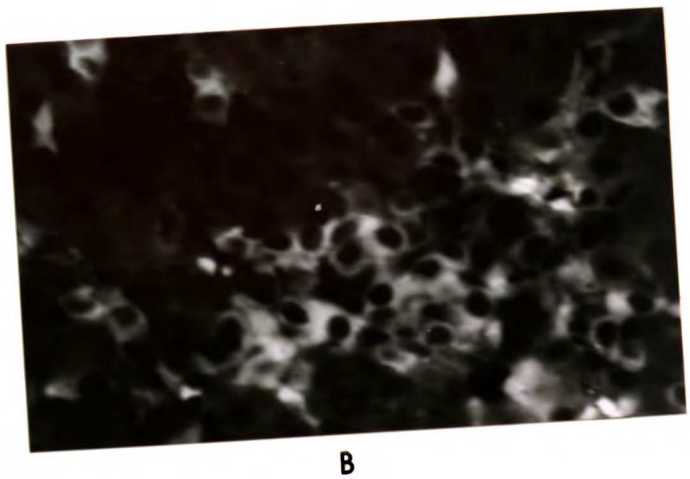


C

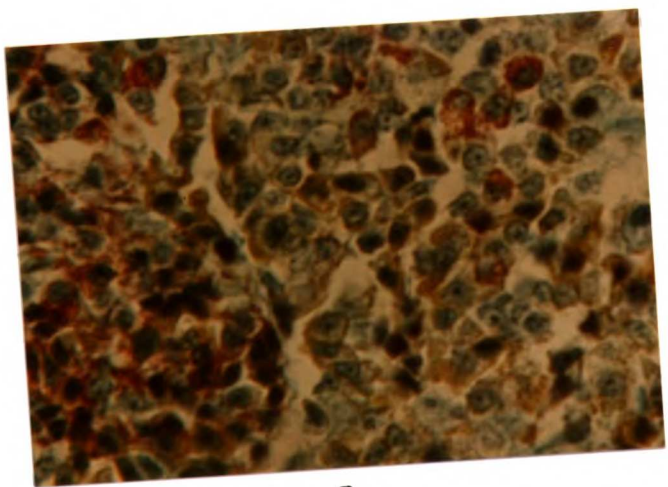
Figure 13. A and C are light photomicrographs taken from a section of an adult female and an adult male rhesus monkey pituitary respectively showing the orangeophils and the large and small sized carminophils. B is a fluorescence micrograph of the identical area as shown in A after immunofluorescent staining using anti-human GH serum. Only the orangeophils are fluorescing identifying them as the GH secreting cells. Magnification 650X.



A

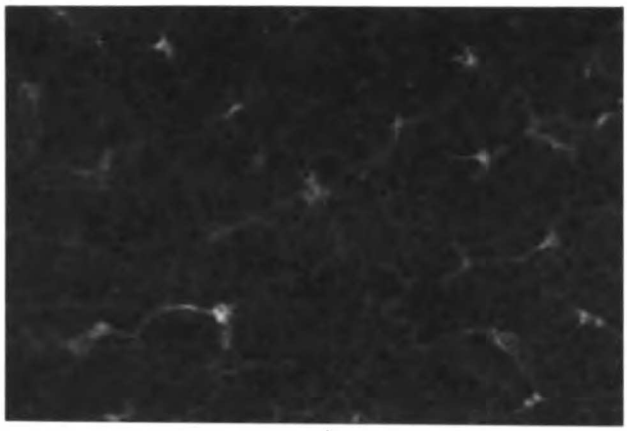


B

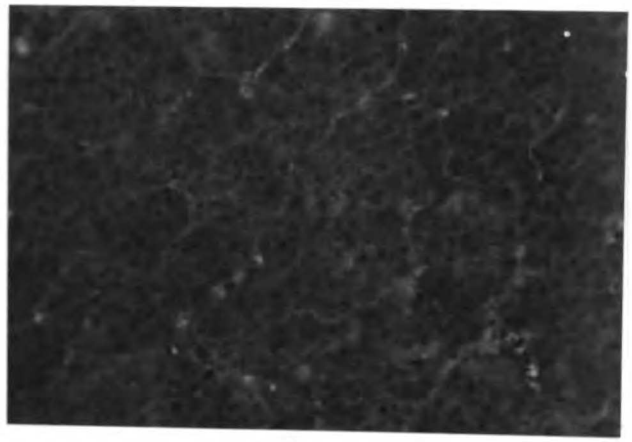


C

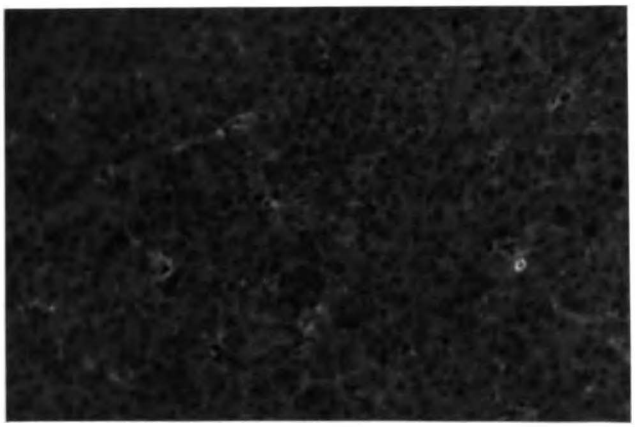
Figure 14. Five fluorescence micrographs showing each of the control experiments outlined in Table I. No specific cellular fluorescence is present in any of the micrographs. Red blood cells and connective tissue fibers are the only brightly fluorescent structures present in the micrographs. A shows the degree of autofluorescence present in pituitary tissue. B shows the amount of nonspecific fluorescence induced by the fluorescein-labelled conjugate alone. C shows the amount of nonspecific fluorescence induced by normal guinea pig serum. D shows the amount of fluorescence following preincubation of the first antibody with its homologous antigen (anti-PRO serum preincubated with purified ovine PRO). E shows the amount of fluorescence following preincubation of the first antibody with unlabelled second antibody (preincubation of guinea pig anti-human GH serum with anti-guinea pig gamma globulin serum). Magnification 300X.



