SHORT TITLE OF THESIS BY

PETER LAM HUM HWANG

HUMAN PROLACTIN : PURIFICATION AND RADIOIMMUNOASSAY

HUMAN PROLACTIN :
ITS PURIFICATION AND RADIOIMMUNOASSAY
WITH APPLICATIONS TO PHYSIOLOGY AND MEDICINE

by

PETER LAM HUM HWANG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH, McGILL UNIVERISTY, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

McGill University Clinic,
Royal Victoria Hospital,
Montreal, Quebec,
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ABSTRACT

HUMAN PROLACTIN : ITS PURIFICATION AND RADIOIMMUNOASSAY WITH APPLICATIONS TO PHYSIOLOGY AND MEDICINE. Peter Lam-Hum Hwang, McGill University Clinic, Department of Experimental Medicine, Royal Victoria Hospital and McGill University.

Prolactin has been purified from human pituitary glands with a yield of 50 μg/gland. The purified product was equipotent with highly purified sheep prolactin in bioassay (30.5 IU/mg) and was sufficiently pure for amino acid sequence analysis, which revealed a striking homology between human and sheep prolactin. Human prolactin has also been partially purified from amniotic fluid.

A specific radioimmunoassay for human prolactin has been developed to measure circulating prolactin concentrations in man. In normal adults, serum prolactin levels usually remained below 30 ng/ml, being slightly higher in females than in males. Serum prolactin concentrations were elevated in the newborn, in pregnant and lactating women, and in most cases of galactorrhea, but not in patients with breast cancer or acromegaly. Chlorpromazine and thyrotropin releasing hormone increased while L-DOPA decreased serum prolactin; these agents may prove useful in evaluating hypothalamic-pituitary function or in treating conditions associated with abnormal prolactin secretion.

RESUME

PROLACTINE HUMAINE: SA PURIFICATION ET SON DOSAGE RADIO-IMMUNOLOGIQUE AVEC APPLICATIONS A LA PHYSIOLOGIE ET LA MEDECINE - Peter Lam-Hum Hwang, Clinique Universitaire de McGill, Département de Médecine Expérimentale, Hôpital Royal Victoria.

La prolactine a été purifiée à partir de glandes pituitaires humaines à raison de 50 µg/glande. Le produit purifié était équipotent avec la prolactine de mouton hautement purifiée en dosage biologique (30.5 UI/mg), et était suffisamment pur pour l'analyse de la séquence d'amino-acides, qui révélèrent une homologie frappante entre la prolactine humaine et la prolactine du mouton. La prolactine humaine fut également partiellement purifiée du liquide amniotique.

Un dosage radio-immunologique de la prolactine humaine a été développé afin de mesurer les concentrations de prolactine circulant chez l'homme. Chez les adultes normaux, les niveaux de serum prolactine demeuraient ordinairement en dessous de 30 ng/ml, étant légèrement plus élevés pour les femelles que pour les mâles. Les concentrations de serum prolactine étaient élevées chez les nouveaux-nés, les femmes enceintes et les mères allaitantes, et dans la plupart des cas de galactorrhée, mais ne l'étaient pas chez les patientes ayant un cancer du sein ou dans les cas d'acromégalies. Le taux de chlorpromazine et d'hormone libérant la thyrotropine augmenta tandis que le L-DOPA réduisit le serum prolactine; ces agents peuvent s'avérer utiles dans l'évaluation de la fonction hypothalamo-pituitaire ou dans le traitement des conditions associées à une sécrétion anormale de prolactine.

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CLAIM TO ORIGINAL WORK OR CONTRIBUTION TO KNOWLEDGE

(1) Prolactin has been purified from human pituitary glands in sufficient quantity and with adequate purity for structural studies, unequivocally establishing its existence in man.

(2) Partial purification of prolactin from human amniotic fluid has been achieved.

(3) A specific radioimmunoassay for human prolactin has been developed, with sufficient sensitivity for measuring prolactin concentrations in the circulation.

(4) Serum concentrations of prolactin in man under different physiological and pathological conditions have been studied. In addition, the effects of several pharmacological agents on prolactin secretion in man have been examined.

ABBREVIATIONS USED

hPRL	Human prolactin
mPRL	Monkey prolactin
oPRL	Ovine prolactin
hGH	Human growth hormone
hPL	Human placental lactogen
PIF	Prolactin inhibiting factor
TRH	Thyrotropin releasing hormone
mPRL-I^{125}	I^{125}-labeled monkey prolactin
anti-	antibodies against

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SECTION I: GENERAL INTRODUCTION

A BRIEF HISTORICAL NOTE ON PROLACTIN IN ANIMALS

In 1928, Stricker and Grüeter (1928) first postulated the existence of a lactogenic principle in the anterior pituitary, after observing that lactation followed the injection of anterior pituitary extracts into pseudo-pregnant ovariectomised rabbits. Their observation was confirmed by others in several species of animals (see Riddle et al, 1933). Soon after, Riddle, Bates and Dykshorn (1932, 1933), using a partially purified fraction of beef or sheep pituitary glands, showed that the lactogenic activity in the anterior pituitary could be dissociated from the other two hormonal activities then known to exist in the pituitary gland, namely those of growth-promotion and gonad-stimulation. On the basis of this observation, they postulated the existence of a new pituitary hormone and called it prolactin. Since then, various other names have been applied to this lactogenic factor, including galactin, galactopoietic hormone, lactogen, lactogenic hormone, mammotropin, mammogenic hormone and others. As a result of a later observation (Astwood, 1941) showing that prolactin was also capable of maintaining corpus luteum function in certain rodents, it has also been called gonadotropin C or III, luteotropin and luteomammotropin.

In 1931, Riddle and Braucher (1931), while studying the reproductive physiology of birds, noted that the extraordinary growth of the crop sac, which normally occurs in pigeons incubating their eggs, was under anterior pituitary control and that the pituitary factor which stimulated crop sac growth was prolactin. This observation

proved to be a milestone in the history of prolactin, for it formed the basis of one of the most widely used quantitative bioassays for this hormone to this day. With the availability of this assay, it became possible to purify prolactin from the pituitary glands of a number of species. Sheep prolactin was first isolated in a highly purified state by Lyons (1936-37) and subsequently by White et al (1937) and Li et al (1940). More recently, prolactin has been purified from cows (Jiang and Wilhelmi, 1965; Kwa et al, 1965), pigs (Eppstein, 1964; Jiang and Wilhelmi, 1965), rats (Kwa et al, 1967a; Groves and Sells 1968; Ellis et al, 1969; Hodges and McShan, 1970; Gala, 1972), and mice (Yanai et al, 1968; Cheever et al, 1969). The chemical isolation of prolactins from these species not only permitted their chemical and biological characterisation, but also led to the development of radioimmunoassays for these hormones.

In recent years extensive work has been done on the chemistry of animal prolactins, and the complete amino acid sequence of sheep prolactin is now known (Li et al, 1969). In addition, the spectrum of biological activities of mammalian prolactin has been examined in many different classes of vertebrates (see Nicoll and Bern 1972), and it appears that prolactin has an extraordinarily wide range of activities. In the review by Nicoll and Bern (1972), over 80 activities were listed, many of which may be of physiological significance in relation to vertebrate growth and metabolism, to reproduction and to osmoregulation. In addition, prolactin also acts on a variety of ectodermal derivatives and acts synergistically with many steroid hormones. An area of particular interest in recent years has been the relationship between prolactin and breast tumors. Studies of Furth (1969), Pearson et al (1969) and Meites et al (1972) indicated that prolactin

might be of primary importance in the development of mammary tumors in at least some species of rodents.

In the past 6-7 years, radioimmunoassays have been developed for prolactin by, among others, Arai and Lee (1967), Bryant and Greenwood (1968), McNeilly (1971), Fell et al. (1972) and Hart (1972) for sheep prolactin; Johke (1969), and Schams and Karg (1969) for beef prolactin; Kwa and Verhofstad (1967a), Niswender et al. (1969), Neill and Reichert (1970) for rat prolactin; and Kwa et al. (1967b), and Sinha et al. (1972) for mouse prolactin. For many years prolactin had been quantitated by the pigeon crop sac and other bioassays, which were not sufficiently sensitive for measuring prolactin concentrations in blood (see Cowie and Tindall, 1971). As a result, information on certain aspects of prolactin physiology, such as the regulation of its secretion, was largely inferential, being based mainly on observations of changes in the prolactin content of the pituitary gland under different physiological circumstances. With the availability of sensitive radioimmunoassays, it became possible to measure directly prolactin concentrations in the circulation under different conditions. Recent radioimmunoassay studies have not only helped to verify conclusions drawn from previous bioassay results but have added considerable insight into the factors controlling prolactin secretion as well as the physiological role of this hormone.

THE PROBLEM OF HUMAN PROLACTIN

In sharp contrast to the enormous amount of information on animal prolactin which has accumulated over the past 4-5 decades, our knowledge of prolactin in primates was, until very recently, very limited. In fact, the very existence of prolactin in man has been seriously questioned (Bewley and Li, 1970). The reasons for the uncertainties about human prolactin were two-fold. In the first place,

attempts made to purify prolactin from human pituitaries invariably resulted in preparations which also showed growth hormone activity (Wilhelmi, 1961; Reisfeld et al, 1963; Apostolakis, 1965). Although among these preparations it was observed that the ratio of prolactin to growth hormone activity varied considerably (Tashjian et al, 1965; Apostolakis, 1965), it had never been possible to dissociate the two activities completely and unequivocally. Moreover, Tashjian et al (1965) and Greenwood (1967) suggested that the variations of prolactin/growth hormone ratio among different human "prolactin" preparations might possibly be accounted for by minor conformational changes of the growth hormone molecule. The second reason for questioning the existence of human prolactin was that Lyons et al (1960, 1961) reported that highly purified human growth hormone had lactogenic activity. Their initial observation was confirmed and extended by many subsequent studies (Chadwick et al, 1961; Ferguson and Wallace, 1961; Li, 1962; Damm et al, 1964; Forsyth et al, 1965; Hartree et al, 1965; Rivera et al, 1967; Peckham et al, 1968), leaving very little doubt that human growth hormone indeed has intrinsic prolactin activity in a variety of species including man. The failure to isolate from human pituitary glands a prolactin that was definitely distinct from growth hormone, and the demonstration that purified human growth hormone has intrinsic prolactin activity led Bewley and Li (1970) to state that "in the human the hormone control of lactation and growth seems to be effected through a single pituitary protein, namely human growth hormone", implying that prolactin might not exist as a separate hormone in man. This notion, however, is inconsistent with a large number of clinical and experimental observations, which may be summarised as follows.

EVIDENCE FOR THE EXISTENCE OF HUMAN PROLACTIN

(a) Clinical Conditions which Suggest the Existence of Human Prolactin

Several clinical observations made in the last 15-20 years make it difficult to accept that growth hormone is the lactogenic hormone in man. As early as 1954, Forbes et al described a syndrome of galactorrhea and amenorrhea which was associated in some cases with pituitary tumors. These patients showed no evidence of acromegaly, indicating that excessive growth hormone secretion was not involved in the pathogenesis of the abnormal lactation. Forbes suggested excessive prolactin secretion as a possible explanation for the syndrome, but evidence for hyperprolactinemia could not be obtained as there was no suitable method for measuring serum prolactin. With the development of radioimmunoassay for human growth hormone (Hunter and Greenwood, 1964), studies were carried out to examine serum growth hormone concentrations in normal postpartum lactation (Roth et al, 1968) as well as in non-puerperal galactorrhea due to various causes (Spellacy et al, 1968; Benjamin et al, 1969). Almost uniformly in these situations, there was no evidence of increased growth hormone concentration in the circulation. Perhaps the most convincing clinical evidence for the existence in man of a separate lactogenic hormone which is distinct from growth hormone came from Rimoin et al (1968) who reported that postpartum lactation was essentially normal in patients with inherited growth hormone deficiency. In these patients growth hormone was undetectable even after provocative tests in the puerperium, and yet lactation was normal.

More direct evidence for the existence of a lactogenic hormone distinct from growth hormone resulted from

studies in which attempts were made to measure lactogenic activity in serum. Canfield and Bates (1965) studied serum prolactin concentrations in 6 patients with nonpuerperal galactorrhea, using a serum extraction procedure and the pigeon crop sac assay. They reported elevated levels of serum prolactin activity in 5 of the 6 cases examined. In a similar study carried out by Roth, et al (1968) on 8 postpartum women and 21 untreated acromegalics, it was observed that all postpartum women showed plasma prolactin levels above the upper limit of normal (> 6 mU/ml in terms of a sheep prolactin standard), whereas their serum growth hormone concentrations were all below 4 ng/ml as determined by radioimmunoassay. The converse was true in the 21 acromegalics, all of whom showed growth hormone concentrations above 10 ng/ml, while only 3 had prolactin levels considered to be above normal. It was of interest to note that these 3 patients showed grossly elevated growth hormone concentrations of above 400 ng/ml. In view of the demonstration that growth hormone has intrinsic lactogenic activity, it was likely that the high "prolactin" activity observed in these patients was due to growth hormone itself.

Although the existence of a distinct lactogenic hormone in man is strongly suggested by the studies described above, its demonstration by means of assays carried out in serum extracts can be criticized because of uncertainties of the extraction procedures employed. In 1970, Frantz and Kleinberg reported a very sensitive <u>in vitro</u> bioassay which was capable of detecting prolactin activity in unextracted human plasma. Breast tissue fragments from mid-pregnant mice were incubated with human plasma for 4 days, after which the secretory activity of the fragments was graded histologically. Human growth hormone was active in this assay, but its lactogen-

ic effect could be entirely neutralised by antiserum to growth hormone. Using this assay, these investigators were able to detect elevated plasma prolactin in all 14 nursing mothers studied, in 14 of 26 patients with non-puerperal galactorrhea and in some patients being treated with psychoactive drugs of the phenothiazine series. In none of these cases was the prolactin activity in plasma neutralizable by antiserum to human growth hormone. This study represents the first unequivocal and direct demonstration of lactogenic activity in unextracted human plasma which is clearly distinct from growth hormone. Other similar in vitro bioassays using either histological or biochemical end-points were developed later by Loewenstein et al (1971), Forsyth and Myres (1971) and Turkington (1971 a). The findings of Frantz and Kleinberg (1970) were largely substantiated in these later studies.

Thus, measurements of serum lactogenic activity and of growth hormone concentration during periods of normal lactation or galactorrhea clearly suggest that a lactogenic factor distinct from growth hormone exists in man.

(b) Histological and Immunofluorescence Studies

In several mammalian species, it is well-established that in the pituitary, two classes of acidophils can be distinguished on the basis of their staining characteristics (see Herlant, 1960, and Pasteels, 1963). One type, which stains with orange G, and is called the orangeophil, is thought to secrete growth hormone (somatotroph). The other type stains red with carmoisine L or erythrosine (carminophil or erythrosinophil) and is considered to be the prolactin-producing cell (lactotroph). The lactotrophs usually undergo marked changes in number and morphology during pregnancy and lactation. In addition,

electronmicroscopic studies reveal that the lactotrophs and somatotrophs can be differentiated on the basis of their secretory granules : those in the lactotrophs are more irregular and larger in size than those in the somatotrophs (see Pasteels, 1972 a).

In man, Erdheim and Stumme (1909) first noted that the pituitary gland during pregnancy contained numerous poorly stained cells which they called "pregnancy cells". These cells were first thought to be chromophobes (Floderus, 1949) but subsequent studies (see Purves, 1966), suggested that they probably were the actively secreting forms of an acidophil subtype. Using the tetrachrome method of staining of Herlant (1960), Pasteels (1972 b) confirmed that it was indeed the erythrosinophils which increased in number and size during pregnancy and lactation in women. Goluboff and Ezrin (1969), in a similar study, showed that carminophils appeared in significant numbers in the human pituitary gland only during pregnancy and the postpartum period, and that during late pregnancy and lactation, these cells might constitute more than 50% of the total acidophils. The similarity which these cells bear to the lactotrophs of lower vertebrates in staining characteristics and their marked increase in number and size during pregnancy suggest they are also prolactin-producing cells.

More direct evidence for the existence of the lactotroph in the human pituitary came from the detailed analysis carried out by Peake et al (1969) on a pituitary tumor removed from a patient with galactorrhea-amenorrhea. The patient had no detectable growth hormone in her plasma prior to removal of the tumor even after arginine infusion. The tumor contained very little growth hormone but had significant pigeon crop sac stimulating activity

which could not be neutralised by antiserum to growth hormone. Histologically, 80-90% of the cells in the tumor were acidophils which stained strongly with erythrosin. The eosinophilic granules in the tumor cells were larger and more irregular in size and shape than growth hormone granules. This study provided good evidence that the tumor arose from prolactin-producing cells.

The development of immunofluorescence techniques contributed significantly to the identification of specific cell types in the pituitary gland in the primate. By a combination of differential staining and immunofluorescence, Herbert and Hayashida (1970) made the important observation that carminophils and only carminophils in monkey pituitary glands fluoresced with antiserum to sheep prolactin. Conversely, antiserum to human growth hormone localised only in the orangeophils. The results indicate that in the carminophils of the primate pituitary, there is a substance which is immunologically related to sheep prolactin and distinguishable from growth hormone, strongly suggesting the existence of a separate prolactin molecule in primate pituitaries. In a similar study with human pituitary glands, Pasteels et al (1972 b) obtained essentially the same findings, that antisera to sheep prolactin and growth hormone localised specifically in carminophils and orangeophils respectively.

These immunofluorescence studies not only suggested the existence of primate prolactin but provided investigators with an immunological tool for distinguishing between prolactin and growth hormone in primates.

(c) Biosynthetic Studies

In the past decade, Pasteels and his colleagues have studied the in vitro secretion of prolactin and growth

hormone of cultured human pituitary glands (Pasteels et al. 1963; Brauman et al. 1964). Their essential finding was that the secretion of immunoreactive growth hormone into the culture medium declined rapidly with time, whereas pigeon crop sac stimulating activity in the medium increased, presumably due to increasing prolactin secretion. This observation clearly indicated that the two hormones were secreted independently. Although Pasteels' work could not be reproduced by Solomon et al. (1969), the independent secretion of growth hormone and prolactin has been confirmed in the rhesus monkey by Nicoll et al.(1970b) and Channing et al. (1970) in in vitro studies.