A

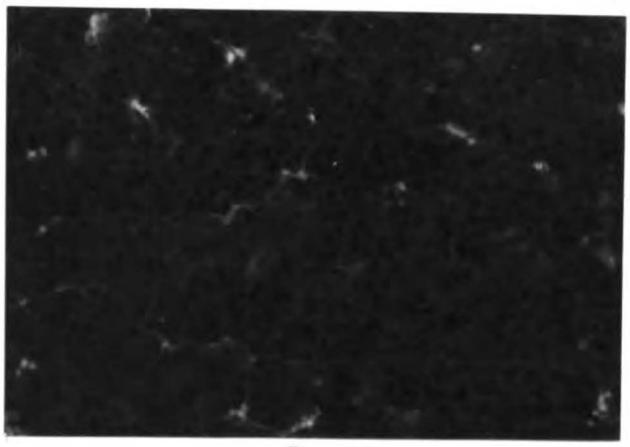


B



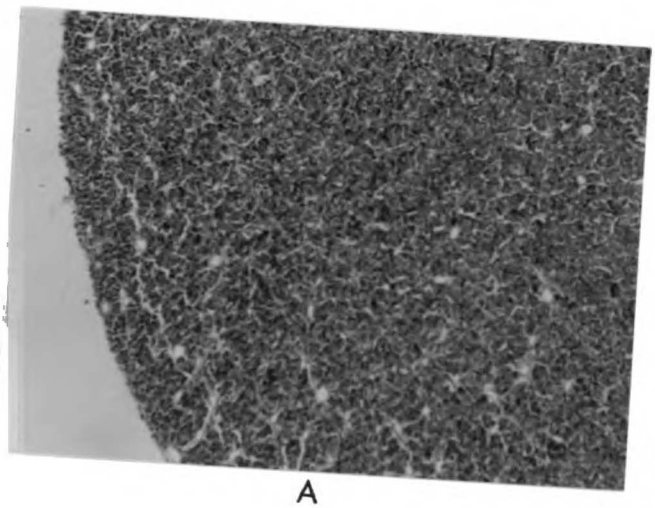
C

D

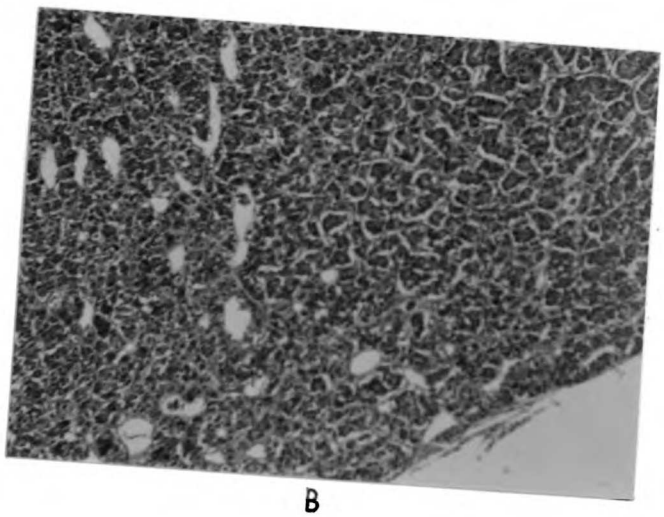


E

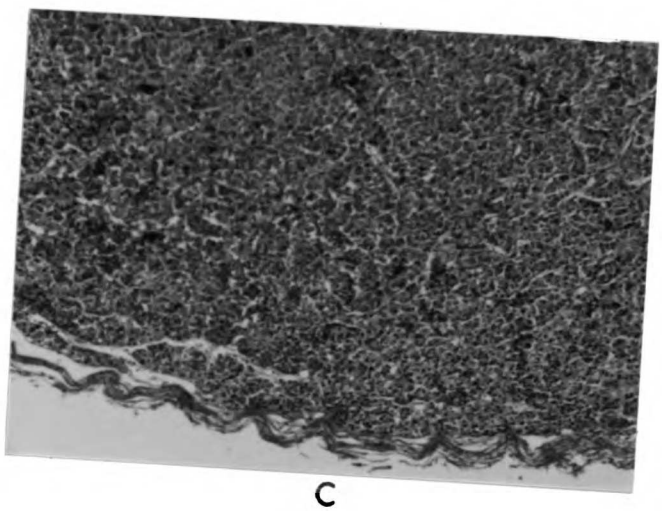
Figure 15. Light photomicrographs illustrating the rating system used to score the relative number of PRO cells present in the primate pituitary gland. A is a section from a juvenile female rhesus monkey with a rating of 0, B is from an adult female with a rating of 1+, C is from an adult male with a rating of 2+, D is from an adult male with a rating of 3+, and E is a section from an adult female with a rating of 4+. Magnification 100X.



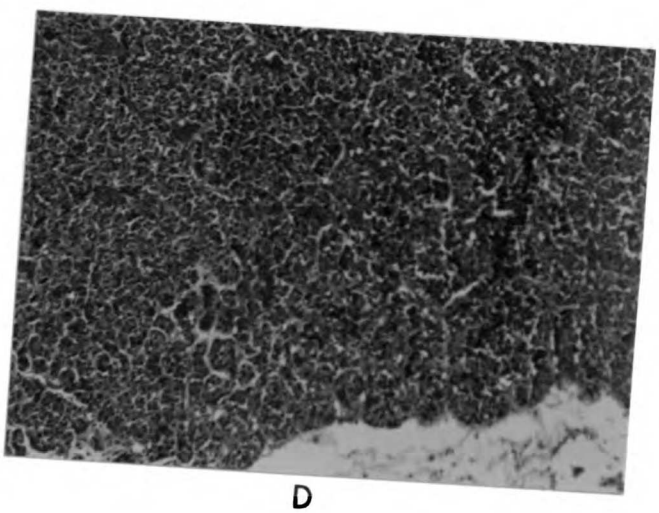
A



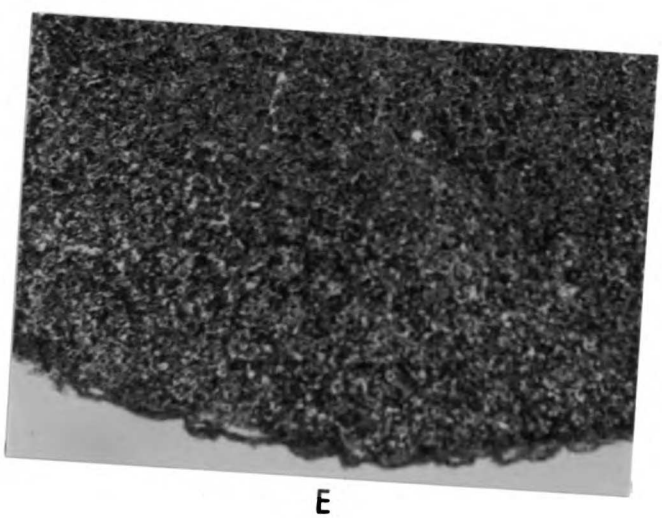
B



C

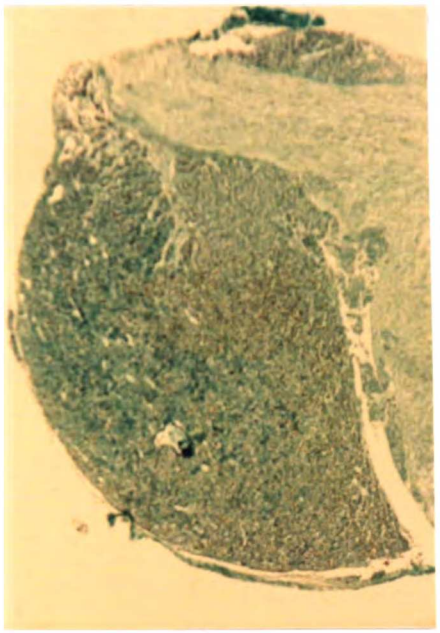


D

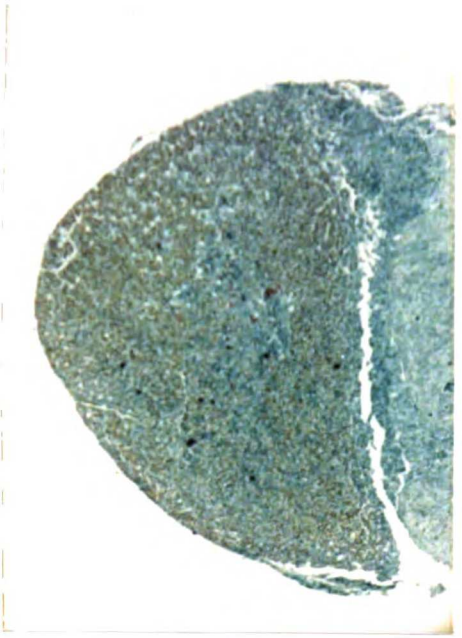


E

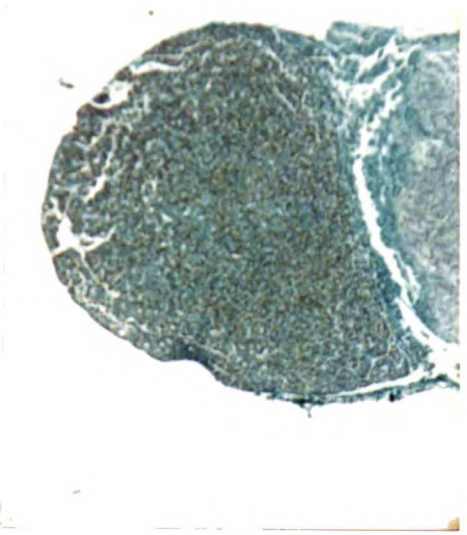
Figure 16. Light photomicrographs of three sagittal sections from a juvenile male rhesus monkey. The sections are oriented with the superior pole towards the top of the photograph and the anterior portion of the gland facing towards the left side of the micrograph. The three sections represent areas near the midline, about half the distance from the midline and near the lateral pole of the pars distalis, respectively. Only a few PRO cells are observed in the anterior (A) and medial regions (B) of the anterior lobe. a rating of 0 was given to the number of PRO cells present in the pituitary. GH cells are quite abundant in all regions of the pituitary except in the anterior (A) and medial (A and B) regions. Magnification 32X.



A

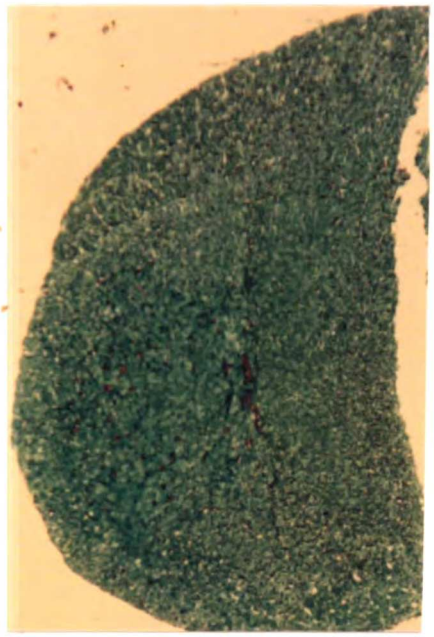


B

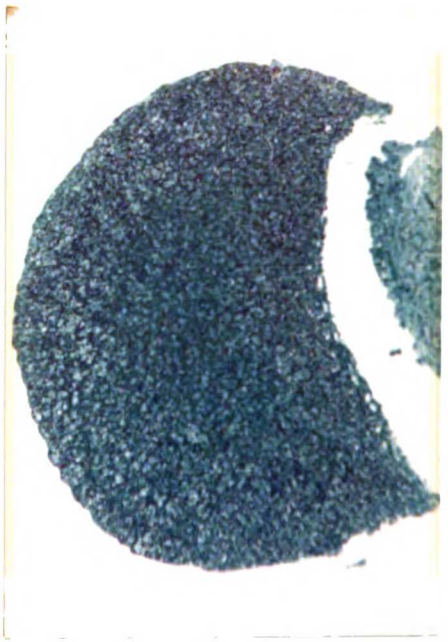


C

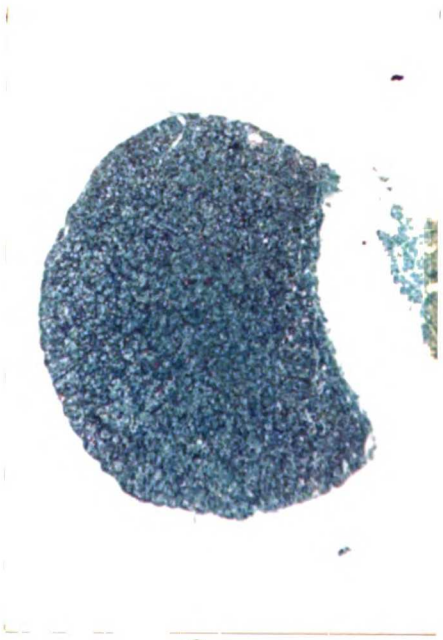
Figure 17. Light photomicrographs from three sagittal sections of a juvenile female rhesus, with the orientation and levels of the sections as in Figure 16. Only a few PRO cells are present in the antero-inferior (A) and medial (B) regions of the anterior lobe. The rating of the number of PRO cells, along with the distribution of the GH cells is identical to that observed in the juvenile male (Fig. 16). Due to technical difficulties, the GH cells are not well illustrated in these micrographs. Their distribution resembled the pattern observed in the juvenile male rhesus monkey as illustrated in Figure 16. Magnification 40X.



A



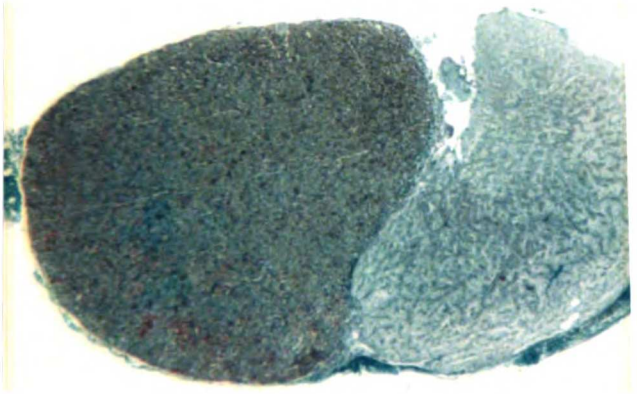
B



C

Figure 18. Light photomicrographs of three sagittal sections from an adult male rhesus monkey, with a rating of 2+ for the number of PRO cells present. The orientation and level of the sections are the same as in Figure 16. The PRO cells are quite abundant in the anterior, antero-inferior and medial regions of the pars distalis. Intermixing of the GH and PRO cells is noted in the extreme anterior (A-C), inferior and lateral (C) regions. The GH cells predominate in the posterior, lateral, inferior and superior regions of the anterior lobe. Magnification 20X.

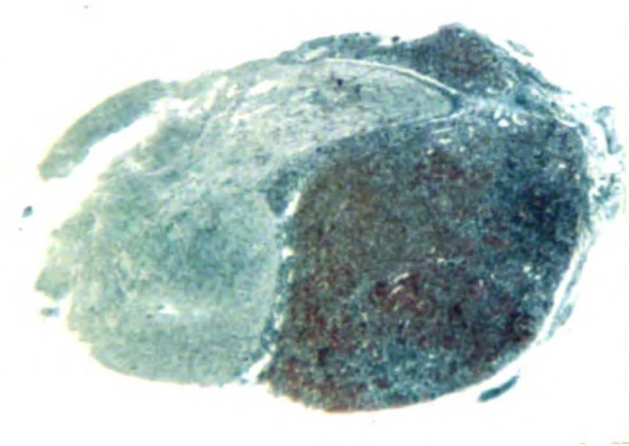
A



B

C

Figure 19. Light photomicrographs of three sagittal sections from an adult female rhesus monkey. The orientation and level of the sections are the same as in Figure 16 except that the anterior region of the pituitary is facing the right side of the micrograph. More PRO cells were observed in this adult female than in any of the other normal rhesus monkeys. The PRO cells are present in nearly every region of this female pituitary except in the more extreme posterior and superior regions. A rating of 4+ was given to the number of PRO cells present. Intermixing of the GH and PRO cells occurs mostly postero-inferiorly, posteriorly and laterally. GH cells predominate in similar regions as indicated for the adult male in Figure 18. Magnification 20X.