A slightly different approach was employed by Friesen and his colleagues in their biosynthetic studies designed to demonstrate the separate existence of primate prolactin. Human (Friesen et al. 1970) and monkey (Friesen and Guyda, 1971) pituitary glands were incubated in vitro for short periods of time in the presence of tritiated leucine. Among the radioactive proteins secreted into the incubation media, they detected a labeled protein which cross-reacted with antiserum to sheep prolactin and which appeared to be most abundant in the media of glands obtained from pregnant or lactating individuals as compared to males or non-pregnant females. They inferred from these studies that the protein which was immunologically related to sheep prolactin probably represented primate prolactin synthesized in vitro. In a similar study with pituitary tumors (Hwang et al.1971), it was found that antiserum to sheep prolactin precipitated 63% of the radioactive proteins in the medium of a tumor removed from a patient with galactorrhea, but only 2% in the medium of a tumor from an acromegalic. An opposite picture was obtained with antiserum to human growth hormone. The later studies of Nasr et al. (1972) and Friesen et al. (1972b) carried out on pituitary tumors associated with galactorrhea

yielded similar results and provided additional evidence for the in vitro biosynthesis of human prolactin.

In summary, evidence for the existence of prolactin in primates comes not only from clinical observations, but also from histological, immunological, and biosynthetic studies, supported by direct measurements of prolactin activity in the circulation. The conclusion is inescapable that prolactin exists in primates and is clearly different from growth hormone.

SECTION II : AIMS OF THE PRESENT INVESTIGATION

The objectives of this investigation were two-fold : (1) to provide unequivocal evidence for the existence of prolactin in man by chemically isolating it from human pituitary glands, and (2) to examine some aspects of the physiology of human prolactin with special reference to the factors which regulate its secretion.

This investigation describes :

(a) the chemical purification of human prolactin from pituitary glands in sufficient quantity and with sufficient purity for structural studies, thus firmly establishing the existence of this hormone in man, and

(b) the development of a sensitive and specific radioimmunoassay for human prolactin, and its application to studies on serum prolactin concentrations in man under various physiological and pathological conditions.

SECTION III : DEVELOPMENT OF A RADIO-IMMUNOASSAY FOR HUMAN PROLACTIN

INTRODUCTION

In the past, various attempts have been made to measure prolactin levels in human urine and plasma under different physiological circumstances. The results obtained have been summarized by Apostolakis (1968). It is difficult to evaluate these reports since there was some uncertainty about the extraction methods employed, and the assays used were often plagued by the problem of non-specificity. In addition, some of the values reported in these earlier studies were at least an order of magnitude higher than those obtained using the more recently developed bioassays or radioimmunoassays, making direct comparisons difficult.

RECENT ATTEMPTS TO MEASURE SERUM PROLACTIN IN MAN

During the course of this investigation, several *in vitro* bioassays with sufficient sensitivity for measuring circulating prolactin concentrations in man were developed independently by other investigators. All these assays were based on the *in vitro* incubation of mammary tissue explants obtained from pregnant or pseudopregnant mice or rabbits. When such breast tissue slices were incubated in the presence of prolactin, certain morphological and biochemical changes occurred; the magnitude of these changes could be quantitatively related to the concentrations of prolactin in the incubation media. Frantz and Kleinberg (1970) and Forsyth and Myres (1971) showed that the secretory changes observed in histological sections of the explants after incubation, when graded on a 0 to 4+ scale, gave a fairly reliable semi-quantitative estimate of the prolactin concentration. Loewenstein et al. (1971) and Turkington (1971a), instead of using histological

criteria, employed as endpoints the increases in N-acetyllactosamine synthetase activity and casein synthesis respectively. In these four bioassays, it was found that with sheep prolactin as standard, a satisfactory standard curve could be obtained, with the lower limit of detectability at 2 to 50 ng/ml. Precision of these assays was low, but with multiple assays, variation was usually within a two fold range and might be as low as 10%. In all these bioassay systems, human growth hormone and human placental lactogen gave positive responses and would interfere with the assay if present in significant amounts unless neutralised by their specific antisera. The major drawback of these various procedures, however, would appear to be the enormous labor involved in assaying multiple samples.

Radioimmunoassays for peptide hormones have been widely employed in the past decade. Their greater sensitivity, precision, specificity and simplicity represent major advantages over bioassays. Radioimmunoassays for sheep, beef, rat, and mouse prolactin have been developed in the past few years (see Cowie and Tindall, 1971). The failure to purify human prolactin was the major stumbling block in the development of a satisfactory immunoassay for this hormone. In the past few years, several groups of investigators attempted to bypass this difficulty by trying to measure prolactin in human serum with immune systems in which the radioactive antigen employed was thought to be immunologically related to human prolactin. These antigens included various animal prolactins and human placental lactogen. Stephensen and Greenwood (1969) attempted to detect human prolactin in the plasma of lactating women using a radioimmunoassay for human placental lactogen. The inhibition of binding which they initially observed proved to be artifactual (Greenwood, 1972).

Josimovich et al, (1971), using chemically modified human placental lactogen as antigen, also claimed to detect immunoreactive material in postpartum sera, but there was insufficient information to be certain of the nature of the material being detected. Midgley (1971) and later Jacobs et al (1972), employing pork or sheep prolactin tracer and antisera to sheep prolactin, detected cross-reacting material in human serum during pregnancy or lactation or in patients with galactorrhea. Their results agreed closely with those obtained by us using the homologous assay which we have developed concurrently, suggesting that the same entity was being measured by all three assays. The ability of heterologous systems in detecting human prolactin appears to be a property peculiar to the particular antisera employed, since other investigators using identical systems have failed to measure human prolactin in plasma. An attempt was made by Bryant et al (1971) to develop a homologous human prolactin radioimmunoassay. They used a protein fraction derived from the culture media of human fetal pituitary glands as antigen. The assay appeared to detect material in human plasma under conditions where a high concentration of human prolactin would be expected.

Recently, radioreceptor assays for lactogenic hormones have also been reported (Turkington, 1971 b; Shiu and Friesen, 1972). These assays are simple and quick to perform and have the possible advantage that only biologically active prolactin is measured, but their sensitivity appears to be somewhat less than that of radioimmunoassays and there are problems of non-specific interference by serum proteins. The value of such assays in clinical situations remains to be determined.

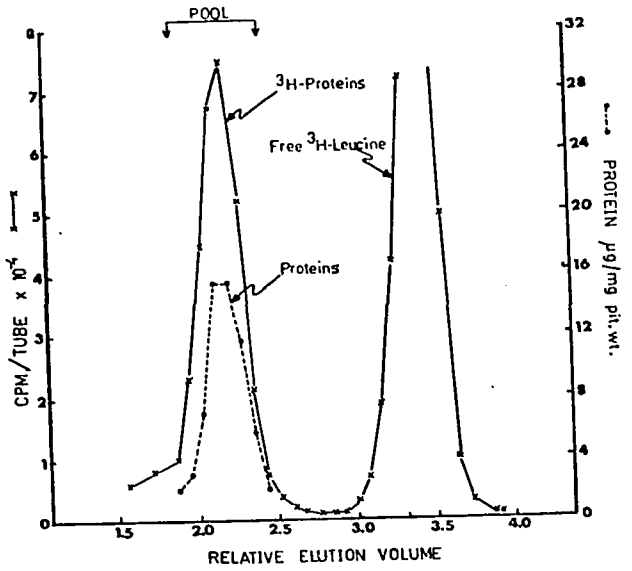
MATERIALS AND METHODS

When work was first initiated by the author to develop a radioimmunoassay for human prolactin, Dr. Harvey Guyda, working in the same laboratory, had already succeeded in partially purifying monkey prolactin. Past attempts to isolate prolactin from human pituitary glands have not met with success, one of the possible reasons being the very low prolactin content of glands obtained at autopsy. Histochemical studies (Goluboff and Ezrin, 1969; Pasteels, 1972 b) clearly demonstrated that prolactin secreting cells were very scanty in human pituitary glands except during pregnancy and lactation. Glands removed from pregnant or lactating subjects would be a rich source of prolactin, but are not readily available. Guyda and Friesen (1971) therefore, employed glands from pregnant and lactating rhesus monkeys as raw material for prolactin purification, hoping that monkey and human prolactin would be similar immunologically so that a radioimmunoassay for monkey prolactin would also detect human prolactin. It was also anticipated that such an assay could distinguish between prolactin and growth hormone since previous immunofluorescence (Herbert and Hayashida, 1970) and biosynthetic (Friesen and Guyda, 1971) studies showed that the two hormones were immunologically distinct in the monkey. With the availability of a simple and specific assay for primate prolactin, the task of purifying human prolactin free from growth hormone would be greatly simplified and the development of a homologous radioimmunoassay for human prolactin should then follow in due course. This approach to the problem of developing a specific radioimmunoassay for human prolactin proved to be successful.

(a) Partial Purification of Monkey Prolactin

The method employed in purifying monkey prolactin by Guyda and Friesen (1971) will be briefly summarised here. Pituitary glands from pregnant or lactating rhesus monkeys were incubated for 24 hours *in vitro* with tritiated leucine. The incubation medium was fractionated on Sephadex G-100. A prominent radioactive protein peak with a relative elution volume of about 2 to 2.5 was observed (Fig. 1a). Previous immunoprecipitation studies (Friesen and Guyda, 1971) have shown that this peak contained a material which cross-reacted with antiserum to sheep prolactin, probably representing monkey prolactin. The only other pituitary hormone found in this peak in significant amounts was monkey growth hormone. The demonstration by Herbert and Hayashida (1970) that monkey prolactin and growth hormone are immunologically distinct suggested that these two hormones might be separated by an immunological procedure. Guyda and Friesen (1971) succeeded in separating these two hormones by passing the fraction obtained from gel filtration through a small column of Sepharose which had been chemically linked to antibodies against human placental lactogen, selectively adsorbing out monkey growth hormone, leaving prolactin to pass through (Fig. 1b). More than 99% of the monkey growth hormone was removed by this procedure. The unadsorbed fraction, when assayed in the pigeon crop sac assay, possessed an average of 3-4 units/mg of biological activity when compared to a highly purified sheep prolactin standard (NIH-PS7, 24.3 IU/mg). If one assumes that monkey and sheep prolactin are equipotent in the pigeon crop sac assay, then the purity of the monkey preparation so obtained would be 10 to 20 %. We did not attempt further purification of this crude prolactin preparation because of the very limited amount of material

(a)



(b)

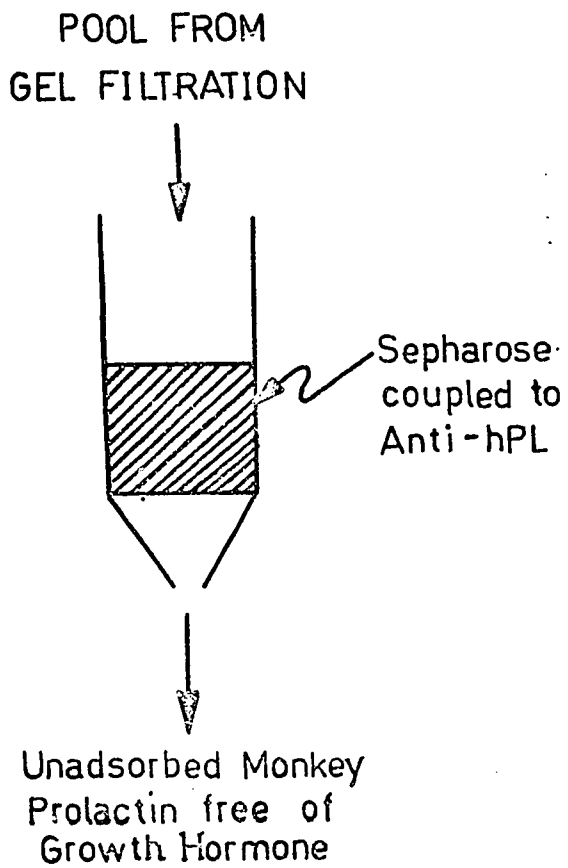


Fig. 1. Partial purification of monkey prolactin. (a) Fractionation of monkey pituitary incubation media on Sephadex G-100 column (2.5 x 90 cm) in 0.1M ammonium bicarbonate. Flow-rate 1 ml/min. (b) Further purification by affinity chromatography using Sepharose coupled to antibodies against human placental lactogen..

available.

(b) Preparation of Labeled Monkey Prolactin

The crude monkey prolactin preparation was iodinated with I^{131} or I^{125} by the method of Hunter and Greenwood (1962). The iodination mixture was applied to a column (1.0 x 0.5 cm) of Sepharose coupled to antibodies against sheep prolactin. Molecules immunologically related to sheep prolactin, presumably monkey prolactin, were retained by the column while other proteins and free iodide were washed through with 50 ml of 0.05 M sodium barbital buffer pH 8.6. Monkey prolactin I^{131} ($mPRL\text{-}I^{131}$) was eluted from the column with 2 ml of 4 M sodium thiocyanate solution which is known to dissociate antigen-antibody complexes (Appendix I). Further purification was carried out on a Sephadex G-100 (2.5 x 45 cm) column which removed damaged hormone was well as the sodium thiocyanate (Fig. 2). Fractions which showed the best binding with antiserum against monkey or ovine prolactin were used in the assay. Thus, even though we iodinated a monkey prolactin preparation which was heavily contaminated by other proteins, the application of immunoadsorbents in the purification of the iodination mixture enabled us to obtain a homogeneous tracer.

(c) Prolactin Standard

At the beginning of this investigation, an arbitrary standard was employed as purified primate prolactin was not available. A postpartum human serum, containing 161 ng/ml sheep prolactin equivalents by bioassay (Kleinberg and Frantz, 1971) and less than 5 ng/ml of growth hormone by radioimmunoassay, was kindly supplied by Dr. A. Frantz for use as standard in our assay. We assumed that sheep and human prolactin were equipotent in the bioassay used

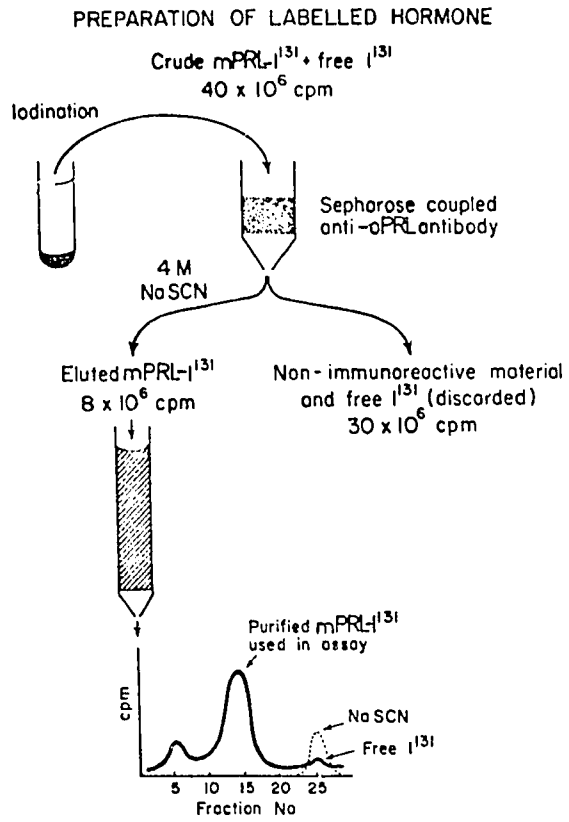


Fig. 2. Purification of labeled monkey prolactin (mPRL-I131) by affinity chromatography and gel filtration.

to measure its activity; hence, the concentration of human prolactin in the standard was also assumed to be 161 ng/ml.

Later, with the purification of human prolactin (see Section V), we employed purified human prolactin as standard. As there was excellent agreement between the results obtained with the two different standards, it was not necessary to apply a correction factor to the earlier data.

(d) Antisera

Even though our crude monkey prolactin preparation was far from pure, we nevertheless used it to immunize rabbits. Two rabbits were each injected subcutaneously at intervals of 1-2 weeks with approximately 100 µg of the crude monkey prolactin in 0.5 ml of 0.1M ammonium bicarbonate mixed with an equal volume of complete Freund's adjuvant. The animals were bled 10 days after the fourth injection and following each subsequent booster injection. Several other animals were immunised with sheep prolactin (Sigma, 30C-0940) and human growth hormone (Raben, 1959) by conventional methods.

(e) Immunoadsorbents

Antibodies to sheep prolactin (anti-oPRL) and to human placental lactogen (anti-hPL) were purified and coupled to Sepharose 4B (Pharmacia, Uppsala Sweden) by the procedure described by Cuatrecasas et al (1968) as detailed by Guyda and Friesen (1971).

(f) Sample Preparation

All dilutions were made in 2.5% bovine serum albumin in 0.05 M barbital buffer, pH 8.6. Serum samples were kept frozen at -20°C until assayed. Certain samples as indicated below were passed through an anti-oPRL or anti-hPL Sepharose column (1.0 x 0.3 cm) at a flow rate of

1 ml/min. and prolactin concentrations were measured before and after immunoadsorption. Fresh frozen human pituitary glands and placental tissue were homogenized by hand in a glass homogeniser in 1 ml 0.1 M ammonium bicarbonate per 20 mg wet weight of tissue. Human placental lactogen was prepared by the method of Friesen (1965) and a reduced alkylated derivative of placental lactogen was kindly prepared by Dr. B. Shome according to the method of Crestfield et al (1963). Human neurophysin was a gift from Dr. K.W. Cheng (Cheng and Friesen, 1972). The oxytocin used was commercial Syntocinon (Sandoz). Lysine vasopressin was obtained from Ferring AB, Sweden, Batch TB2215. A human pituitary gonadotropin preparation (LER 907) was obtained through the Endocrine Study Section, NIH. Human chorionic gonadotropin was a preparation from Ayerst Laboratories (1000 IU/mg). Human growth hormone was prepared according to Raben (1959), or obtained from NIH (Lot HS 1394). Insulin was from Sigma Chemicals, Lot 10C-1600 (24 IU/mg).