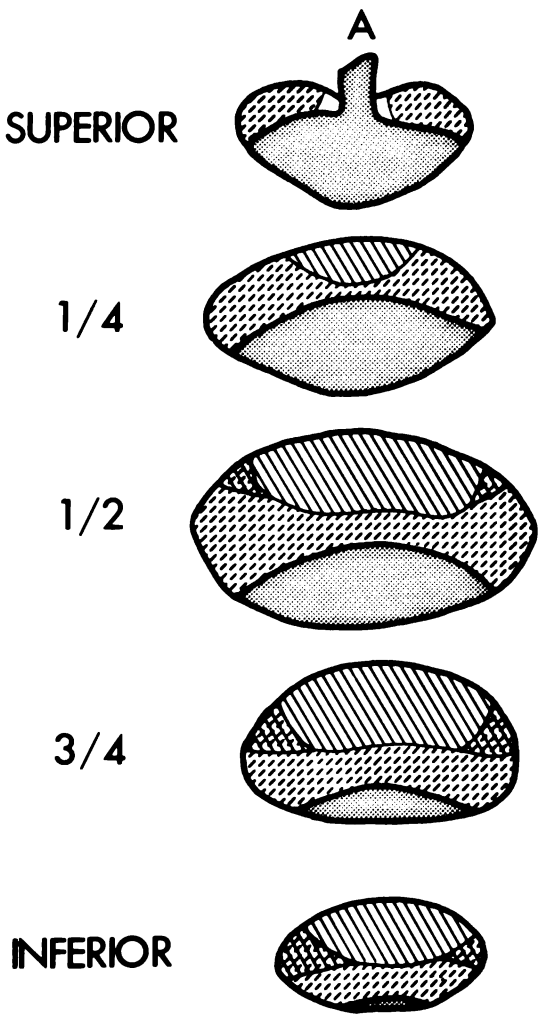
A

B

C

Figure 20. The areas in which the two acidophils, the PRO and GH cells predominate are illustrated in horizontal and sagittal sections. The areas shown by the diagonal and broken lines are the regions in which the lactotrophs and somatotrophs, respectively, are observed most frequently. The region in which both the diagonal and broken lines are located represent areas where the greatest intermixing of the PRO and GH cells occurs. The intermediate and posterior lobes are shown by the stippled areas. PRO cells predominate in the anterior, antero-inferior, and medial regions while the GH cells predominate in the posterior, lateral, superior and inferior regions of the anterior lobe. Intermixing of the two cell types occurs mainly in the inferiór and lateral regions.

HORIZONTAL SECTIONS



SAGITTAL SECTIONS

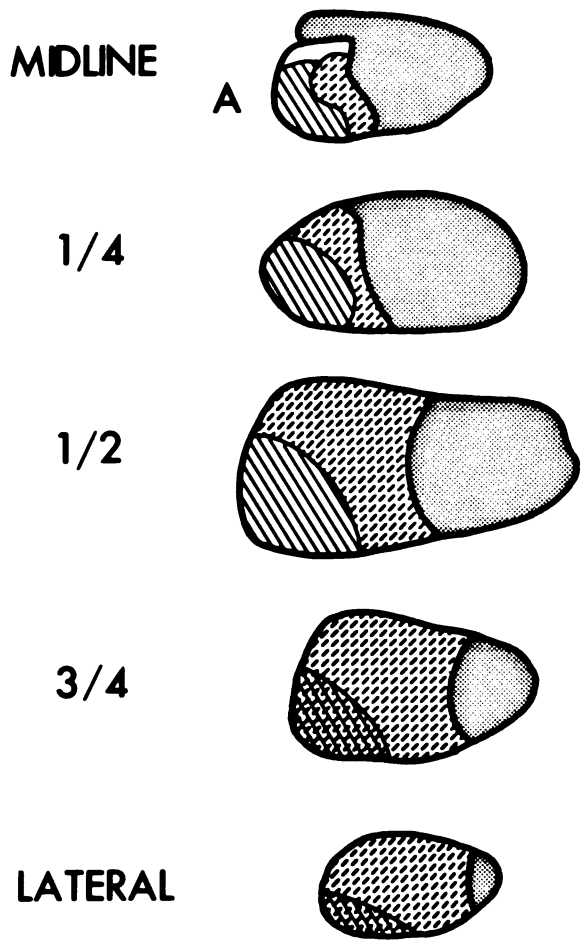
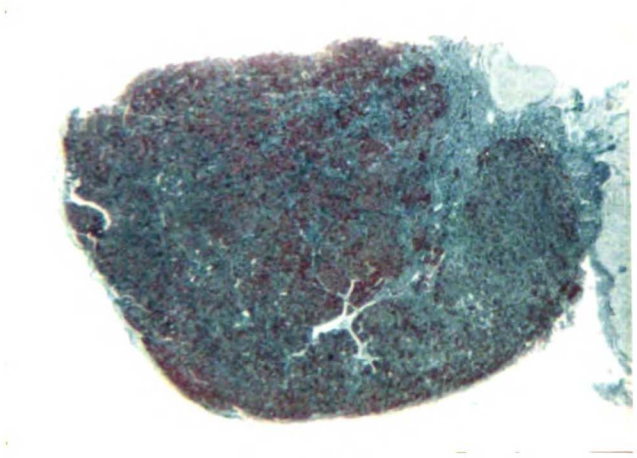
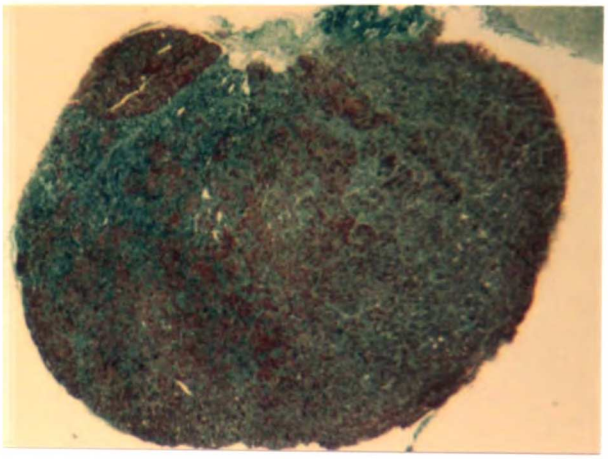


Figure 21. Light photomicrographs of three sagittal sections from a pregnant rhesus female monkey. The orientation and level of the sections are the same as in Figure 16. Only PRO cells are readily observed in all areas of the anterior lobe including those regions where the GH cells are observed in the juvenile and adult rhesus monkeys. Magnification 20X.