(g) Assay Procedure

A double antibody radioimmunoassay (Beck et al, 1965) was used in which 50 µl of standard or assay sample and 0.1 ml of appropriately diluted antiserum were added to 0.5 ml of 2.5% bovine serum albumin containing about 20,000 cpm of mPRL-I^{131} or mPRL-I^{125} per assay tube. After 72 hours of incubation at 4°C a suitable quantity of sheep anti-rabbit gamma globulin antiserum was added to precipitate antibody-bound mPRL-I^{131}. The precipitate which formed after 24 hours was centrifuged and counted in a Packard Autogamma counter (Model 3002). Human growth hormone was measured by a similar procedure, using Wilhelmi hGH HS-1394 as standard.

RESULTS AND COMMENTS

(a) Antisera Testing

Antisera raised against our crude monkey prolactin preparation (anti-mPRL) as well as those against sheep prolactin (anti-oPRL) were tested for their ability to bind labeled monkey prolactin (mPRL-I^{131}). As controls we initially employed several antisera against human growth hormone (anti-hGH). To our surprise we found that some, but not all, anti-hGH antisera bound significant amounts of mPRL-I^{131}. We therefore examined several more anti-hGH antisera for their ability to bind mPRL-I^{131}. The results are shown in Table I. At a final dilution of 1:75, all the anti-mPRL and anti-oPRL antisera and three of eight anti-hGH antisera bound more than 50% of the tracer. Those antisera which bound more than 70% of the tracer were further tested for their suitability for use in the radioimmunoassay. Of these, two antisera (anti-mPRL #60-4 and anti-hGH # 11) were found to be best in terms of sensitivity because 20 µl of the serum standard (having a presumed prolactin content of 3 ng) gave the greatest inhibition of binding of mPRL-I^{131}. These two antisera behaved very similarly in the assay at final dilutions of 1:15,000 (#60-4) and 1:500 (#11).

(b) Characterisation of the Assay

Fig. 3 shows a representative standard curve. The minimum amount of prolactin which could be detected with confidence was of the order of 0.25 to 0.5 ng. Using a sample volume of 50 µl, a sample concentration of 5 ng/ml could readily be detected. However, the sample volume could be increased to a maximum of 250 µl, making it possible to detect a serum concentration of 1 ng/ml. With this degree of sensitivity we have been able to detect prolactin in more than 90% of the subjects studied

TABLE I

BINDING OF LABELED MONKEY PROLACTIN BY DIFFERENT ANTISERA

ANTISERUM	ANTIGENS USED FOR IMMUNIZATION	BINDING OF mPRL I^{131} (%)		
		ANTISERUM DILUTION 1:75	ANTISERUM DILUTED TO BIND 50-65% mPRL-I131	
			No hPRL	+3 ng hPRL
Anti-oPRL #13	Commercial Sheep prolactin : Sigma, Lot. No. 30C-0940	80	58	55
15		85	50	43
72		55	-	-
73		70	63	59
Anti-mPRL #60-2	Crude mPRL Preparation	60	-	-
60-4		85	60	20
Anti-hGH # 2	Human growth hormone prepared according to Raben. (1959)	28	-	-
10		53	-	-
11		77	58	23
21		77	55	30
23		46	-	-
25		37	-	-
26		10	-	-
27		33	-	-
Normal rabbit serum	-	5	-	-

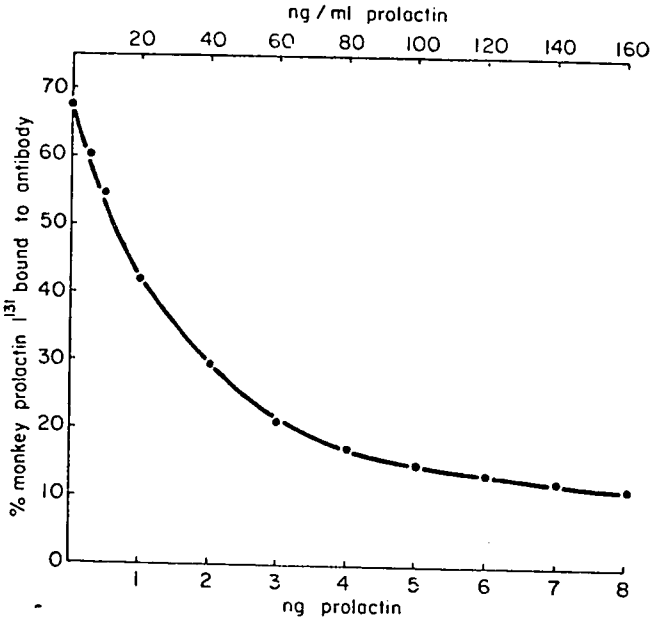


Fig. 3. Standard curve of human prolactin radioimmunoassay. The concentrations indicated on the upper abscissa refer to a sample volume of 50 µl.

(see Section IV). In the most recent 10 assays, the "50% intercept" (Rodbard et al 1970), that is the amount of prolactin required to give a 50% displacement of binding of tracer, averaged 1.12 ± 0.07 (SD) ng per tube. The useful range of the assay was from about 5 ng/ml to 60 ng/ml with sample volume at 50 µl.

Table II shows the within-assay and between-assay variability. For prolactin concentrations between 10 and 50 ng/ml, the intra-assay coefficient of variation was less than 10%, while the interassay coefficient was less than 20%.

The specificity of the assay depended on the homogeneity of the labeled hormone. Although the material used for iodination and immunization was an impure monkey prolactin preparation heavily contaminated by other proteins, the homogeneity of the tracer was assured by purification with affinity chromatography on an anti-oPRL Sepharose column which adsorbed only those molecules immunologically related to sheep prolactin, presumably monkey prolactin.

A variety of substances have been tested for cross-reactivity in the assay (Fig. 4). The crude monkey prolactin preparation was the most potent cross-reacting material, 3 ng giving a 50% inhibition of binding of the tracer. Sheep prolactin cross-reacted but in a non-parallel manner. A human pituitary extract gave a curve parallel to that of the standard serum, showing that in the human pituitary gland the cross-reacting material was immunologically indistinguishable from that in the serum. The Raben growth hormone preparation cross-reacted but only very weakly, requiring 1,250 ng to give a displacement equal to that produced by 3 ng of crude monkey prolactin. That this weak displacement by growth hormone was due to prolactin contamination was shown by the observation that after passage through an anti-oPRL Sepharose column all the cross-reacting material was removed without affecting the growth hormone content as measured by radioimmunoassay.

TABLE II

ASSAY VARIABILITY

Four serum pools (I-IV) were each assayed in duplicate in 9 separate assays. The intra-assay and between-assay variances and standard deviations were calculated according to Rodbard et al. (1970).

Assay No.	Sample No.							
	I		II		III		IV	
1	9.5	12.0	16.7	16.0	25	30	43	46
2	11.2	12.0	18.5	22.5	27	29	50	49
3	12.0	11.5	17.2	18.2	29	29	48	55
4	9.0	9.5	16.0	14.2	22	24	41	47
5	10.0	9.0	14.5	16.5	23	23	39	47
6	10.0	11.5	18.3	19.0	28	31	45	47
7	10.0	9.3	14.5	17.0	23	24	44	47
8	11.7	12.0	19.0	21.5	31	31	54	51
9	12.5	14.0	20.0	20.5	32	32	54	55
Mean	10.94		17.77		27.39		47.89	
Within-assay Variance	0.704		2.11		2.39		13.5	
S.D.	0.839		1.45		1.55		3.67	
Between-assay Variance*	1.49		4.45		10.20		15.59	
S.D.*	1.22		2.11		3.19		3.95	

*Variance and standard deviation of the means of each pair of estimates.

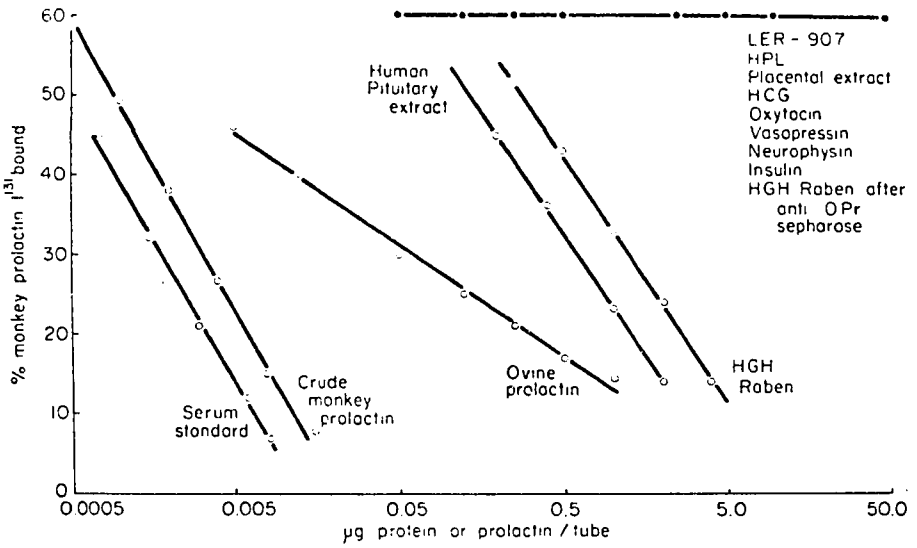


Fig. 4. Cross-reactivity of different materials in the assay : The inhibition curve of the standard serum is plotted on the assumption that it contained 161 ng/ml of human prolactin. Other curves are plotted on the basis of the amount of protein added. Among the substances showing no cross-reaction, only some (as specified in the text) have been tested up to a concentration of 50 µg. Frozen human pituitary gland or placental tissue was extracted with 1 ml of 0.1 M ammonium bicarbonate per 20 mg tissue wet weight. LER-907, human pituitary gonadotropin; HCG, human chorionic gonadotropin; anti-OPr Sepharose, Sepharose coupled to antibodies against sheep prolactin.

Human pituitary gonadotropin LER 907 (1 μg), human neurophysin (5 μg), oxytocin (0.5 U), lysine vasopressin (10 μg), a placental tissue extract (20 mg/ml wet weight), hPL (50 μg) and its reduced alkylated derivative (50 μg), human chorionic gonadotropin (100 IU), and insulin (10 μg), all did not cross-react in the assay.

Binding of mPRL-I^{131} by several anti-hGH antisera possibly could be due to the presence in these antisera of antibodies against human prolactin. To test this possibility, we obtained sera from pregnant and postpartum subjects and from patients with galactorrhea and acromegaly. As shown in Fig. 5, the binding of mPRL-I^{131} to anti-hGH (#11) was readily inhibited by sera from pregnant and postpartum subjects and from a patient with galactorrhea, but not by sera from the acromegalic patient or a normal subject. In addition, an even more significant finding was that the serum of the galactorrhea patient, which inhibited binding of the tracer to the antiserum, no longer did so after passage through an anti-oPRL Sepharose column, indicating that the cross-reacting substance had been removed.

As undiluted sera were assayed in most of our studies and concentrations were determined from standards diluted in 2.5% bovine serum albumin, it was necessary to determine whether this difference would produce any systematic error due to non-specific serum effects. Different amounts of the standard were diluted to 1 ml with bovine serum albumin or a human serum sample which contained 5 ng/ml human prolactin as measured by the assay. The concentrations of prolactin in these dilutions were determined and the results (Table III) indicate that the recovery was excellent, showing that the presence of serum proteins did not significantly affect the assay results.

To determine whether low levels of prolactin concentration

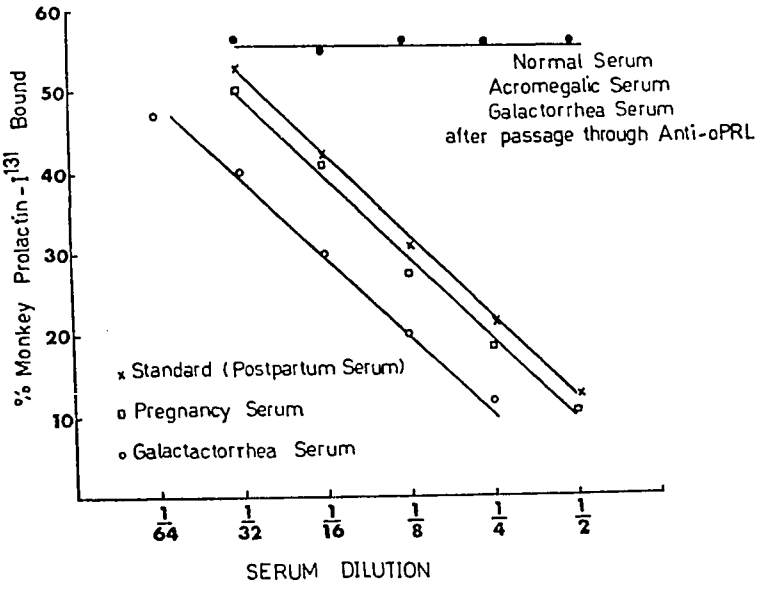


Fig. 5. Inhibition of binding of prolactin tracer by serial dilutions of different types of serum samples.

TABLE III

RECOVERY OF HUMAN PROLACTIN IN BSA* AND HUMAN SERUM

Amount of Serum Added (161 ng/ml) added		Observed hPRL	
		in BSA	in Human Serum
µl	hPRL (ng)	(ng/ml)	(ng/ml)
0	0	0	5
10	2	2	6
25	4	4	10
50	8	8	12
75	12	11	17
100	16	18	22
150	24	27	30
300	48	45	56

*BSA, bovine-serum albumin.

(5-10 ng/ml) were real or artifactually produced by non-specific serum effects, we passed a number of serum samples with prolactin concentrations in this range through a column of anti-oPRL Sepharose. Invariably all cross-reacting material in such samples was removed by this immunoadsorbent but not by a column of anti-hPL Sepharose.

DISCUSSION

The radioimmunoassay described has been carefully evaluated with regard to its specificity. While ovine prolactin cross-reacted in the assay, it gave a non-parallel response. No other hormone examined, including human growth hormone and human placental lactogen, cross-reacted in the assay significantly. The weak cross-reaction of human growth hormone has been shown to be due to contamination by small amounts of prolactin. The ability of the assay to distinguish prolactin from growth hormone or placental lactogen makes it possible to measure serum prolactin concentrations in conditions such as pregnancy or acromegaly in which high circulating levels of placental lactogen or growth hormone make it difficult to apply the recently described in vitro bioassays (Kleinberg and Frantz, 1971; Loewenstein et al 1971; Forsyth and Myres, 1971; Turkington, 1971a).

We attribute the surprising finding that a number of anti-hGH antisera bound mPRL-I^{131} to the presence of low titer anti-prolactin antibodies in these anti-hGH antisera. The demonstration that prolactin was a contaminant of growth hormone preparations makes it likely that rabbits immunized with growth hormone would also develop antibodies against prolactin. In addition, the binding of mPRL-I^{131} by anti-hGH antisera was specifically inhibited by sera from pregnant and postpartum subjects and from a

patient with galactorrhea but not by serum from a patient with acromegaly. These observations suggest that prolactin and not growth hormone was detected by the system, even though the antiserum used was obtained from an animal immunized with growth hormone.

We were particularly concerned about the finding that prolactin could be detected in the sera of most of our patients, including those with hypopituitarism (Section IV), whereas other investigators using in vitro bioassays (Kleinberg and Frantz, 1971; Loewenstein et al. 1971; Forsyth and Myres, 1971; Turkington, 1971a)have reported that most normal men and non-pregnant non-lactating women had no measurable prolactin in their sera. However, in these bioassays, serum from normal males was added routinely to the incubation media containing the prolactin standard, making it impossible to detect concentrations at or below that of the serum pool added to the standards. Our demonstration that, when serum samples with low prolactin concentrations were passed through an immunoadsorbent column of anti-sheep prolactin Sepharose, no prolactin could be detected in the eluant, strengthens our belief that even the low values were real and not due to non-specific interference by serum proteins.

Recently, using the in vitro bioassay which is sufficiently sensitive to detect circulating levels of prolactin in unextracted plasma, especially when the levels of prolactin are somewhat elevated, Frantz et al. (1972a)carried out an extensive study which showed that there was excellent agreement between results obtained by his bioassay and results obtained by our radioimmunoassay. Although similar correlative studies have not been made to compare our radioimmunoassay results with those of other bioassays or heterologous radioimmunoassays developed during the course of this work, the data that have emerged from the

different laboratories are in general agreement (see Section IV), providing good evidence that the radioimmunoassay we have developed measures human prolactin.

A HOMOLOGOUS RADIOIMMUNOASSAY FOR HUMAN PROLACTIN

Having a radioimmunoassay which specifically detects primate prolactin, it became feasible to embark on the purification of prolactin from human pituitary glands. This was accomplished by the procedure outlined in Section V. With the availability of a highly purified human prolactin preparation, we were able to develop a completely homologous radioimmunoassay for human prolactin. The behavior of this assay in terms of specificity and sensitivity is essentially the same as the monkey prolactin system.

Recently, a homologous radioimmunoassay for human prolactin has also been reported by Sinha et al. (1973) but the details have not yet been published.

SECTION IV : PHYSIOLOGICAL AND CLINICAL STUDIES

INTRODUCTION

The development of a radioimmunoassay for human prolactin has enabled us to measure serum prolactin concentrations in a large number of subjects. The results obtained reveal considerable similarity as well as interesting differences in animal and human prolactin physiology. Since the scope of this part of the present investigation was largely guided by what was known about prolactin in animals, a brief review of some aspects of animal prolactin physiology is given below.

REVIEW OF PHYSIOLOGY OF PROLACTIN IN ANIMALS

Control of Prolactin Secretion in Animals.- Prolactin secretion is predominantly under the control of the hypothalamus, though a number of agents may act directly on the pituitary gland to alter prolactin secretion. In mammals, the hypothalamus normally exerts an inhibitory effect on prolactin secretion. The evidence for this concept has been adequately summarised, among others, by Meites and Nicoll (1966), McCann and Porter (1969), and Meites (1972). Briefly, it has been shown that placement of lesions in the median eminence, pituitary stalk section, or transplantation of the pituitary gland away from its hypothalamic connection led to increased prolactin secretion as indicated by either a rise in serum prolactin concentration as measured by radioimmunoassay or the manifestation of effects usually associated with increased prolactin secretion, namely the initiation of lactation or the stimulation of corpus luteum function. Furthermore, prolactin inhibiting activity has been demonstrated in hypothalamic extracts or hypophyseal portal blood by both *in vitro* and *in vivo* methods. In addition to this inhibitory control, recent evidence indicates that there may also be a stimulatory component to the hypothalamic regulation of prolactin secretion (Nicoll et al.1970a; Krulich et al.1971; Valverde-R et al.1972).

The presumptive hormone mediating hypothalamic inhibition of prolactin secretion has been named 'prolactin inhibiting

factor' or PIF (Talwalker et al.1963). The chemical nature of PIF is unknown, though it appears to be a small molecule. Its activity in the hypothalamus appears to be under the control of catecholamines (see Meites et al.1972). Chemical agents which increase hypothalamic catecholamine activity, such as L-DOPA and the monoamine oxidase inhibitors, also increase PIF activity in the hypothalamus and decrease prolactin levels in the serum. Conversely, agents such reserpine and chlorpromazine,which diminish hypothalamic catecholamine activity, also lower PIF activity and increase serum prolactin. Kamberi and coworkers recently demonstrated that infusion of dopamine into the third ventricle of rats led to the appearance of PIF activity in hypophyseal portal blood (Kamberi et al.1971b) and a lowering of serum prolactin (Kamberi et al. 1971a), whereas direct perfusion of the pituitary gland with dopamine had no effect. On the basis of these observations, it appears reasonable to conclude that dopaminergic fibers, which are found in the hypothalamus, control PIF activity and hence prolactin secretion. A further finding of Kamberi et al.(1971c), showing that serotonin infusion into the third ventricle of rats was followed by an elevation of serum prolactin, raises the possibility that prolactin secretion may be controlled by two opposing systems in the hypothalamus, namely dopaminergic and serotoninergic fibers.

Many stimuli which increase or decrease prolactin secretion do so by acting via the hypothalamus. Agents which have been found to decrease hypothalamic PIF activity include the suckling or milking stimulus, several tranquillizing drugs, and various steroid hormones including estradiol (see Meites, 1972). Agents which increase PIF activity and reduce prolactin secretion include L-DOPA and monoamine oxidase inhibitors (Lu and Meites, 1971), ergot derivatives (Wuttke et al.1971), and prolactin itself (Chen et al.1967; Clemens and Meites, 1968; Voogt and Meites, 1971). Whether or not any of these agents also act by influencing 'prolactin releasing activity' in the hypothalamus is unknown.

Prolactin secretion is also affected by a number of agents acting directly on the pituitary gland. In vitro studies indicate that prolactin secretion may be stimulated by estrogens (Nicoll and Meites, 1962), thyroxine (Nicoll and Meites, 1963), low doses of catecholamines (Koch et al.1970), as well as thyrotropin releasing hormone (TRH) (Tashjian et al.1971; LaBella and Vivian, 1971; Nicoll, 1972). High doses of catecholamines (MacLeod, 1969; Birge et al.1970), ergot derivatives (Lu et al.1971), and a number of other drugs inhibit prolactin secretion in vitro (see Gold and Ganong, 1967). Many of these effects may be pharmacological and have no physiological significance. However, at least in the female rat, estrogen is thought to be an important physiological stimulus of prolactin secretion at the onset of puberty, at proestrus and estrus, and possibly at the time of parturition (Meites et al.1972)

Serum Prolactin Levels in Animals.- Following the development of radioimmunoassays, serum prolactin concentrations have been measured in the rat, mouse, sheep, goat, and cow under different physiological circumstances. Meites et al. (1972) have recently reviewed in detail the studies carried out in the rat. The data obtained by other investigators in other species resemble those in the rat in many respects.

(a) Before Puberty : Few studies have been carried out in immature animals. Prolactin levels are high in the bovine fetus but fall sharply in the neonatal period (Oxender et al. 1972). Young mice show a progressive increase in serum prolactin concentration from day 2 to day 20 after birth (Sinha et al.1972), whereas in the rat prepubertal levels remain low up to day 36, showing a sharp rise with vaginal opening on day 37 (Voogt et al.1970).

(b) During Reproductive Life : In the female rat, circulating prolactin concentrations are low during diestrus and high at proestrus and estrus (see Meites et al.1972). A similar cyclic variation has also been found in the sheep (Reeves et al.1970; Cumming et al.1972), goat (Bryant and Greenwood, 1968), mouse (Kwa and Verhofstad, 1967b), and cow (Raud et al. 1971).

(c) During Pregnancy and Lactation : Except for an early transient rise in some species, serum prolactin levels during pregnancy in most of the species examined remain low until about the time of parturition when a sharp increase occurs, persisting into the postpartum period (see Meites et al. 1972). The low levels observed during most of pregnancy may have to be revised in view of the recent demonstration (Freeman and Neill, 1972) that in the pseudopregnant rat prolactin might be released in surges at night for up to 11 days. More frequent blood sampling at appropriate times may reveal a different pattern of prolactin secretion during pregnancy. The rise of serum prolactin occurring at the time of parturition is probably associated with increased estrogen secretion and possibly with the stress of parturition (Meites et al. 1972). In the postpartum period, suckling or milking is a powerful stimulus to prolactin secretion (see Meites et al. 1972).

(d) Males : Little data are available on prolactin levels in male animals. Studies in the male rat indicate that there is no cyclical variation in serum prolactin concentration and that the levels are generally as low as or lower than in the female during diestrus (Niswender et al.1969; Amenomori et al.1970). A recent study (Convey et al.1971) reports a rise of prolactin levels in bulls shortly after ejaculation.

Physiological Role of Prolactin.- While prolactin has been found to exhibit more than 80 biological activities in vertebrates (Nicoll and Bern, 1972), only some of these have established physiological significance in mammals.

Prolactin is best known for its function in mammary growth and lactation. The classical studies of Lyons et al.(1958) clearly showed that prolactin forms part of the hormone complex required for full mammary development and lactation. In most species studied, it is thought that postpartum lactation is initiated by a rise of prolactin and adrenocorticotrophin secretion at the time of parturition, accompanied by a fall in estrogen and progesterone secretion. The evidence for this

concept has been reviewed (Meites, 1967; Cowie and Tindall, 1971). In the postpartum period, the suckling-induced release of prolactin helps to maintain lactation (see Cowie and Tindall, 1971).

Prolactin also appears to have physiological effects on ovarian function in some species. Meites and his associates have presented convincing evidence that the rise of prolactin secretion at proestrus and estrus in rats (Wuttke and Meites, 1971) and mice (Grandison and Meites, 1972) is responsible for the morphologic regression of corpora lutea formed during the previous estrous cycle. Prevention of this increase in prolactin secretion at proestrus by administration of ergot alkaloids led to persistence of the corpora lutea, an effect which could be reversed by the simultaneous administration of sheep prolactin. Traditionally, however, prolactin is better known for its luteotropic activity. It helps to maintain the function of the corpus luteum in early pregnancy or pseudopregnancy in certain species of rodents (see Lyons and Dixon, 1966). It appears that the functional state of the luteal tissue at the time when a rise in serum prolactin occurs determines whether prolactin acts to induce luteolysis or to maintain corpus luteum function (Malven and Sawyer, 1966; Meites et al.1972).

The physiological role of prolactin in males is unknown. Although there are reports that prolactin may stimulate growth of male accessory sex organs, influence testicular enzyme activity, affect water and electrolyte balance, in addition to having growth hormone like effects in mammals (see Nicoll and Bern, 1972), it remains to be determined how many of these effects are of physiological significance.

MATERIALS AND METHODS

Sample Collection and Preparation.- Most of the samples examined by radioimmunoassay in this part of the study had been collected for other purposes in the past and stored frozen in our laboratory for periods of up to 3-4 years. Some samples were obtained more recently in collaborative studies with

Drs. Barbeau, Belanger, Bowers, Desjardin, Faiman, Gilette, Guyda, Hardy, Samaam, Shibata, Singer, Tyson, VanCampenhout, Volpe, and Wilansky. In these latter studies, blood was usually drawn at varying times of the day and no overnight fast was imposed. The experimental conditions through which certain individuals were put are indicated below. Blood samples were left to clot overnight at 4°C, after which serum was recovered by centrifugation and stored at -20°C until assayed. Amniotic fluid was obtained at induction of labor or abortion or during diagnostic amniocentesis and stored frozen. Certain serum and amniotic fluid samples and an extract of a frozen human pituitary gland were subjected to gel filtration on a Sephadex G-100 column (2.5 x 90 cm) equilibrated with 0.1 M ammonium bicarbonate. Fractions were collected at room temperature and assayed for prolactin. Some serum samples were passed through columns of either anti-sheep prolactin Sepharose or anti-human placental lactogen Sepharose, and the prolactin concentrations measured before and after immunoadsorption.

Radioimmunoassays.- Radioimmunoassays for prolactin and growth hormone were carried out by the double antibody procedure as described in Section III.

RESULTS AND COMMENTS

Serum Prolactin Concentrations in Different Physiological States.-

(a) Variations with Age and Sex : The adults and children included in this study were either normal volunteers or electively hospitalised patients in no obvious distress, having no suspected endocrinopathy, and not being treated with drugs known to affect prolactin secretion. The newborns had full term normal deliveries and were free from any obvious neonatal pathology.

Table IV summarises the results obtained. The average serum concentration of prolactin among normal men was 6.0 ± 0.6 (SEM) ng/ml, while that in non-pregnant non-lactating women was 8.1 ± 0.8 (SEM) ng/ml, the difference being statistically significant ($p < 0.05$ by the Student's t-test). Individual

TABLE IV

VARIATION OF SERUM PROLACTIN CONCENTRATIONS WITH AGE AND SEX

GROUP	AGE	NO.	SERUM PROLACTIN ng/ml Mean ± S.E.M.
Adults			
males	16-84	68	6.0 ± 0.6
females+	16-85	46	8.1 ± 0.8
Children*	1-15	48	6.5 ± 0.7
Newborn*	at birth	19	257 ± 23.0
	24 hr	7	192 ± 51.0
	1-2 wk	7	84 ± 25.0
	5-6 wk	5	20 ± 2.9

+Pre- and post-menopausal subjects have similar levels.

*No significant difference was observed between male and female.

values for both males and females varied from less than 2.5 ng/ml to 30 ng/ml; variation appeared to be greater among females. It was unusual to observe values above 30 ng/ml in normal adults at rest. In our experience, the great majority of normal subjects had clearly measurable prolactin levels in their sera, there being less than 10% of normal individuals having serum prolactin levels < 2.5 ng/ml. The possibility that low levels might be artifactually produced by non-specific serum protein interference was tested by passing serum samples with low but measurable prolactin concentrations through a column of anti-sheep prolactin Sepharose. Invariably all cross-reacting material in such samples was removed by this immunoadsorbent but not by a column of anti-human placental lactogen Sepharose.

In children in the age group 1-15 years, levels comparable to those in the adult male were found, there being no difference between males and females in this group.

In the newborn, in the first 24 hours of life, serum prolactin concentrations were of the order of 200 ng/ml. The concentration gradually fell with increasing age so that at the end of 6 weeks the average concentration had reached 20 ng/ml. Again there was no sex difference in this age group.

We have insufficient data to know whether serum prolactin levels in postmenopausal women differ signficantly from the premenopausal age group.

(b) Prolactin Levels during the Menstrual Cycle : In 9 adult female volunteers with normal menstruation, serum prolactin was assayed throughout the cycles. As can be seen in Fig. 6 the average levels were fairly constant at about 10 ng/ml throughout the cycle, no definite elevation being seen either at midcycle or in the luteal phase. In individual subjects, however, serum prolactin fluctuated considerably from day to day, accounting for the large standard deviations observed. Measurement of luteinising hormone in the same samples showed the usual mid-cycle ovulatory peak in all cases.

(c) Prolactin Levels during Pregnancy and the Puerperium :

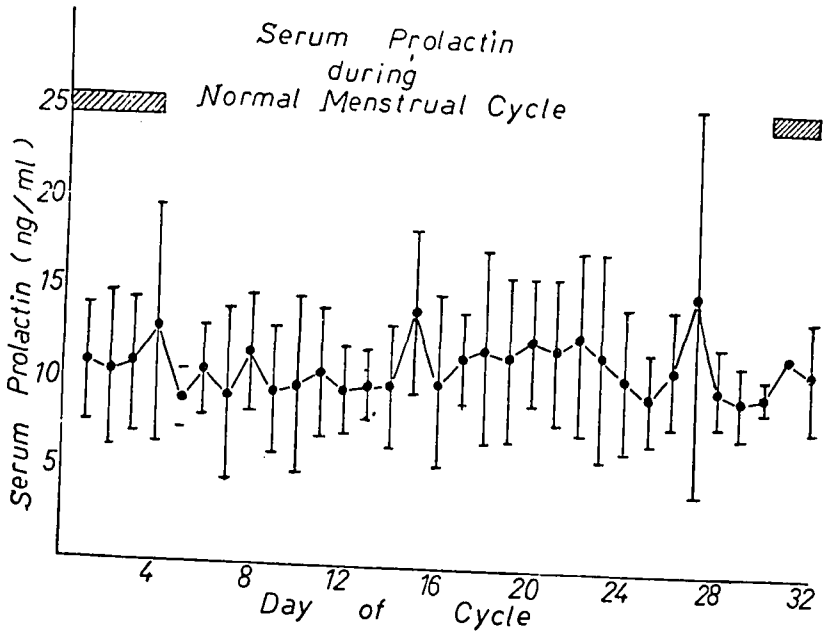


Fig. 6. Serum prolactin concentrations during the menstrual cycle. The mean ± SD is shown for 9 subjects.

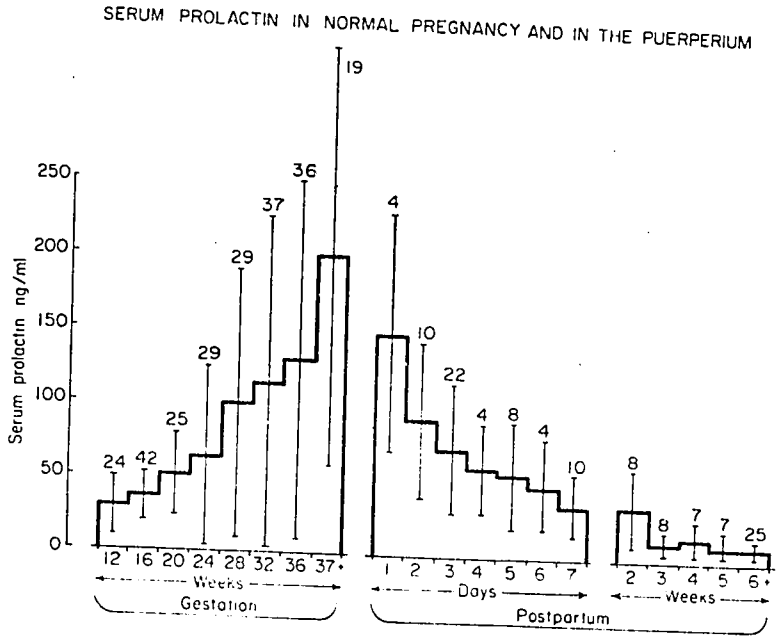
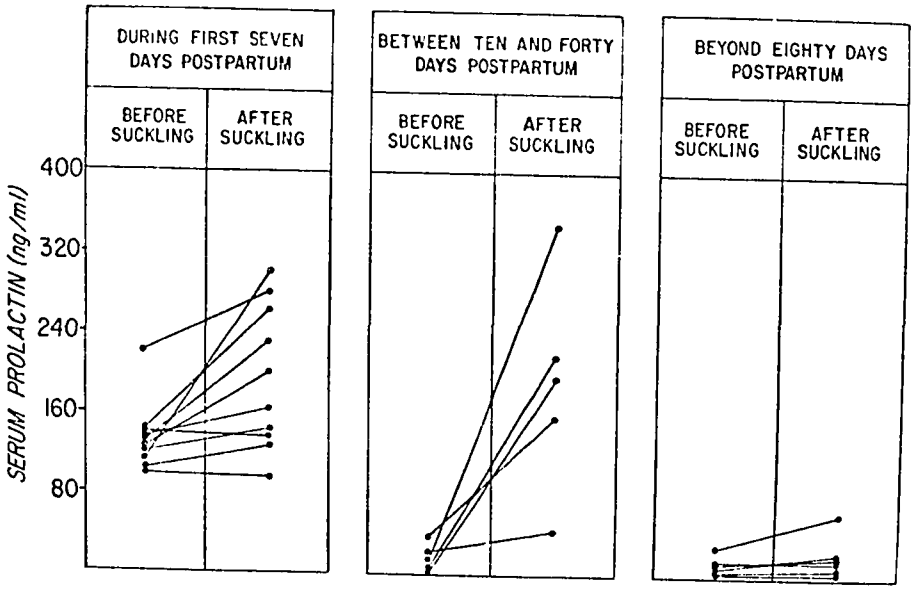


Fig. 7. Serum prolactin concentrations during pregnancy and puerperium. The prolactin level is given as mean ± SD. The number of subjects in each group is indicated.