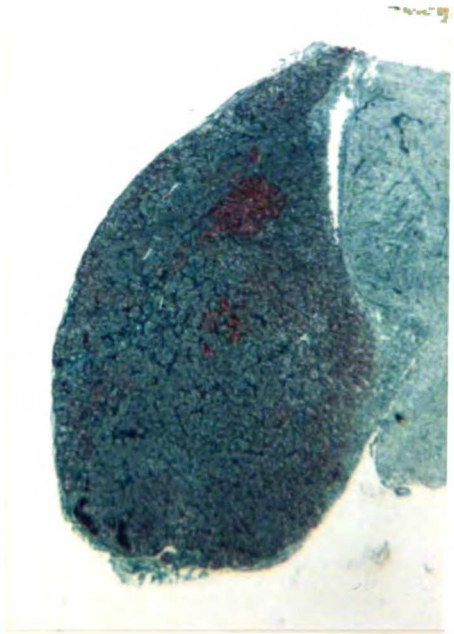
A



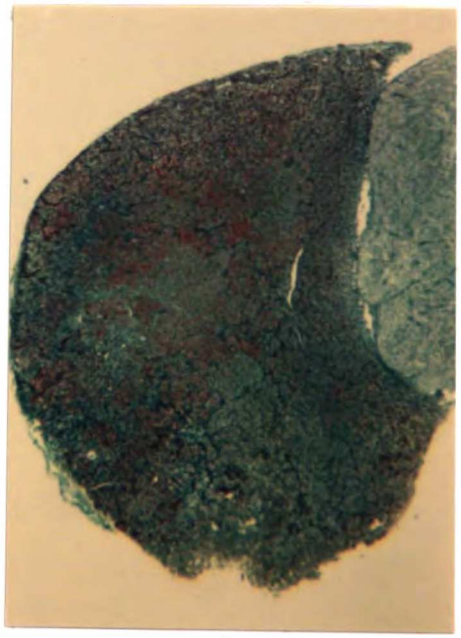
B

C

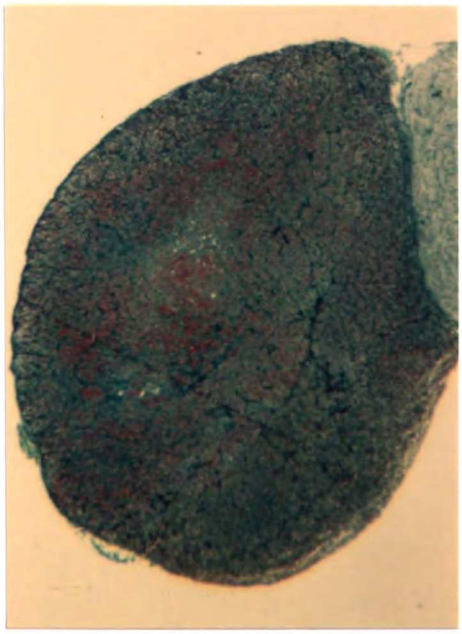
Figure 22. Light photomicrographs of three sagittal sections from a lactating rhesus female monkey, with the orientation and level of the sections being the same as in Figure 16. The histology is identical as that observed in the pregnant female rhesus shown in Figure 21, with the PRO cells being present in all regions of the anterior lobe. Magnification 20X.



A

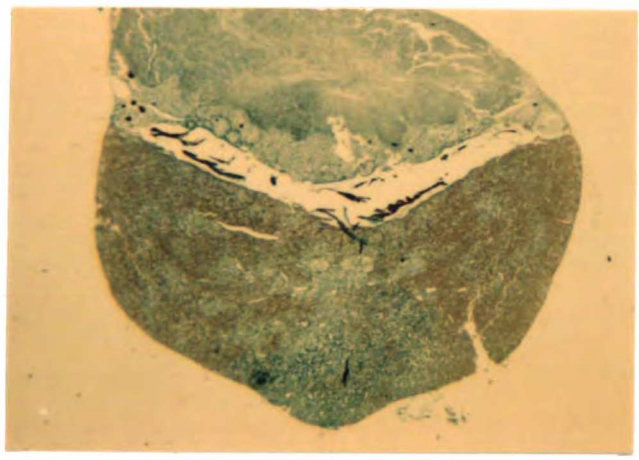


B

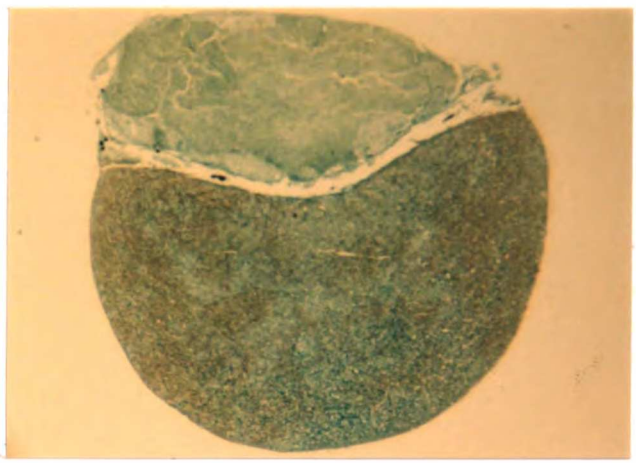


C

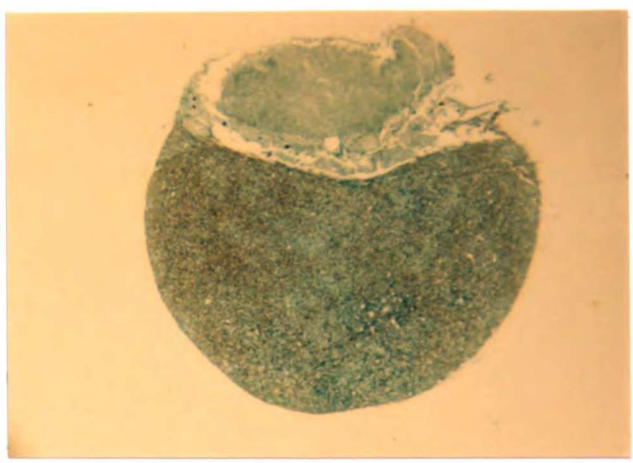
Figure 23. Light photomicrographs showing three horizontal sections taken from an adult male capuchin monkey. The orientation is with the lateral regions facing the right and left sides, and the anterior region facing the bottom of the micrograph. The three areas represented were taken from near the superior pole (A), about half the distance between the superior and the inferior poles (B), and near the inferior pole (C). Only an occasional PRO cell is present in the most anterior regions while the GH cells are found in the same areas as in the rhesus monkeys. Magnification 16X.



A

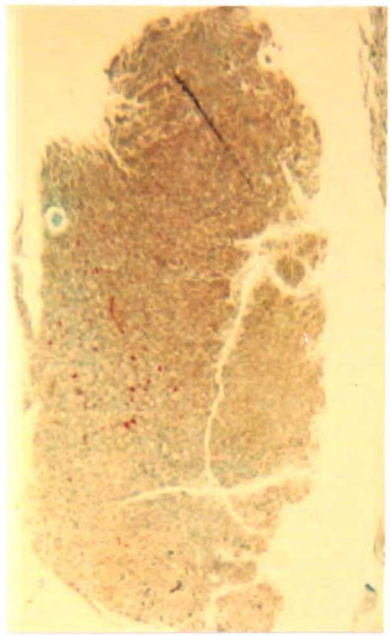


B



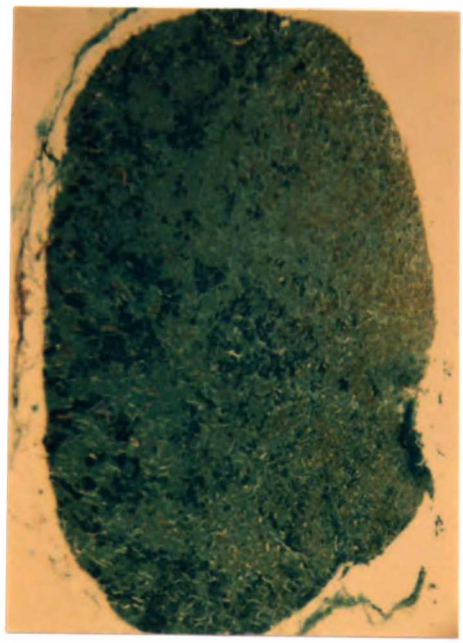
C

Figure 24. Light photomicrographs illustrating a section from an adult male gorilla (A), a sagittal section from a juvenile male orangutan (B) and a horizontal section from a juvenile female chimpanzee (C). The plane of section is unknown on the gorilla pituitary. Very few PRO cells were observed in any of these species of apes, which cannot be seen in these micrographs. GH cells predominate in the same areas as in the rhesus monkeys. The GH cells are quite deeply stained in the gorilla (A) and are observed in the upper half of the tissue section. In the orangutan (B), the GH cells are not stained well but were primarily observed posteriorly. Magnification 16X.



A

B



C

Figure 25. Standard curves from two strains of mice, White-Swiss and CD-1, used in bioassay studies. Verticle bars represent the standard error for each point. The curves are plotted semilogarithmically.