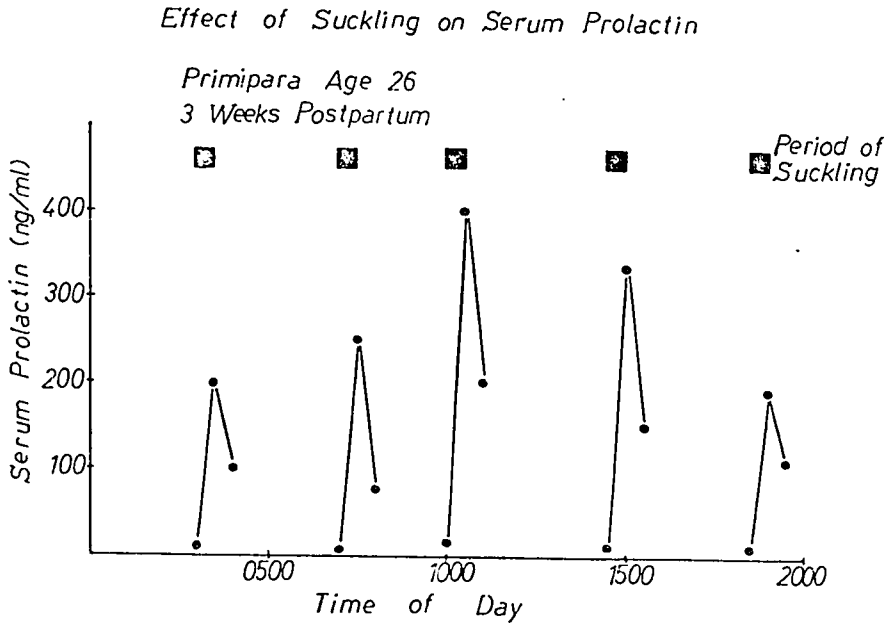
Fig. 7 shows the results of a study on serum prolactin in more than 300 subjects during various stages of pregnancy and the immediate postpartum period. Elevations of serum prolactin were evident as early as 8 weeks gestation, the average concentration in the first trimester being about 30 ng/ml. With advancing pregnancy, serum prolactin concentration progressively increased to a maximum of about 200 ng/ml at term. Postpartum, in the absence of breast-feeding, the serum prolactin fell fairly rapidly and by the end of the first week had returned to 30 ng/ml. There were large variations at each stage of pregnancy, particularly in the third trimester where values ranged from 40 to 600 ng/ml.

(d) Effect of Suckling on Serum Prolactin Concentration : In the postpartum period, suckling was a potent stimulus to prolactin secretion in women. Fig. 8a shows the changes in serum prolactin levels occurring in nursing mothers 30 minutes after feeding their infants. During the first postpartum week, serum prolactin increased from an average basal concentration of about 100 ng/ml before nursing to about 200 ng/ml after nursing. Between 10 and 40 days postpartum, the increases were far more dramatic, commonly ranging from 10 to 20 fold above the basal level. However, beyond 80 days, suckling had little or no effect on serum prolactin even though in these individuals lactation appeared to be well maintained. In a single nursing mother in her third postpartum week, blood was sampled 5 minutes before and 30 and 60 minutes after each episode of nursing during the course of a day. Fig. 8b shows that serum prolactin levels increased 10-20 fold at 30 minutes after the start of breast feeding. The subsequent fall was equally rapid, suggesting that the half time disappearance of prolactin in such individuals is less than 30 minutes.

Serum Prolactin Levels in Pathological Conditions.- In addition to studying physiological variations of serum prolactin, we have examined a number of patients with either hypothalamic-pituitary diseases or conditions possibly associated with



(a)



(b)

Fig. 8. Effect of suckling on serum prolactin. In (a), the increases after suckling were significant for the first two periods ($p \leq 0.05$).

abnormal prolactin secretion. The results are summarised in Fig. 9.

A total of 24 patients with galactorrhea were studied. Among these, 8 had pituitary tumors, 4 developed galactorrhea following contraceptive medication, and one after surgery (hysterectomy). In the rest there was no obvious cause for the symptom. Serum prolactin concentrations were distinctly elevated in 21 of the 24 patients, being 30 ng/ml or higher (Fig. 9). In 3 cases, two of whom had idiopathic galactorrhea and the third developed galactorrhea after contraceptive therapy, serum prolactin was less than 20 ng/ml, which was well within the normal range. The only male patient in this group had a serum prolactin of 60 ng/ml with slight gynecomastia and moderate galactorrhea. The cause of galactorrhea in his case was unknown. In the group as a whole, there did not appear to be any correlation between the serum prolactin and the severity of galactorrhea, nor was there any clear relationship between prolactin concentration and the presumed etiology of the condition, though patients with pituitary tumors generally had higher levels; the highest level (1500 ng/ml) was found in a patient with a chromophobe adenoma.

Nineteen cases of acromegaly, some of whom had been previously treated by conventional pituitary irradiation, were examined. With the exception of a single case, all had serum prolactin levels in the normal range (Fig. 9). However, their basal growth hormone levels were all elevated above 10 ng/ml. The case having a prolactin concentration of 200 ng/ml was a four year old girl, with gigantism but no galactorrhea or gynecomastia, in whom the serum growth hormone was also extraordinarily high at 500 ng/ml. There was no deficiency in thyroid or adrenal function. A small pituitary adenoma (chromophobe) was later removed from the patient.

Nine patients with breast cancer not responding to conventional therapy were studied (Fig. 9). After all medication except analgesics had been withdrawn for one week, blood was sampled for prolactin assay. In three cases, an oral dose of

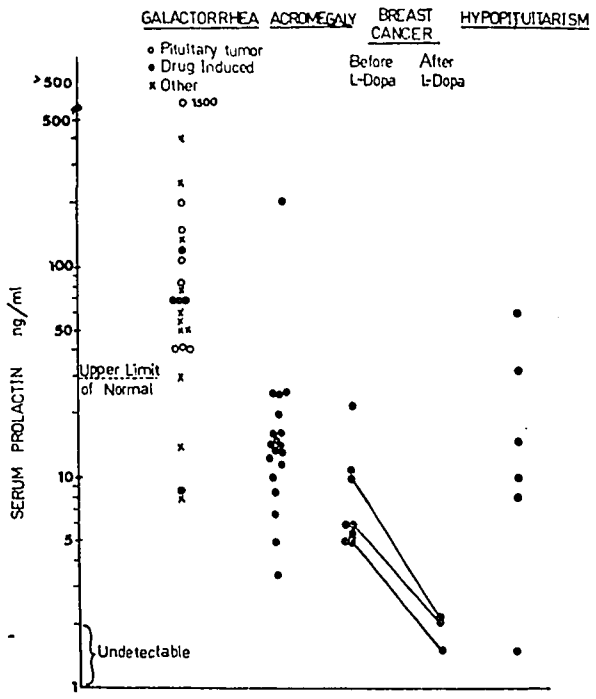


Fig. 9. Serum prolactin concentrations in cases of galactorrhea, acromegaly, breast cancer and hypopituitarism. Three patients with breast cancer were given 500 mg of L-DOPA orally and prolactin was measured before and after L-DOPA administration (see text).

500 mg of L-DOPA was given and blood was withdrawn 0, 1, 2, 3, and 4 hours later. All nine patients had serum prolactin levels within the normal range; the mean concentration of serum prolactin before L-DOPA administration was 9.0 ± 1.8 (SEM) ng/ml, which was not significantly different from that found in normal women (Table IV). In the three patients subjected to the L-DOPA test, a fall in serum prolactin was observed, the maximum percentage decrease being greater than 60% in all three cases.

Among the hypopituitary patients, the surprising finding was that prolactin was detected in 5 of the 6 cases examined. In 4 of these 5 cases, there was growth retardation due to growth hormone deficiency. The patient with abnormally elevated prolactin level of 60 ng/ml had a craniopharyngioma which had been removed. The only patient in this group having no detectable circulating prolactin was a patient who had recently undergone hypophysectomy for breast cancer. That prolactin, and not some non-specific substance, was being measured in the serum samples of this group of hypopituitary patients was shown by the complete removal of the cross-reacting substance in their sera by anti-sheep prolactin Sepharose.

Modification of Prolactin Secretion by Pharmacological Agents in Man.- This part of the study, which examined the effects of thyrotropin releasing hormone (TRH), chlorpromazine, and L-DOPA on prolactin secretion in man was carried out in collaboration with Drs. Bowers and Tyson, who kindly provided the blood samples for radioimmunoassay. The results are summarized in Fig. 10.

Synthetic TRH (25 μg) was given intravenously as a single bolus and blood was drawn for prolactin estimations immediately before and 15 and 30 minutes after TRH administration. The response in 28 normal individuals (14 males and 14 females) were shown in Fig. 10. TRH caused a 3 to 6 fold increase in serum prolactin which was maximum at 15 minutes but remained elevated at 30 minutes. The response was greater in females than in males, but large individual variations were noted.

Eleven normal females (aged 18-21) were given 25 mg of

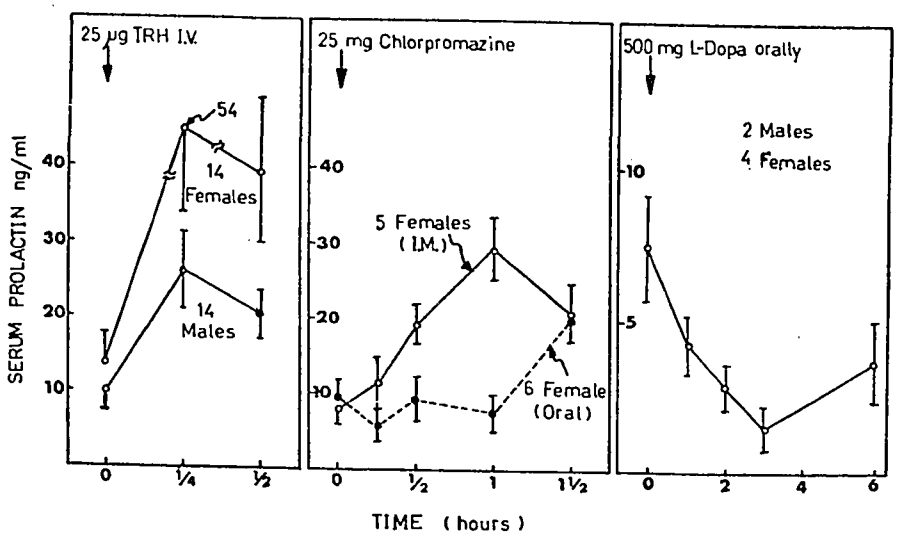


Fig. 10. Effects of thyrotropin releasing hormone (TRH), chlorpromazine, and L-DOPA on serum prolactin. The altered serum prolactin levels after treatment were significantly different from "0" time values for all three drugs ($p < 0.01$).

chlorpromazine either orally or intramuscularly and blood samples were taken 0, 30, 60, and 90 minutes after administration of the drug. Fig. 10 shows that after oral chlorpromazine no consistent change in serum prolactin occurred until 90 minutes whereas intramuscularly the drug caused a more rapid increase in serum prolactin. The peak was reached at 60 or 90 minutes and in all subjects there was at least a two-fold increase in serum prolactin.

A single oral dose of L-DOPA was given to 6 normal volunteers, 2 males and 4 females, and blood samples were taken at -30 minutes, and 0, 1, 2, 3, 4, and 6 hours later. Fig. 10 shows that after L-DOPA administration, there was a fall in serum prolactin with the lowest values occurring at about 3 hours, after which the prolactin levels gradually rose again.

Prolactin in Amniotic Fluid.- An incidental observation we have made in the course of this work is that human amniotic fluid is extremely rich in prolactin. Table V shows the prolactin concentrations in amniotic fluid at different stages of pregnancy in comparison to those in maternal serum at the same times. It is clear that the amniotic fluid concentrations of prolactin were 6 to 85 times higher than those in maternal serum, the difference being greatest in early pregnancy.

Serial dilutions of amniotic fluid gave inhibition curves parallel to that of the pituitary prolactin standard in the radioimmunoassay, indicating that there is no obvious immunological difference between the two prolactins. In addition, the elution volume of amniotic fluid prolactin on gel filtration was very similar to those of pituitary and serum prolactin, showing that they were of very similar size and excluding the possibility that amniotic fluid prolactin was a small fragment of pituitary prolactin. Furthermore, as described in Section V, purified amniotic fluid prolactin has the same electrophoretic mobility and amino terminal residue (leucine) as pituitary prolactin.

DISCUSSION

With the availability of a specific and sensitive radio-

TABLE V

PROLACTIN IN HUMAN AMNIOTIC FLUID AND MATERNAL SERUM

DURATION OF PREGNANCY WEEKS	NO. STUDIED	PROLACTIN (ng/ml)		
		AMNIOTIC FLUID	MATERNAL SERUM	AF/SERUM
10-14	3	2,550	30	85
15-20	7	2,000	50	40
21-28	4	1,800	100	18
29-35	4	1,000	150	6
36-38	11	1,210	137	9

immunoassay for human prolactin, it is now possible to explore the secretion of prolactin in human health and disease with some facility. Previous attempts to measure prolactin levels in serum or urine of patients by bioassay required the extraction of large volumes of urine or blood before a positive response by the pigeon crop sac assay was obtained. Despite the technical difficulties of the extraction procedures and the problem of non-specific inflammatory reactions which follow the injection of crude extracts, various estimates of serum and urine prolactin levels have been reported in patients who were pregnant, lactating, or who had galactorrhea. These data have been reviewed by Apostolakis (1968). A direct comparison of the results obtained in many of these studies with our own is difficult because of the very large variations in prolactin levels reported in these studies. Results obtained by investigators using the more recently described in vitro bioassays and heterologous radioimmunoassays are in general in better agreement with our data.

(a) Physiological Variations of Prolactin Concentration.-

The serum prolactin levels we have found for normal subjects of different ages and sexes are in reasonable agreement with other reports which have appeared during the course of this study. One point of apparent discrepancy which needs to be clarified is the difference between males and females. In our earlier study (Friesen et al.1972c)which was confined to hospitalized patients, as well as in the study of Frantz et al. (1972a) on normal healthy individuals and in the more recent studies of L'Hermite et al.(1972) and Sinha et al.(1973) in unselected subjects, no significant difference was found between males and females. However, the present study carried out among normal volunteers and certain selected hospital patients confirms the sex difference recently reported by Jacobs et al.(1972). The discrepancy between the different studies may be related to choice of subjects, time of blood sampling, the assays employed, and possibly other factors.

The high prolactin concentrations observed in the newborn, also noted by L'Hermite et al.(1972), indicate that the pituitary gland of the neonate is probably very active in the synthesis and secretion of prolactin, since preliminary studies showed that prolactin did not readily cross the placental barrier in monkeys (Friesen et al.1972a; Tyson, personal communication, 1972). The physiological significance of this finding remains to be determined, but the intriguing observation of Sinha et al.(1971) that injection of anti-prolactin antiserum into newborn mice led to a high mortality suggests that prolactin might serve a critical but as yet unknown role during the neonatal period.

The pattern of prolactin concentration we have observed during the menstrual cycle has also been observed by Midgley (1972), and confirmed by L'Hermite et al.(1972), though in the latter study there was some suggestion for a slightly higher level during the luteal phase compared to the follicular phase. The absence of any definite elevation of serum prolactin during the menstrual cycle is somewhat surprising since in the female of several species of animals a cyclical pattern of prolactin secretion was observed (see Meites et al.1972). It is possible that more frequent blood sampling may reveal a different pattern since prolactin secretion in man may occur in surges (L'Hermite et al.1972) and exhibits a diurnal rhythm (Sassin et al.1972; Nokin et al.1972). The proestrus surge of prolactin secretion in the rat and mouse has been shown to be responsible for the regression of the corpora lutea formed during the previous cycle (Wuttke and Meites, 1971; Grandison and Meites, 1972). In women it is not known what causes luteolysis, but the absence of any clear elevation of serum prolactin during the menstrual cycle would suggest that prolactin is probably not involved. This suggestion is supported by Midgley (1972) who observed by radioautography that labeled human prolactin failed to localise in monkey ovarian tissue.

The progressive increase in serum prolactin during human pregnancy, an observation also noted by Midgley (1972) and confirmed by Jacobs et al.(1972), is also strikingly different from

the pattern observed in many animals, including the rat (Amenomori et al.1970; Linkie et al.1972), sheep (Arai and Lee, 1967; Saji, 1967; McNeilly, 1971), cow (Schams and Karg, 1969, 1970), goat (Bryant and Greenwood, 1968), and monkey (Friesen et al. 1972a). In these species, serum prolactin remains low through most of pregnancy until near parturition when a sharp rise occurs. The reason for the species difference is not known. The physiological significance of the rise in serum prolactin in pregnant women is also not clear. In some rodents, prolactin is necessary for the maintenance of corpus luteum function in early pregnancy, but in man there is no evidence that prolactin is luteotropic. In view of the known mammotrophic effect of prolactin in animals, however, it might be suggested that prolactin, in synergism with other hormones, promotes the development of the human breast during pregnancy. There does not appear to be any doubt, however, that prolactin is required for both the initiation and maintenance of lactation in women. Recent studies demonstrated that suppression of prolactin secretion with 2-Br-α-ergocryptine not only prevented the initiation of lactation in women (Varga et al.1972; del Pozo et al.1972) but also abolished established lactation (Brun del Re et al. 1973).

Suckling in postpartum women, as in animals, is a potent stimulus for prolactin secretion, a finding confirmed by Frantz et al.(1972a). While it appears likely that prolactin release following suckling helps to maintain postpartum lactation, it is not clear whether the very high levels attained after nursing are required. Beyond 80 days postpartum, in the few subjects we have studied there was little change in serum prolactin with suckling, but lactation appeared to be well maintained. It is possible that a low basal secretion might be sufficient to maintain lactation once it is established.

(b) Serum Prolactin Levels in Pathological Conditions.-

Among our patients with galactorrhea, the majority, but clearly not all, had elevated serum prolactin concentrations. This was also the experience of Frantz et al.(1972a) but not of Turkington (1971a) who found raised prolactin levels in all

cases of galactorrhea. The finding of elevated prolactin levels in cases of galactorrhea implicates excessive prolactin secretion in the pathogenesis of the condition. This suggestion is supported by the successful treatment of a high proportion of these cases with pharmacological agents which suppress prolactin secretion (Lutterbeck et al.1971; Malarkey et al.1971; Besser et al. 1972; Turkington, 1972a; Varga et al.1973). However, the finding of normal prolactin levels in some cases of galactorrhea, as well as the absence of galactorrhea in some patients with high prolactin levels associated with pituitary tumors (Frantz et al. 1972a; Friesen et al.1972a) indicates that the relationship between prolactin and galactorrhea may be more complex.

The results we obtained for <u>acromegalics</u> not only confirm the separate identity of human prolactin and growth hormone, but also provide additional support for the specificity of our radioimmunoassay. The finding of extremely high prolactin and growth hormone levels in one patient with a pituitary tumor suggests the possibility that some pituitary tumors may secrete excessive amounts of more than one hormone, although the possibility that the high prolactin level was due to interference with the normal hypothalamic inhibition of prolactin secretion could not be excluded.