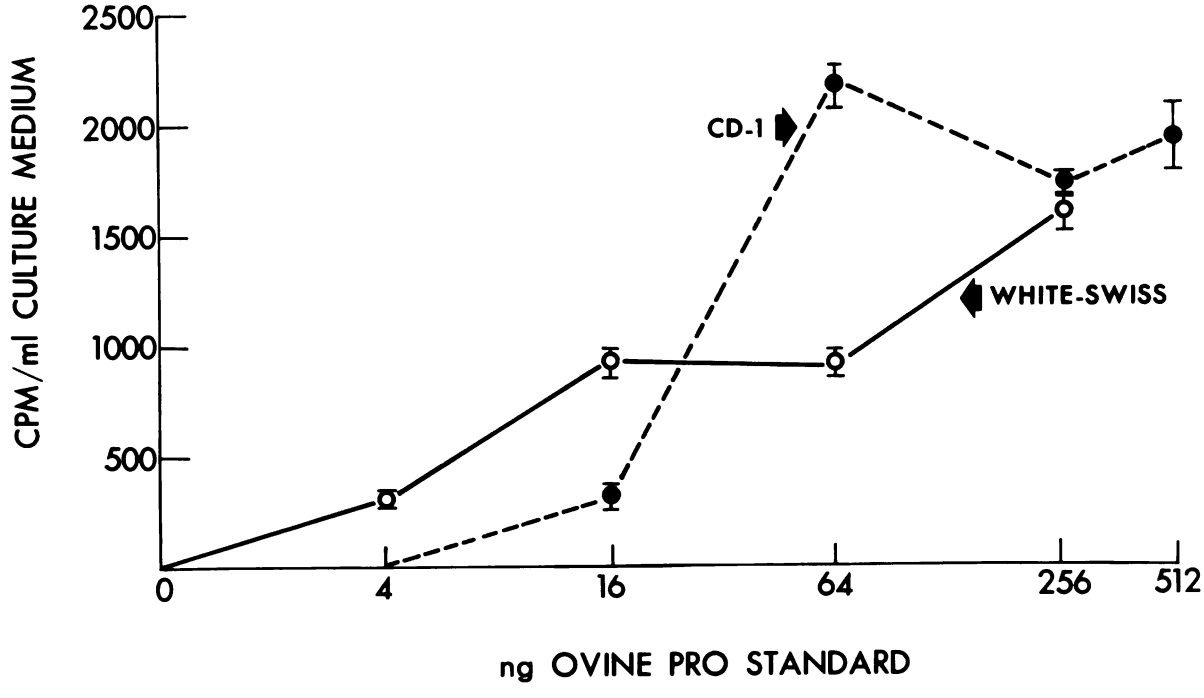

2500
2000
1500
1000
500
CPM/ml CULTURE MEDIUM
CD-1
WHITE-SWISS
0
4
16
64
256
512
ng OVINE PRO STANDARD

Figure 26. Agar gel studies showing the immunochemical relatedness of the precipitin reactions observed between guinea pig #8 anti-ovine PRO serum and the PRO in PE pools from the estradiol benzoate treated juvenile female rhesus monkeys. The center cell contains 150 µl of the antiserum, with 10 µg of ovine PRO (SPRO) and 2.5 mg of each of the three PE pools in the peripheral wells. A precipitin reaction is present from the normal juvenile PE pool (substituted for the control PE pool), as well as from the low and high dose PE pools which are giving reactions of partial identity with ovine PRO. Photographed at 74 hrs.

Figure 27. Agar gel studies showing the immunochemical relatedness of the precipitin reactions observed between guinea pig #30 anti-human GH serum and the GH in the same PE pools as used in Figure 26. A volume of 150 µl of antiserum was placed in the center well, and 15 µg of human GH (HGH) was placed in one of the peripheral wells. The other peripheral wells contain 0.75 mg of the low and high dose PE pools and 1.5 mg of the normal juvenile female PE pool (substituted for the control PE pool). Precipitin reactions are present from the three PE pools which give reactions of qualitative identity with each other and with purified human GH. Photographed at 50 hrs.


LOW DOSE
SPRO
JUVENILE ♀
HIGH DOSE
SPRO

26


LOW DOSE
HGH
JUVENILE ♀
HIGH DOSE
HGH

27

Figure 28. Radioimmunoassay studies showing the degree of cross-reaction of the PE pools from the three groups of female juvenile rhesus monkeys in the estradiol benzoate experiment in a heterologous human PRO assay system. RIA results are graphed using logarithmic logistic transformation.

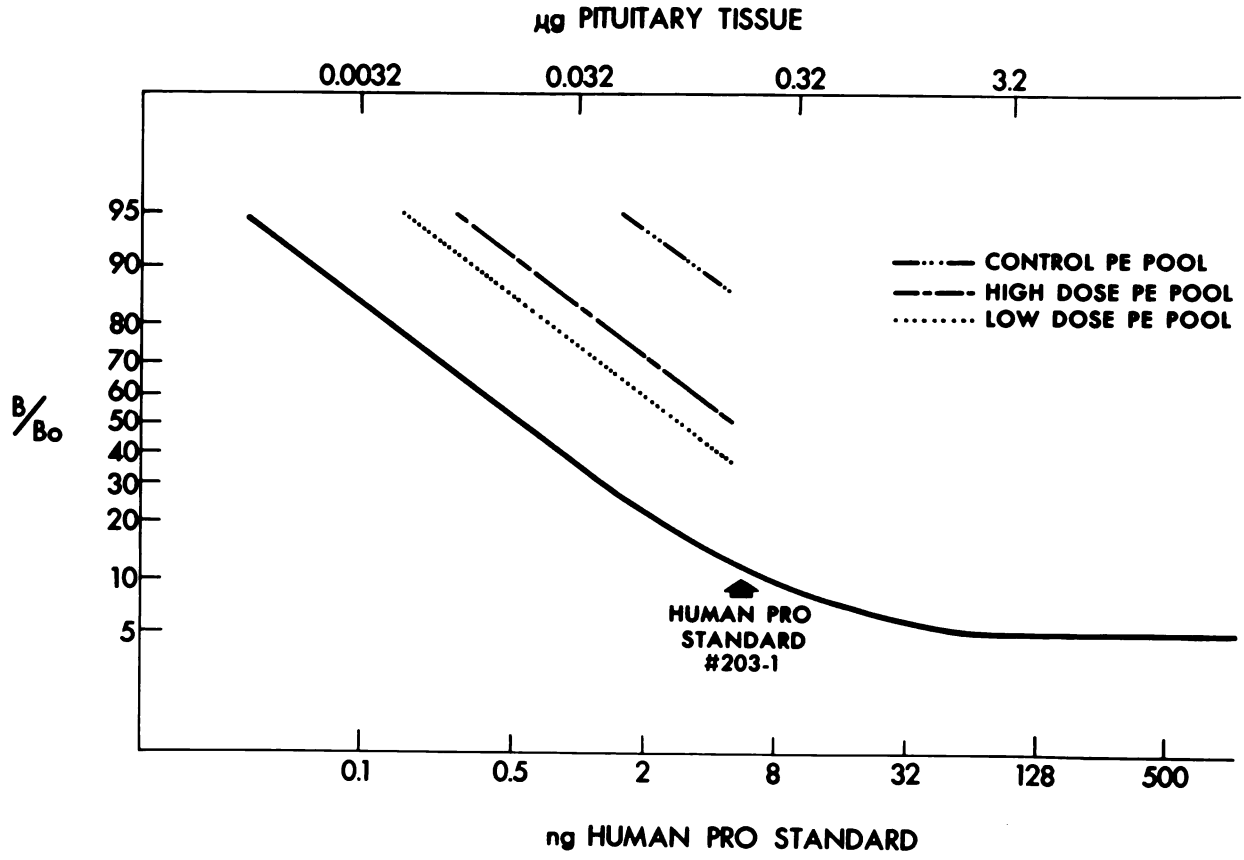

µg PITUITARY TISSUE
0.0032
0.032
0.32
3.2
95
90
80
70
60
50
40
30
20
10
5
B/Bo
CONTROL PE POOL
HIGH DOSE PE POOL
LOW DOSE PE POOL
HUMAN PRO STANDARD #203-1
0.1
0.5
2
8
32
128
500
ng HUMAN PRO STANDARD

Figure 29. Radioimmunoassay studies showing the extent of cross-reaction of the PE pools from the three groups of female juvenile rhesus monkeys in the estradiol benzoate experiment in a homologous human GH assay system. The results are shown by logarithmic logistic transformation.