Our study of <u>breast cancer</u> patients must be considered preliminary since the patients studied belonged to a highly selected group : those who did not respond or had stopped responding to conventional therapy. Superficially our results would suggest that breast cancer patients were not different from normal subjects in their 'basal' prolactin levels and their response to L-DOPA administration. Other investigators (Forrest, 1972; Frantz et al.1972b) have also reported similar findings, but Murray et al.(1972) reported that breast cancer patients had slightly elevated basal prolactin levels which were less readily suppressible by L-DOPA treatment compared to controls. In addition, Berle and Voigt (1972) recently claimed that 26 of 64 patients with breast cancer had detectable prolactin in their

sera by a local pigeon crop sac assay whereas only 6 of 66 controls had measurable levels. The discrepancies among the different studies might be related to different assay systems employed, but deserve further examination as animal studies have provided abundant evidence implicating prolactin in the development and growth of mammary tumors (Furth, 1972; Meites et al.1972; Pearson et al.1969). In the past 1-2 years, several attempts have been made to treat human breast cancers with drugs which suppress prolactin secretion. The results so far are encouraging but somewhat conflicting (Stoll, 1972; Dickey and Minton, 1972; Minton and Dickey, 1972; Heuson, 1972; Pearson et al.1972; Frantz et al.1972b).

In studying patients with hypopituitarism we were surpised to find normal or even elevated serum prolactin levels in 5 of 6 cases. We were satisfied that the values measured were real since the cross-reacting material in their sera was completely and specifically adsorbed by antibodies to sheep prolactin. Clearly, many patients judged to be hypopituitary by other criteria may not be deficient in prolactin secretion. Indeed, in some patients prolactin levels might be elevated above normal (see also Tolis et al.1973), suggesting hypothalamic involvement by the disease process. An elevated prolactin level in cases of 'idiopathic' hypopituitarism would suggest a primary hypothalamic disorder with deficiency in hypophysiotropic hormones.

(c) Effects of Drugs on Prolactin Secretion.-

Our observation that TRH and chlorpromazine stimulate and L-DOPA suppresses prolactin secretion in man has largely been substantiated by other studies (Kleinberg et al.1971; Jacobs et al.1971; Bowers et al.1971; Turkington, 1972b; Sachson et al. 1972) and generally agree well with studies in experimental animals (Tashjian et al.1971; Meites et al.1972; LaBella and Vivian, 1971; Nicoll, 1972; Schams, 1972; Convey et al.1973, Kelly et al.1973, Davis and Borger, 1972); the rat may be an exception in that it apparently does not respond with an increase in serum prolactin following an acute dose of TRH (Lu

et al.1972). TRH apparently acts directly on the pituitary gland to stimulate prolactin secretion (Tashjian et al.1971; LaBella and Vivian, 1971; Nicoll, 1972), whereas chlorpromazine and L-DOPA act mainly via the hypothalamus by altering catecholamine and PIF activity (Meites et al.1972; Kamberi et al.1971b).

The availability of pharmacological agents which alter prolactin secretion by different mechanisms may be of considerable value in the diagnosis of hypothalamic-pituitary disorders, especially cases of hypopituitarism. A positive response to TRH indicates the presence of functional pituitary tissue. A failure to respond to chlorpromazine or L-DOPA would suggest a primary hypothalamic lesion if the response to TRH is normal. The value of these tests in diagnosing such cases of 'hypothalamic hypopituitarism' is obvious : administration of the appropriate releasing hormone should correct the defect.

Although at present information on the regulation of prolactin secretion in man is incomplete, the observation that chlorpromazine and L-DOPA respectively increases and decreases serum prolactin levels in both man and animals suggests that the mechanisms controlling prolactin secretion in man might be very similar to that in other mammals. The possibility that TRH might also be the presumptive 'prolactin releasing factor' has been considered (Bowers et al.1971). However, the recent demonstration that pig and rat hypothalami extracts contained a prolactin releasing activity distinct from TRH (Valverde-R et al.1972) favors the concept that the prolactin releasing activity of TRH may be a pharmacological effect.

(d) Amniotic Fluid Prolactin.-

The finding that prolactin is present in extremely high concentrations in human as well as monkey amniotic fluid (Friesen et al. 1972a) raises many unanswered questions. The chemical characteristics of human amniotic fluid prolactin would indicate that it is probably identical to human pituitary prolactin though small molecular differences could not be excluded and at present its biological activity has not yet

been tested. It is not clear where it comes from and its function in the amniotic sac, if any, remains a mystery. It has been speculated that since prolactin is important in osmoregulation in certain types of fish (Nicoll and Bern, 1972), it might also play a similar role for the human fetus during its existence in an aquatic environment in utero (Friesen et al. 1972a). Indeed, there is some limited evidence that prolactin might be related to water and electrolyte balance in human adults (Horrobin et al.1971; Buckman et al.1973).

SECTION V: THE PURIFICATION OF HUMAN PROLACTIN

INTRODUCTION

The chemical isolation of human prolactin had been elusive for many years. Although physiological evidence was overwhelming for its existence, the repeated failures in its purification (Wilhelmi, 1961; Li, 1962; Tashjian et al, 1965; Apostolakis, 1968), together with the observation that human growth hormone has intrinsic prolactin-like activity (Lyons et al, 1961) led to the speculation that prolactin might not exist as a separate hormone in man, and that in man growth-promoting and lactogenic functions were both mediated by growth hormone (Bewley and Li, 1970). In the past two years, human prolactin has been purified independently by Lewis et al, (1971) and by us (Hwang, Guyda, and Friesen, 1972), establishing unequivocally that it exists as a distinct molecule in man. In addition, recent studies carried out by Niall et al, (1973) with the human prolactin purified in our laboratory provided considerable information on the structure of this molecule.

MATERIALS AND METHODS

Starting Materials:- Several possible sources of raw material for prolactin purification have been examined: (1) Pituitary glands removed at autopsy and stored frozen at -20°C were kindly provided by the National Pituitary Agency, USA; (2) Acetone-dried human pituitary powder was obtained from Nordic Chemicals (Canada); (3) A side-fraction obtained during the purification of human growth hormone by the Raben procedure (1959) was kindly provided by Dr. Raben. It was derived from the

supernatant which remained after growth hormone was precipitated with ethanol at pH 8.5. Cold acetone was added to the supernatant to raise the organic solvent concentration to 80% and the precipitate was collected by filtration on a Buchner funnel; (4) Amniotic fluid was collected during the induction of delivery or abortion and kept frozen at -20°C. Drs Josimovich and Guyda have kindly furnished most of the amniotic fluid employed in this study.

Primate Prolactin Preparations:- Monkey and human prolactin preparations were obtained from Drs. Peckham (Peckham and Nicoll, 1971) and Lewis (Lewis et al, 1971) respectively for the purpose of comparison.

Prolactin Assay:- A modification of the assay described in Section III was used to facilitate quick analysis. The following reagents were incubated for 6-12 hours at room temperature:

0.5 ml of 0.5% bovine serum albumin
0.1 ml of an appropriately diluted antiserum to human prolactin,
0.1 ml of labeled human prolactin, with about 20,000 cpm,
0.05 ml of standard or unknown sample.

The optimum amount of antiserum required was determined by incubating serial dilutions of the antiserum with labeled hormone. The dilution which gave approximately 50% binding after 6-12 hours was used. Separation of bound from free hormone was achieved by adding to each tube 1 ml of dextran-coated charcoal (12.5 gm activated charcoal and 1.25 gm dextran-250 in 1 liter of 0.05 M phosphate buffer pH 7.4). The tubes were centrifuged and the sedimented charcoal counted. For more precise estimations, the double antibody technique described in Section III was employed.

Growth Hormone Assay:- Radioimmunoassay for growth hormone was also carried out by a charcoal separation method similar to that described for prolactin.

Bioassays:- Bioassays for prolactin were very kindly performed by Dr. Frantz with an *in vitro* assay using mouse mammary tissue culture (Kleinberg and Frantz, 1971) and by Dr. Nicoll using the local pigeon crop sac assay (Nicoll, 1967).

Protein Measurement:- Protein concentrations of the fractions collected during purification were estimated by measuring the absorbance at 280 nm, making the assumption that one unit of absorbance was equivalent to a protein concentration of 1 mg/ml. For low protein concentrations, an Aminco-Bowman Spectrofluorometer was employed, using human growth hormone as standard; excitation was at 278 nm and emission at 350 nm. The final purified product was weighed after lyophilisation, and its protein content per unit weight determined by Lowry's procedure (Lowry et al, 1951), using bovine serum albumin as standard.

Electrophoresis:- Polyacrylamide gel electrophoresis was carried out as described by Davis (1964) and Reisfeld et al, (1962). Starch gel electrophoresis was performed according to the method of Ferguson and Wallace (1963). In one of the latter experiments, the channel containing prolactin was divided longitudinally into two halves. One half was stained, the other half was cut serially, and the individual segments were eluted by hysteresis for prolactin radioimmunoassay.

N-Terminal Amino Acid Analysis:- The 1-dimethylaminophthalene-5-sulphonyl chloride (dansyl chloride) method of Gray (1967) was used. The dansylated protein was hydrolysed in 6 N HCl for 18 hours at 110°C. The dansylated amino acid of the N-terminal residue was identified by comparison

with dansylated amino acid standards on two dimensional thin layer chromatography on polyamide layers as described by Woods and Wang (1967).

PURIFICATION PROCEDURES

The methodology for the purification of prolactin from frozen glands will first be described. Modifications of the method which were required for Raben's side fraction and amniotic fluid will be detailed subsequently. We were unsuccessful in our attempt to purify prolactin directly from acetone-dried pituitary powder.

All procedures were carried out at 0-4°C unless otherwise specified. The following are descriptions of representative runs.

(a) Purification of Prolactin from Frozen Glands

(1) Extraction and Organic Solvent Precipitation

Five hundred glands (219 gm) was homogenised in 1 liter of 0.05 M ammonium acetate with a Virtis homogenizer (Model 45) for 3 minutes at about 20,000 rpm. After the pH had been adjusted to 5.0 with glacial acetic acid, the homogenate was stirred for 1 hour and then centrifuged at 27,000 x g for 30 minutes. The residue was extracted for 12 hours in 1 liter of 0.1 N NaOH, the pH being adjusted to 10.5 with 5 N HCl at the beginning of extraction. After filtration through glasswool to remove tissue debris, 100 ml of 1 M Tris was added to the extract and the pH lowered to 8.5 with 5 N HCl. Cold ethanol was then added slowly with stirring to a final concentration of 25%. The precipitate was removed by centrifugation. More ethanol was added to the supernatant to raise the organic solvent concentration to 85%. The flocculent precipitate which formed was either recovered immediately by centrifugation or allowed to settle for 1-2 days and recovered by decantation followed by centrifugation.

(2) Sephadex Gel Filtration

The 85% ethanol precipitate was suspended in 180 ml of distilled water. With constant stirring, 20 ml of 1 N NaOH was added. The precipitate dissolved readily and an almost clear solution was obtained. The pH was immediately lowered to 10.0 by the addition of 1 N HCl. Gel filtration was then carried out on a Sephadex G-100 column (8.5 x 90 cm) equilibrated with 0.01 M Tris-HCl buffer pH 9.0. The fractions collected were analyzed for protein and prolactin. Fractions rich in prolactin were pooled for chromatography on DEAE cellulose.

(3) DEAE-Cellulose Chromatography

The pool from the Sephadex column was adjusted to pH 8.5 with 5 N HCl and applied to a column (4 x 20 cm) of diethylaminoethyl cellulose (Whatman DE-32) previously equilibrated with 0.01 M Tris-HCl pH 8.5. After washing the column with 400 ml of starting buffer, elution was carried out with 0.06 M and 0.5 M NaCl in the buffer.

(4) CM-Cellulose Chromatography

Fractions from DEAE cellulose chromatography which were rich in prolactin were pooled, diluted with one half volume of distilled water and adjusted to pH 5.6 with 1 N acetic acid and applied to a column (1.4 x 15 cm) of carboxymethyl cellulose (Whatman CM-23) equilibrated with 0.01 M ammonium acetate pH 5.6. The column was washed with 0.05 M NaCl in 50 ml of starting buffer before elution was carried out by increasing concentrations of NaCl. Highly purified prolactin was eluted and was concentrated in an Amicon cell and lyophilised after desalting on Sephadex G-25.

(b) Purification of Prolactin from Raben's Side Fraction

Attempts made to purify prolactin directly from acetone dried human pituitary powder were largely unsuccessful

(unpublished). However, when a discard fraction obtained during the purification of human growth hormone from acetone powder (Raben, 1959) was used as the starting material, chemically homogeneous and biologically active prolactin could be obtained. In Raben's procedure for growth hormone purification, growth hormone is precipitated at pH 8.5 by the addition of ethanol to 50% concentration. The supernatant which remains is normally discarded. If, however, acetone is added to this supernatant to raise the final organic solvent concentration to 80%, a precipitate is obtained which contains about 1% by weight of prolactin (Hwang, Guyda and Friesen, 1972).

About 5 gm of this material were obtained from a batch of 10,000 glands. The precipitate was recovered by filtration on a Buchner funnel (medium) and dried by additional washes of acetone. It was then extracted for about 2 hours with 250 ml 0.1 N NaOH, with the pH lowered to 10.5 by the addition of 5 N HCl at the beginning of extraction. Any insoluble residue was removed by centrifugation at 27,000 x g for 30 minutes. Chromatography was then carried out on Sephadex G-100, DEAE-cellulose and CM-cellulose exactly as described above for the frozen glands. The bed volumes of the columns employed for 5 gm of starting material were the same as those employed for 500 frozen glands.

(c) Purification of Prolactin from Amniotic Fluid

Owing to its high prolactin content (Friesen et al, 1972a), we have explored the possibility of purifying prolactin from amniotic fluid. At the time of writing, limited success has been achieved in the purification of prolactin from amniotic fluid obtained during early pregnancy. Conventional chemical methods were used.

(1) Ammonium Sulphate Precipitation

A sufficient amount of solid ammonium sulphate

was added to 3 liters of amniotic fluid to achieve 50% saturation. The pH was then lowered to 5.0 with glacial acetic acid. The precipitate was either recovered immediately by centrifugation at 27,000 x g for 10 minutes or allowed to settle for 2 days and recovered by decantation and centrifugation. The supernatant was discarded.

(2) Ethanol Fractionation

The 50% ammonium sulphate precipitate was suspended in 200 ml distilled water and sufficient 10 N NaOH was added to raise the pH to 10.5. Extraction was carried out at room temperature for 1 hour, after which 30 ml of 1 M Tris was added and the pH lowered to 8.0 with 6 N HCl. Cold ethanol was then added to a final concentration of 70%. The precipitate was removed by centrifugation and discarded. More ethanol was added to the supernatant to raise the ethanol concentration to 90%. The precipitate was allowed to settle for 2 days and recovered by decantation and centrifugation. It was extracted with 0.1 N NaOH with the pH adjusted to 10.5 with 6 N HCl. Any insoluble material was removed by centrifugation at 27,000 x g for 30 minutes.

(3) Column Chromatography

The clear supernatant was subjected to Sephadex G-100, DEAE-cellulose and CM-cellulose chromatography as described for frozen glands. For 3 liters of amniotic fluid, the column sizes employed were 4 x 60 cm for Sephadex G-100, 2 x 8 cm for DEAE-cellulose, and 1 x 8 cm for CM-cellulose.

RESULTS

Prolactin Content of Starting Materials

Table VI shows the prolactin content of the different starting materials we have employed in our attempt to

TABLE VI

PROLACTIN CONTENT OF PITUITARY GLANDS AND AMNIOTIC FLUID

MATERIAL	PROLACTIN CONTENT*
Acetone-dried powder	0.5 - 1 mg per gram of powder or 5 mg per 100 glands.
Frozen glands (autopsy material)	15 - 27 mg per 100 glands
Raben's pituitary side fraction**	5 - 10 mg per gram of powder
Amniotic fluid first trimester	3 mg per liter
third trimester	1 mg per liter

*As determined by radioimmunoassay. Pituitary materials were extracted with 0.1 N NaOH with pH adjusted to 10.5 with HCl.

**See text.

purify human prolactin. It appears that fresh glands obtained at autopsy and stored frozen at -20°C were a far richer source of prolactin than acetone-dried pituitary powder on a per gland basis; acetone treatment apparently led to a 80% loss of activity. The side fraction derived from Raben's procedure for the isolation of human growth hormone from acetone-dried powder proved to be about 10 times as rich in prolactin as the original acetone dried powder when compared on a weight basis. Human amniotic fluid, particularly if obtained during early pregnancy, contains very high concentrations of prolactin and may be a useful alternative when pituitary glands are not readily available.

Prolactin Purification from Frozen Glands

Several conditions were tested to determine the best procedure to use in the initial extraction of prolactin from frozen glands. As shown in Table VII, by far the greatest amount of prolactin was extracted with 0.1 N NaOH adjusted to pH 10.5, 405 µg being obtained per gram of gland wet weight (or about 200 µg per gland). Acidic solutions were much less effective than alkaline solutions in solubilizing prolactin. Fig. 11A shows the rate at which prolactin went into solution during extraction with 0.1 N NaOH adjusted to pH 10.5. A maximum amount of prolactin was extracted in 12 hours. More prolonged extraction periods were actually associated with a gradual decrease of the prolactin concentration in the extract. The rate of disappearance of prolactin increased if the alkaline extract was kept at room temperature. After ethanol had been added to 25% concentration, however, loss of prolactin became negligible even at room temperature (Fig. 11B). It appeared important that the alkaline extraction of glands should be carried out in the cold for a controlled

TABLE VII

EXTRACTION OF FROZEN PITUITARY GLANDS

Glands were Homogenized in 2 volumes of Extracting Fluid and Stirred overnight at 4°C.

EXTRACTING FLUID	pH	PROLACTIN EXTRACTED µg/gm wet weight
0.1N NaOH*	10.5	405
0.1N NH_4OH + 0.1M NH_4HCO_3	9.5	225
0.1M NH_4HCO_3	8.6	48
0.05M ammonium acetate	5.0	<1
0.1N acetic acid	3.0	<1
0.1N HCl	2.0	12

*pH lowered to 10.5 with 5N HCl.