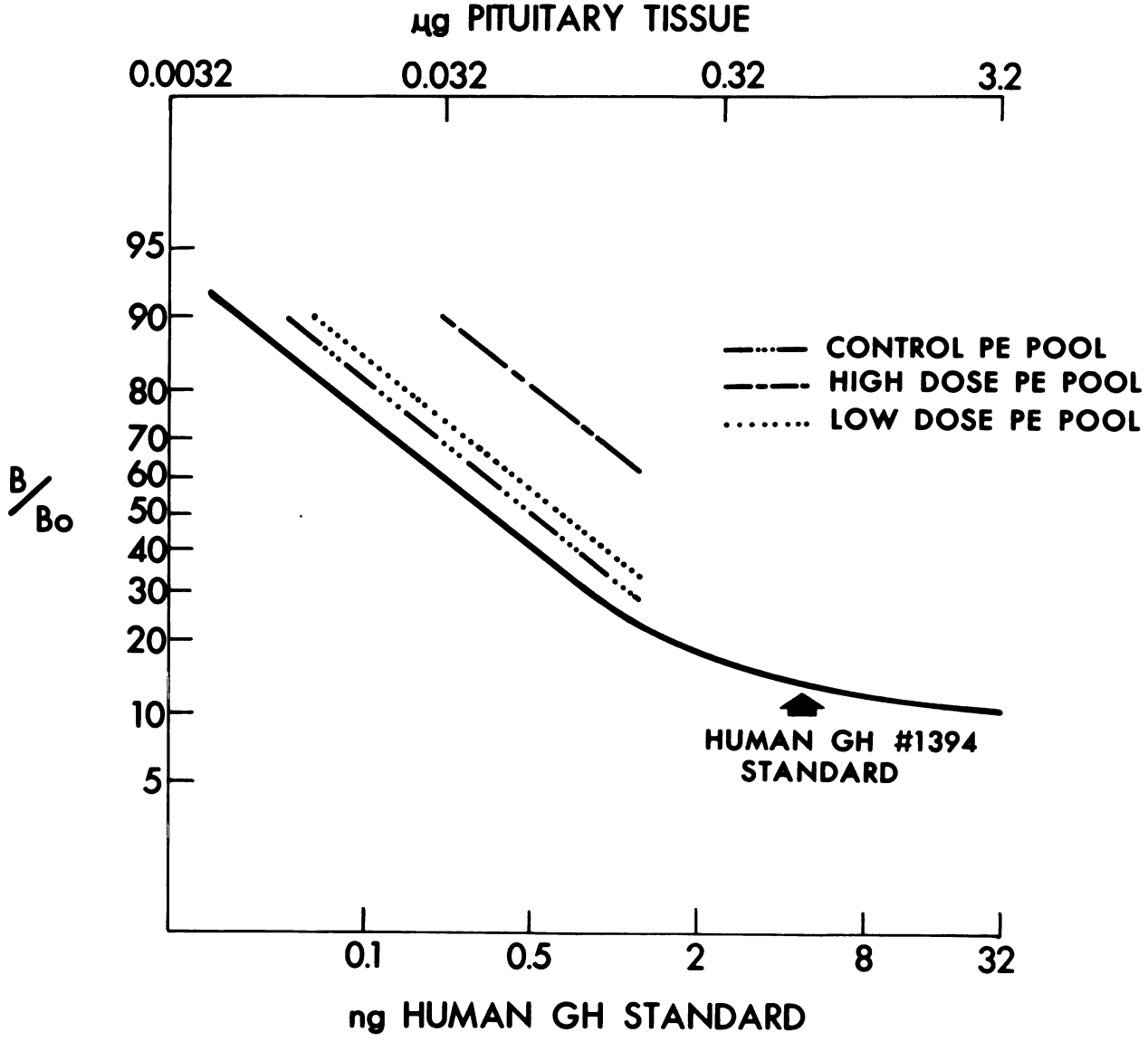
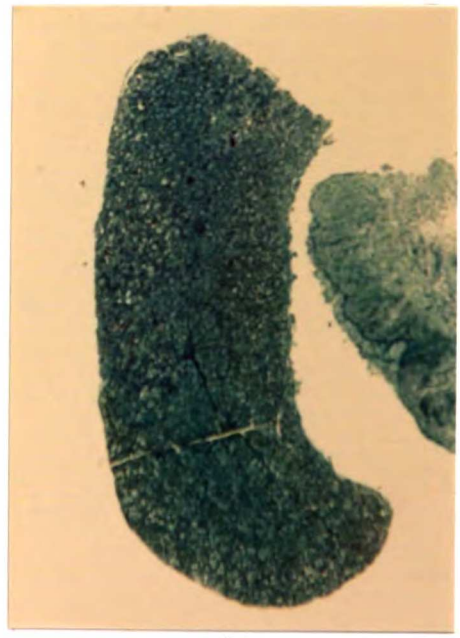

µg PITUITARY TISSUE
0.0032
0.032
0.32
3.2
95
90
80
70
60
50
40
30
20
10
5
B/Bo
CONTROL PE POOL
HIGH DOSE PE POOL
LOW DOSE PE POOL
HUMAN GH #1394
STANDARD
0.1
0.5
2
8
32
ng HUMAN GH STANDARD

Figure 30. Light photomicrographs of sagittal sections from one of the control animals in the estradiol benzoate study. The orientation and levels of the sections are the same as in Figure 16. Only a few PRO cells are present in the medial regions (B) of the pars distalis. Magnification 32X.

A

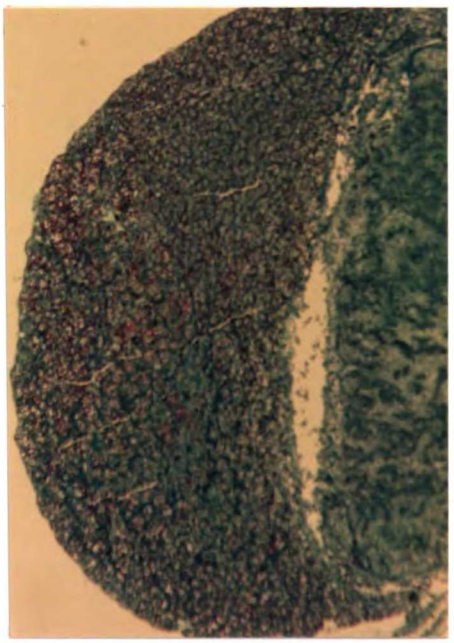


B

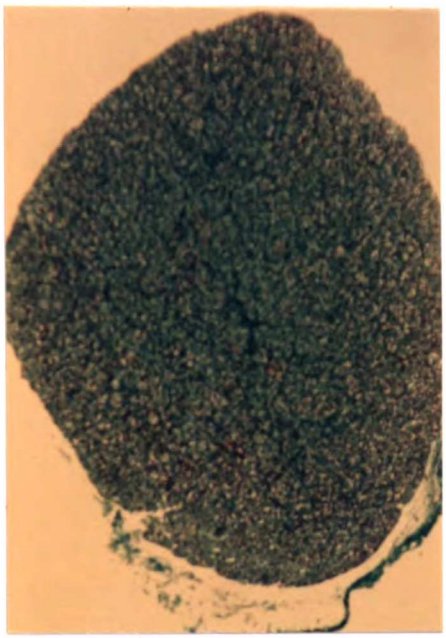
C

Figure 31. Light photomicrographs of sagittal sections from one of the animals which received the low dose (15 ug/day) of estradiol benzoate. The orientation and levels of the sections are the same as in Figure 16. More PRO cells are present than in either the control or the high dose treated animals. These cells are mostly observed anteriorly, medially (A and B) and antero-inferiorly (C). The few GH cells observed in the animals treated with the low dose of estradiol benzoate were located posteriorly (A and B) and laterally (C). This is not well illustrated in these three micrographs, due to technical difficulties. Magnification 40X.

A



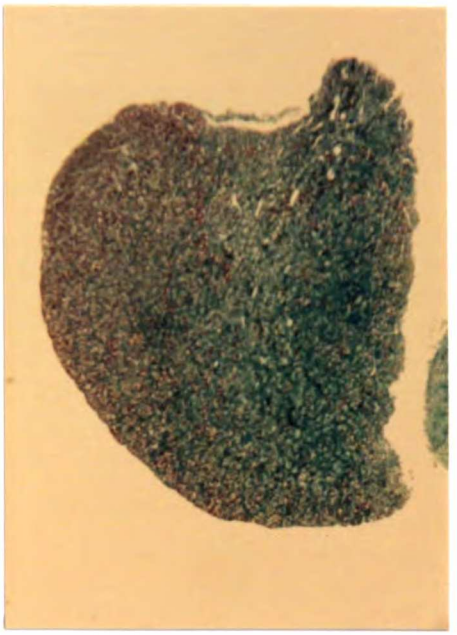
B



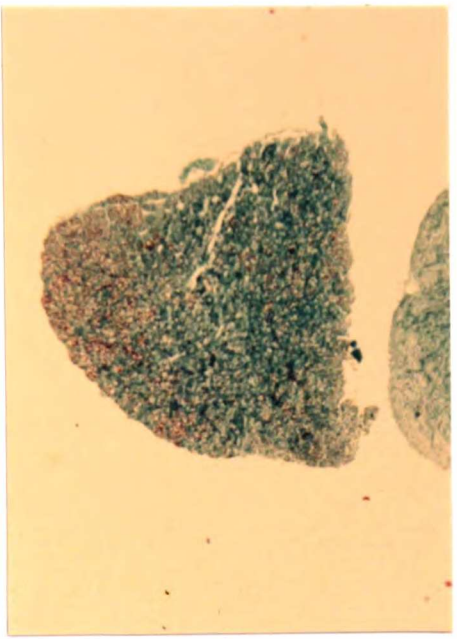
C

Figure 32. Light photomicrographs of sagittal sections from one of the monkeys treated with the high dose (150 ug/day) of estradiol benzoate. The orientation and level of the sections are the same as in Figure 16. PROcells are more abundant than in the control animals and are present anteriorly, medially (A-C) and posteriorly (B). Fewer lactotrophs, however, are present in the high dose treated animals than are observed in the animals treated with the low dose of the steroid. The few GH cells observed in the animals given the higher dose of estradiol benzoate were primarily located medially (A) and posteriorly (A-C). This is not well illustrated in these micrographs, due to technical difficulties. Magnification 32X.

A

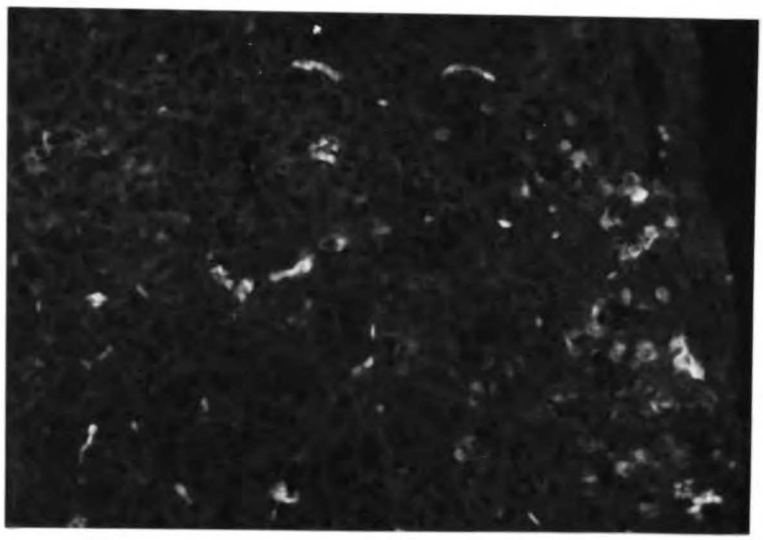


B

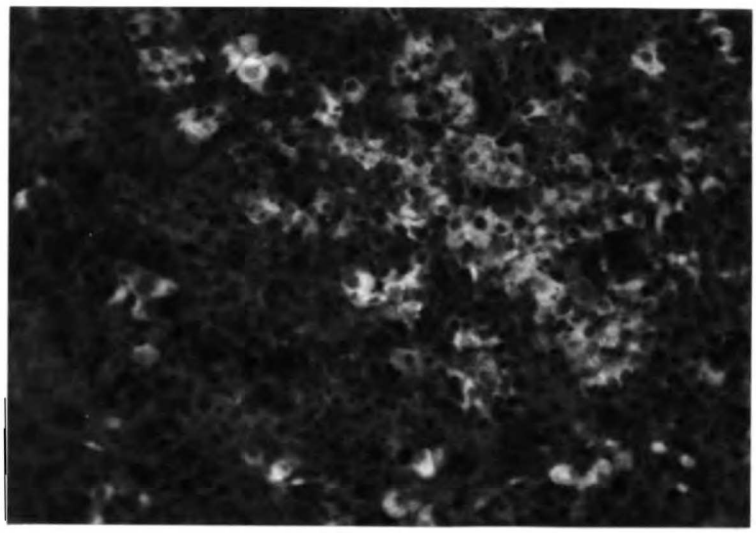


C

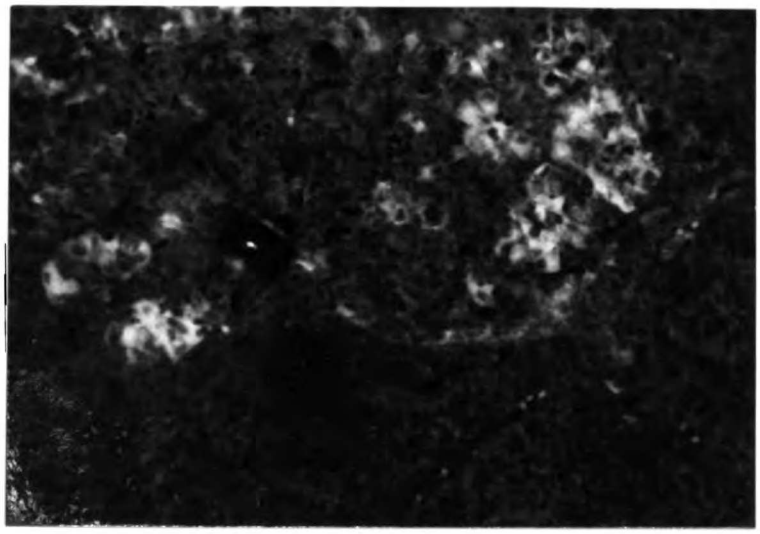
Figure 33. Fluorescent micrographs from a representative member of each of the three groups of female juvenile rhesus monkeys in the estradiol benzoate experiment after immunofluorescent staining with anti-ovine PRO serum. The number of fluorescing PRO cells are compared in each of the three groups of animals. Considerably more PRO cells are fluorescing in the low (B) and high dose (C) steroid treated animals than in the controls (A). Magnification 300X.



A



B



C

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