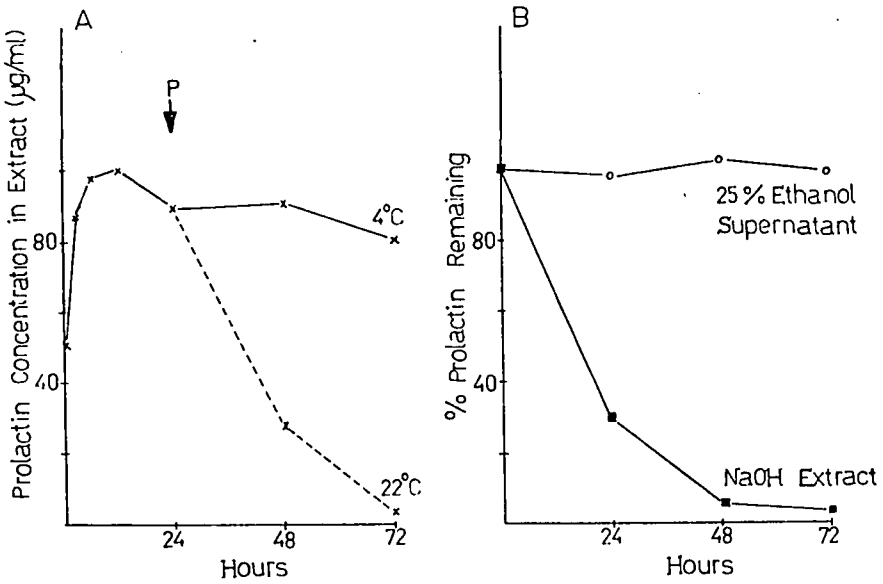


Fig. 11. (A) Quantity of prolactin extracted as a function of extraction time. Pituitary glands were extracted at $4^{\circ}C$ with 0.1N NaOH adjusted to pH 10.5 with 5N HCl. Aliquots of the extract were taken at intervals for measurement of prolactin concentration. At point P, some of the extract was exposed to room temperature and prolactin concentration was measured after varying periods of time. Note the rapid disappearance of prolactin at the higher temperature. (B) Rates of disappearance of prolactin in the alkaline extract and the 25% ethanol supernatant at room temperature.

period of time.

Fig. 12 shows the distribution of protein and prolactin after Sephadex gel filtration. About 90% of the prolactin emerged in a single peak with a relative elution volume of about 2.5 (Fraction A_2). Only a small amount of prolactin was found in the unretarded peak (Fraction A_1). In contrast to this distribution, if the NaOH extract was chromatographed on Sephadex without prior precipitation by ethanol, about 40% of the total immunoreactive prolactin emerged in the void volume peak (Fig. 12 inset); the large molecular weight species, the nature of which was not clear, was almost completely precipitated by 25% ethanol.

Fig. 13 shows the results of fractionating Fraction A_2 on DEAE-cellulose. The bulk of the prolactin was eluted with 0.06 M NaCl in Fraction B3 which was chromatographed on CM-cellulose as shown in Fig. 14. Highly purified prolactin was eluted from the CM-cellulose column by step-wise increases in NaCl concentration to 0.2 M, 0.5 M, and 1.0 M (Fraction C_3).

Table VIII shows a flow-sheet of the purification scheme and Table IX summarises the distribution of protein, prolactin, and growth hormone in the different fractions obtained during purification. As shown in Table IX, the pH 5 extract contained negligible amounts of prolactin but quite substantial quantities of protein, indicating the usefulness of pre-extracting the glands with ammonium acetate at pH 5 before alkaline extraction. At pH 10.5, a total of 85 mg of protein was obtained from 500 glands, giving an average protein content of 170 µg per gland. When ethanol was added to the alkaline extract to 25% concentration, only 44 mg of the prolactin remained in the supernatant, the other 41 mg having been precipitated. However, it is clear from Fig. 12 that most of the precipitated prolactin represented large molecular weight material which was not further purified. From the 85 mg of prolactin present in

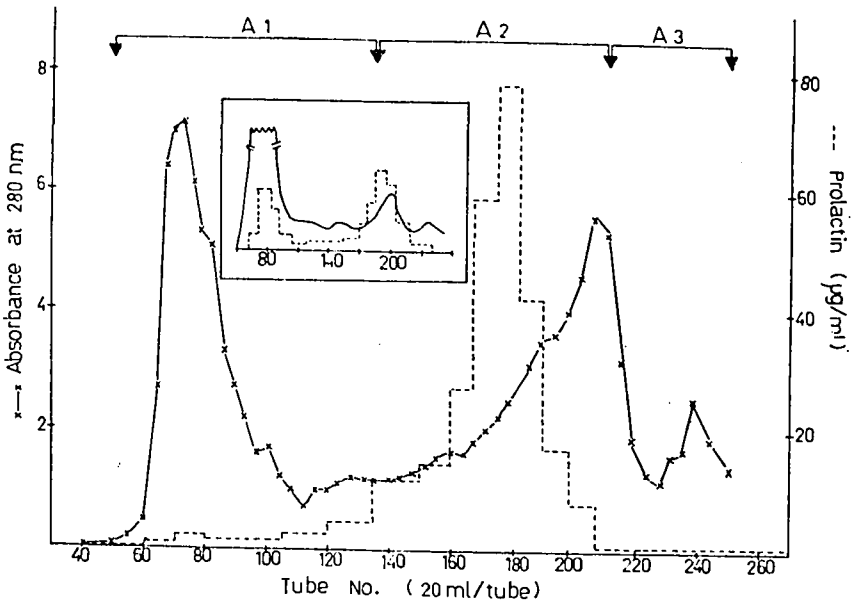


Fig. 12. Sephadex G-100 chromatography of the 85% ethanol precipitate. Flow rate 3 ml/min. Vo = 1400 ml, Vt = 5100 ml, Kav for the prolactin peak = 0.59. Inset shows the protein and prolactin distribution observed when the NaOH extract was fractionated directly without prior ethanol precipitation.

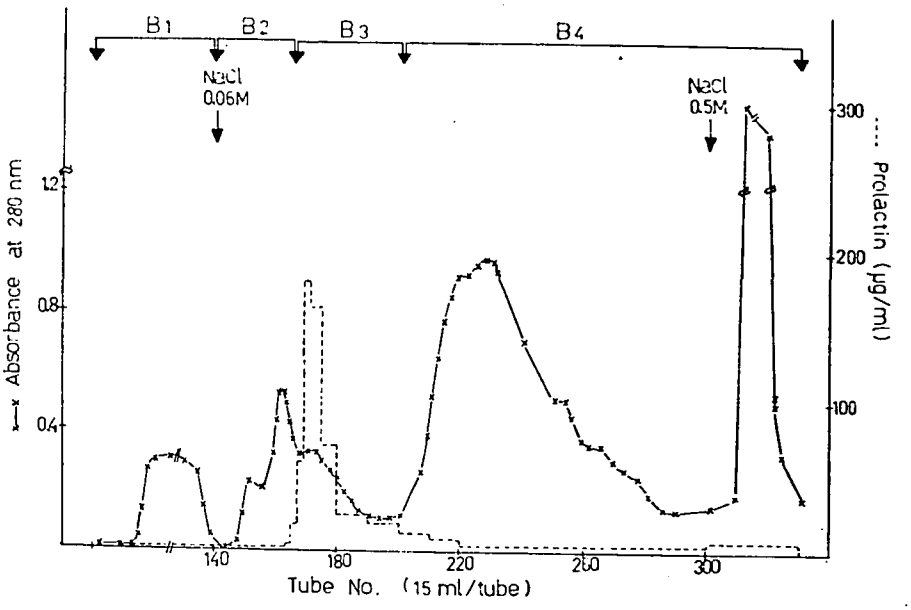


Fig. 13. DEAE-cellulose chromatography of Fraction A2 from the Sephadex column in Fig. 12. Flow rate, 1 ml/min.

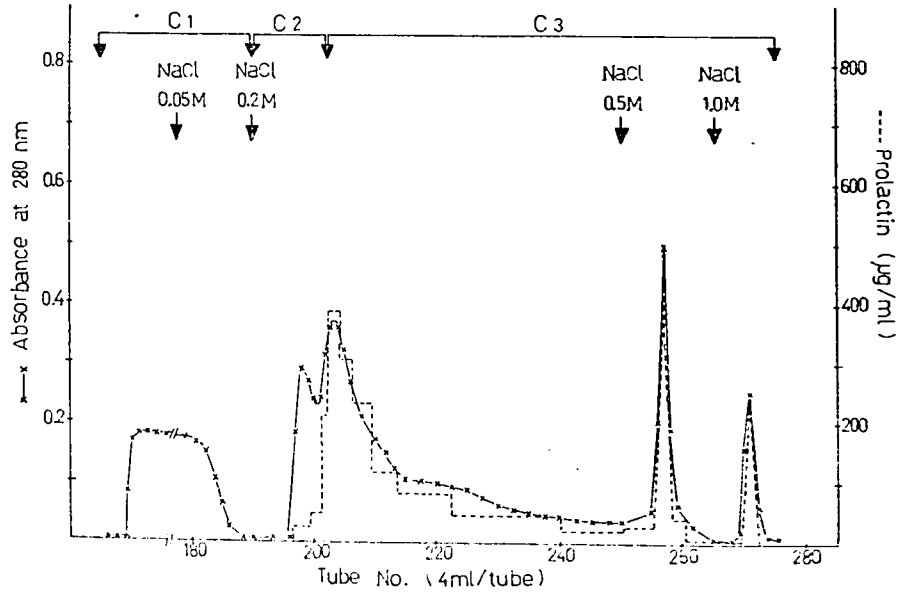


Fig. 14. Fractionation of Fraction B3 from the DEAE-cellulose column in Fig. 13 on carboxymethyl cellulose. Flow-rate, 0.5 ml/min.

TABLE VIII

FLOW CHART FOR HUMAN PROLACTIN PURIFICATION FROM FROZEN PITUITARY GLANDS

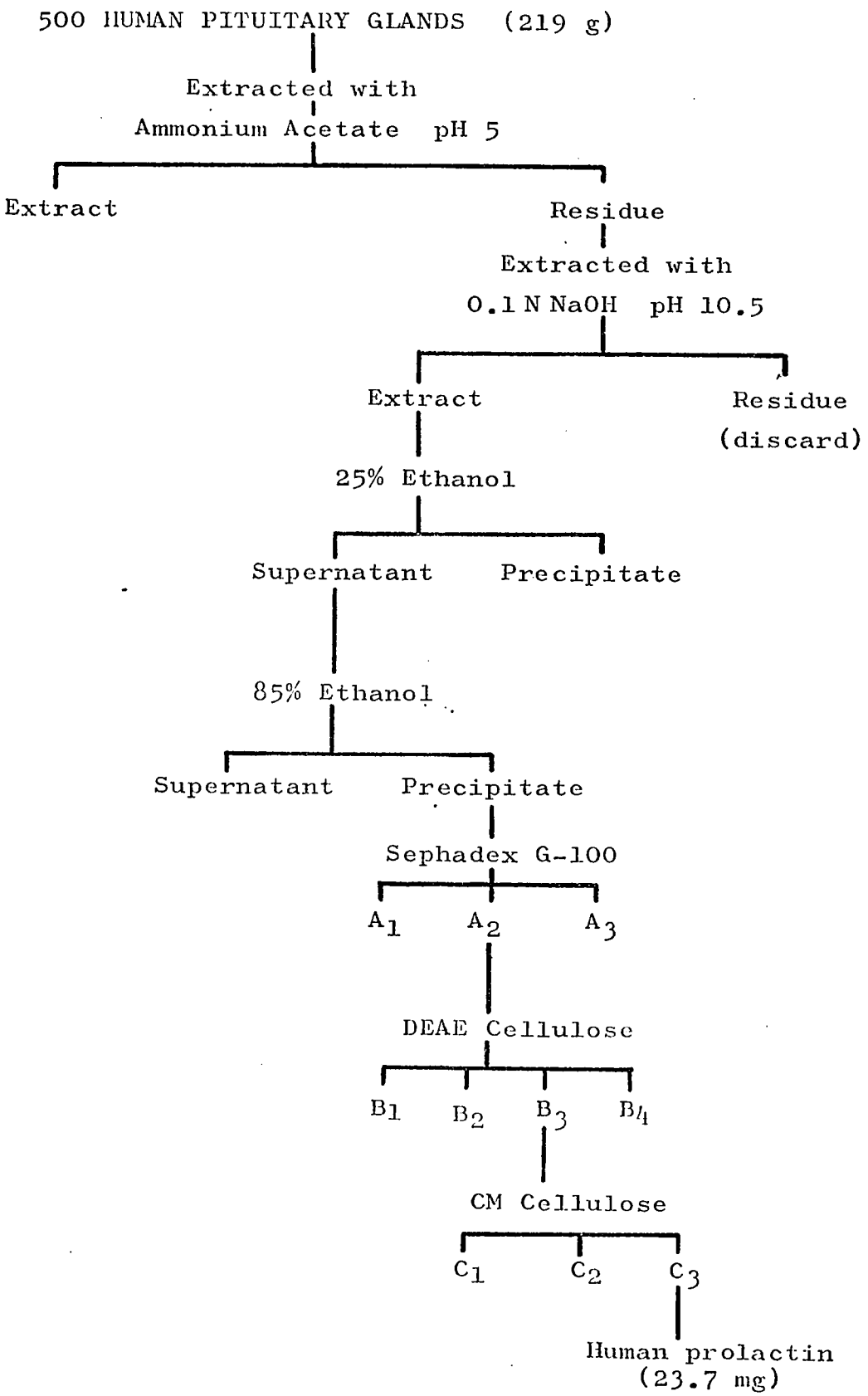


TABLE IX

DISTRIBUTION OF PROTEINS, PROLACTIN AND GROWTH HORMONE DURING PURIFICATION OF PROLACTIN FROM FROZEN PITUITARY GLANDS

FRACTION	PROTEINS (mg)	PROLACTIN (mg)	GROWTH HORMONE (mg)
pH 5 Extract	8000	< 0.3	2100
pH 10.5 Extract	NA*	85	4240
25% Ethanol Supernatant	10000	44	2000
Sephadex G-100			
A1	3940	3.5	192
A2	3980	42	1980
A3	1570	1.5	33
DEAE Cellulose			
B1	130	< 0.5	< 1.0
B2	84	0.5	< 1.0
B3	98	31	1.4
B4	3700	7	1750
CM Cellulose			
C1	60	< 0.1	1.0
C2	6.2	1.5	< 0.1
C3	25.7	23.7	0.1

* NA, not available.

the original extract, a total of 23.7 mg of highly purified prolactin was obtained, giving an overall recovery of about 30%. The yield per gland was 47 µg. This estimate of the yield, based initially on radioimmunoassay, was confirmed by weighing the material after lyophilisation and by Lowry's procedure. Table IX shows also that, during precipitation by organic solvents and gel filtration, growth hormone was closely associated with prolactin. Clear separation of the two hormones was achieved only after ion-exchange chromatography. Growth hormone contamination of the purified prolactin (Fraction C3) was less than 0.5% by radioimmunoassay. In some of the side fractions such as B4, approximately half of the protein was growth hormone.

Prolactin Purification from Raben's Side Fraction

Table X summarises the quantitative aspects of purification from Raben's side fraction. About 13 mg of prolactin could be obtained from 5 gm of starting material.

Prolactin Purification from Amniotic Fluid

Table XI shows the recovery and yield of prolactin during purification from amniotic fluid. Although approximately a 1,000 fold purification was achieved by the method described, the recovery was only 17% and the purity of the final product as measured by radioimmunoassay was somewhat less than 50%.

N-Terminal Amino-Acid

N-terminal amino acid analysis of two different preparations of pituitary prolactin showed leucine to be the N-terminal amino acid. The prolactin purified from amniotic fluid also has leucine at the amino terminal (Hugh Niall, personal communication, 1972).

Electrophoretic Mobility

Fig. 15 shows the electrophoretic patterns in polyacryl-

TABLE X

RECOVERY AND YIELD OF PROLACTIN DURING PURIFICATION FROM RABEN'S DISCARD PITUITARY FRACTION*

Stage of Purification	Protein (mg)	Prolactin (mg)	% Purity +
Starting material	4500	60	1.3
Gel filtration	730	35	4.8
DEAE cellulose chromatography	170	21	12
CM cellulose chromatography	16	13	81

+Amount of prolactin as determined by radioimmunoassay as a percentage of the weight of total proteins.

*Figures obtained from a representative run.

TABLE XI

RECOVERY AND YIELD OF PROLACTIN DURING PURIFICATION FROM AMNIOTIC FLUID

Stage of Purification	Protein (mg)	Prolactin (mg)	% Purity*
Amniotic fluid 3 l	18,000	9.0	0.05
$(NH_4)_2SO_4$ ppt'n	10,000	8.8	0.09
Ethanol Fractionation	1,000	8.0	0.8
Sephadex Chromatography	114	4.0	3.5
DEAE Cellulose Chromatography	29	3.1	11
CM Cellulose Chromatography	3.5	1.5	43

*Amount of prolactin as determined by radioimmunoassay as a percentage of the weight of total proteins.

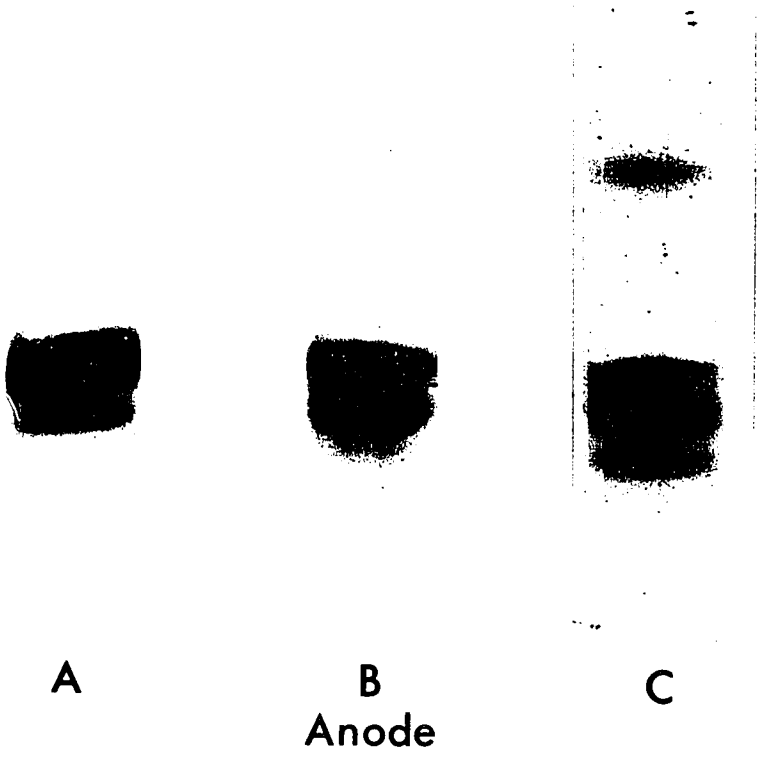


Fig. 15. Polyarylamide gel electrophoresis of prolactin prepared from frozen glands (A), Raben's side fraction (B), and human amniotic fluid (C). Gel concentration 7.5%; pH 9.5, The Rf of the most cathodal band in both A and B was 0.5.

amide gel at pH 9.5 for prolactin derived from frozen glands, from Raben's side fraction, and from amniotic fluid. In all cases, 2-3 bands with Rf values between 0.5 and 0.6 were observed; in all cases, immunoreactive prolactin was detected in this zone. The amniotic fluid prolactin preparation clearly showed a major contaminant band with a Rf of about 0.3. Fig. 16 shows the electrophoretic pattern of pituitary prolactin on starch gel electrophoresis. Three bands were also observed, the slowest (most cathodal) of which was clearly cathodal to the slowest moving band of human growth hormone. To determine whether the two bands which moved faster represented deaminated forms of prolactin or contaminant proteins, individual segments of the starch gel were eluted and assayed for prolactin. All three components were found to be immunoreactive (Fig. 16), and the quantities of prolactin recovered by elution corresponded roughly to the intensities of staining observed. In addition, when serial dilutions of the eluates from these segments were made, the response curves observed in the radioimmunoassay showed complete parallelism to that of the standard. Upon electrophoresis of prolactin at pH 3.6, in polyacrylamide gel according to the method of Reisfeld et al, (1962), only one component was observed, moving distinctly faster than human growth hormone towards the anode (Fig. 17).

Immunological and Biological Activity

Table XII shows the immunological and biological potencies of several purified prolactin preparations. The potency estimates by radioimmunoassay are made relative to the Medical Research Council Research Standard A (#71/222) which was a partially purified human prolactin preparation supplied by us earlier (Hwang, Guyda and Friesen, 1972) as a temporary reference standard. Preparation hPRL 71-9-4 was derived from Raben's side fraction and was 2.3 times as

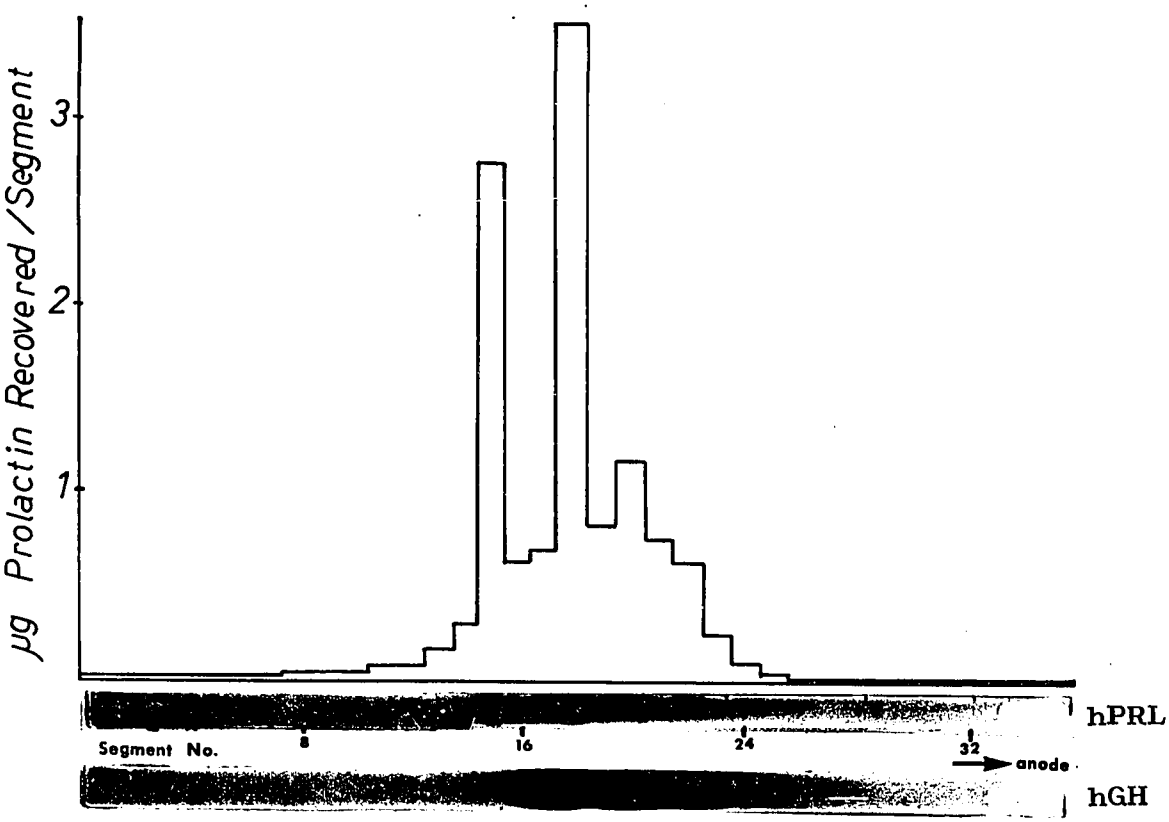


Fig. 16. Starch gel electrophoresis of human pituitary prolactin and human growth hormone : the Rf values of the components of prolactin are 0.41, 0.50, and 0.56, and those of growth hormone are 0.48, 0.55, and 0.62. The quantity of prolactin recovered from each segment was determined by radioimmunoassay.

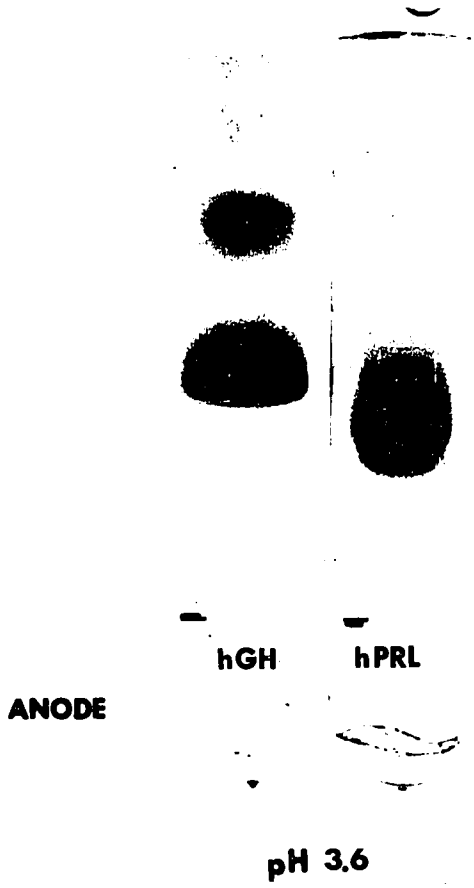


Fig. 17. Polyacrylamide gel electrophoresis of human prolactin and growth hormone at pH 3.6, gel concentration 7.5%. The Rf of prolactin is 0.7 while that of growth hormone is 0.65.

TABLE XII

RELATIVE POTENCY OF DIFFERENT PROLACTIN PREPARATIONS AS MEASURED BY RADIOIMMUNOASSAY AND BY BIOASSAYS

PREPARATION	SOURCE	RELATIVE IMMUNO-REACTIVITY*	BIOLOGICAL ACTIVITY (IU/mg)+
MRC Research Std A (#71/222)	Acetone-dried powder**	1.0	11.4 (8.3-15.6)
hPRL 71-9-4	"	2.3	30.5 (23.8-37.2)
hPRL 72-3-26	Frozen glands	3.0	-
hPRL 72-4-9	"	2.4	-
hPRL B	"	3.1	-
Dr. Lewis's Prep. (#201-161-1)	"	2.2	24.0 (19 - 29)

*Taking the immunological potency of MRC Research Standard A as 1.0. The confidence limits of these values are provided by that of the radioimmunoassay, that is ± 20%.

+Bioassays were carried out with sheep prolactin as standard in the local pigeon crop sac assay (MRC Std.A and Dr. Lewis's preparation) or in the in vitro assay of Frantz and Kleinberg (1970) using mouse mammary tissue cultures. Figures in parentheses indicate the 95% fiducial limits.

**Refers to Rabens's side fraction (see text) rather than the crude acetone-dried pituitary powder.

potent as Research Standard A in the immunoassay. All other preparations, including that of Dr. Lewis, were derived from frozen glands and had essentially the same immunological potency as hPRL-71-9-4, within the limits of experimental error. Serial dilutions of these prolactin preparations gave completely parallel inhibition curves in the radioimmunoassay. Three of the preparations listed in the table had been bioassayed. There appears to be a good correlation between biological and immunological activity. Preparation hPRL 71-9-4 was as active as the most highly purified sheep prolactin preparations currently available, having an activity of 30.5 ± 6.7 IU/mg.

A NOTE ON THE CHEMISTRY OF HUMAN PROLACTIN

Dr. Hugh Niall of Harvard University, using our preparation of purified human prolactin, has been able to obtain considerable information on the chemical structure of this hormone (Niall et al, 1973). Preliminary studies carried out by him, as well as those of Lewis et al. (1972), showed that human prolactin is similar in many respects to sheep prolactin. It has approximately the same size and very similar amino acid composition. The presence of six half cystine residues would suggest that it also contains three disulfide bridges as in sheep prolactin. Amino acid sequence analysis of human prolactin has not yet been completed. Fig. 18 shows the first 40 amino terminal amino acids of human prolactin in comparison to those of sheep prolactin. The continuous lines indicate identical amino acids, and the broken lines those amino acids which are considered to be highly acceptable replacements. It can be seen that about 90% of the amino acids in this region of the two molecules are either identical or very similar. Fig. 18 also shows the amino terminal sequences of two other human hormones which have lactogenic activity, namely human

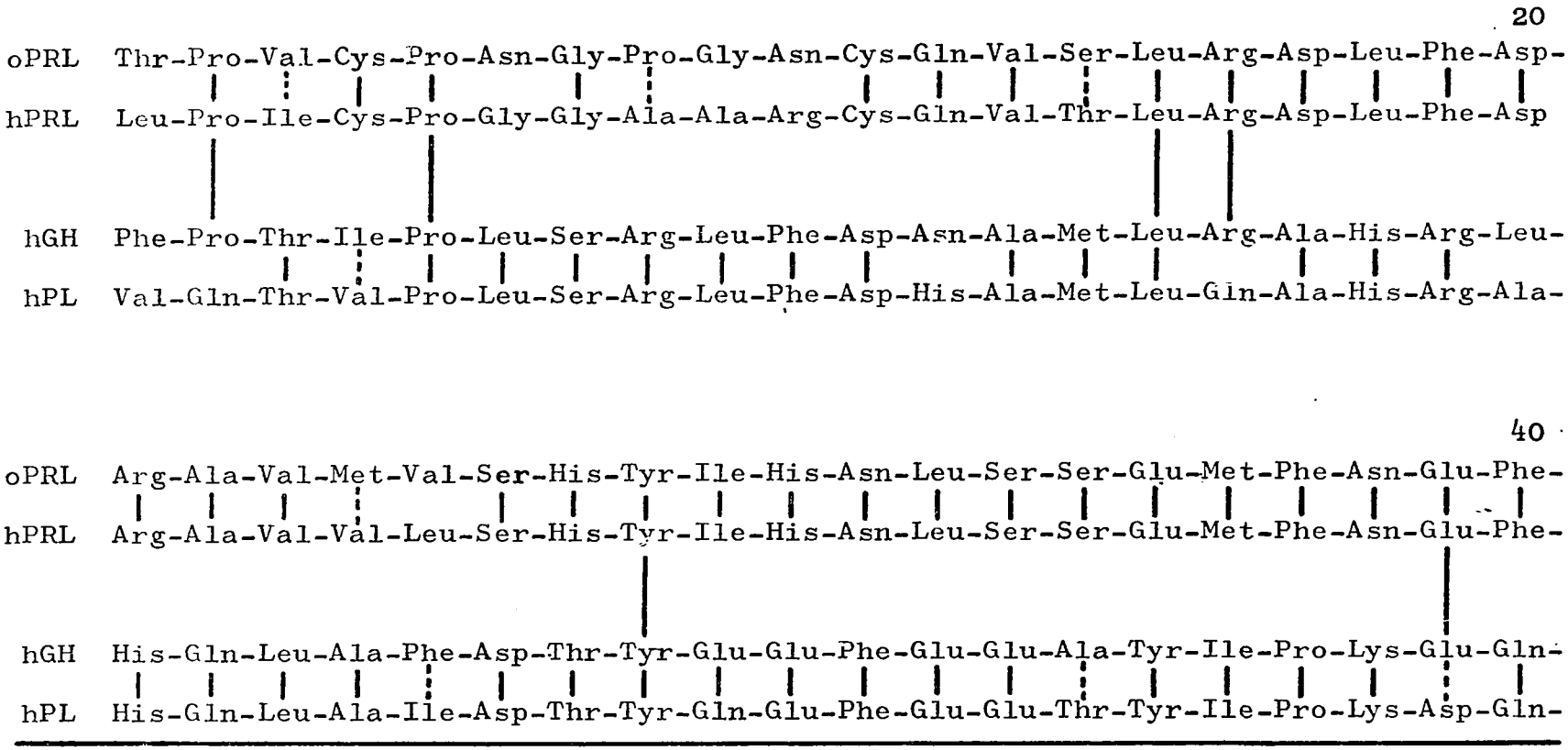


FIGURE 18 - Comparison of the amino terminal sequences of human prolactin (hPRL), sheep prolactin (oPRL), human grcwth hormone (hGH), and human placental lactogen (hPL). Continuous lines (——) indicate identical amino acids, interrupted lines (----) indicate highly acceptable substitutions.(Niall et al. 1973).

growth hormone and human placental lactogen. It is clear that while human growth hormone is very similar to placental lactogen, it is definitely a different molecule from prolactin.

DISCUSSION

In retrospect, it would appear that the main reasons for past failures to purify human prolactin are two-fold, namely the relatively low prolactin content in human pituitary glands and the absence of a specific assay for monitoring prolactin during purification. In the several species of animals whose prolactin has been purified, it appears that the pituitary content of prolactin is much greater than in human pituitaries. From one gram of fresh tissue, the amount of highly purified animal prolactin which could be obtained ranges from 800 μg in the bovine (Jiang and Wilhelmi 1965) to 7mg in rats (Groves and Sells, 1968), whereas in human glands the total prolactin content per gram tissue is only about 400 μg from which only 100 μg of purified prolactin could be obtained. In previous attempts to purify human prolactin, the pigeon crop sac assay was usually employed to monitor prolactin distribution during fractionation. It has been demonstrated that human growth hormone is active in the pigeon crop sac assay to varying degrees (Damm et al, 1964; Tashjian et al, 1965; Rivera et al, 1967; Peckham et al, 1968; Li, 1968). Since, as it now appears, the growth hormone content in human pituitary glands is 50-100 times higher than that of prolactin, the pigeon crop sac assay would inevitably follow the distribution of growth hormone rather than prolactin. The development of a radioimmunoassay which distinguishes clearly between prolactin and growth hormone was instrumental in our finding a successful scheme for the chemical purification of human prolactin free from growth hormone. Similarly, the successful isolation of human prolactin by Lewis et al, (1971) could be largely attributed to the application of

polyacrylamide gel electrophoresis to distinguish between prolactin and growth hormone.

It is clear that pituitary glands stored frozen are far richer in prolactin than acetone dried powder. Freezing at -20°C therefore appears to be the preferred method of storage. We do not yet have information on whether the prolactin content of frozen glands progressively decreases with prolonged storage as in the case of animal glands (see Lewis et al, 1972). When available, frozen glands would appear to be the best starting material for prolactin purification. We have not been successful with acetone-dried pituitary powder in purifying prolactin, and Raben's side fraction is not usually available in bulk. Amniotic fluid at present therefore appears to be a promising alternative source of human prolactin although the method for isolating the hormone from it has not yet been entirely worked out and the isolated material has not yet been adequately characterized. It is in general much easier to obtain amniotic fluid in quantity than pituitary glands, and the prolactin content of amniotic fluid can be considerable: 10 liters of early pregnancy amniotic fluid contain the amount of prolactin found in 100 frozen glands.

In our initial attempts to purify prolactin from frozen glands we encountered a number of difficulties. One major problem was that the prolactin appeared to disappear rather rapidly during the course of purification. In one particular experiment which extended over 2 weeks, only 0.4 mg of prolactin was recovered from 150 glands with an estimated total prolactin content of 30 mg (unpublished). The cause for the progressive loss of prolactin was not clear. Other investigators have observed proteolysis of pituitary hormones in crude extracts of the pituitary gland (Adams and Smith, 1951; Lewis, 1962), and it is likely that

in our experiment prolactin was degraded by enzymes liberated during homogenisation. Our observation that the rate of loss was temperature-dependent is consistent with this interpretation. More studies are needed to confirm this. In the purification procedure for prolactin described by Lewis et al, (1971), an enzyme inhibitor was used during extraction, but it is not clear to what extent this helped in reducing losses. Another difficulty encountered during the purification of prolactin from frozen glands was that the alkaline extract was a turbid and viscous suspension which could not be cleared by centrifugation or filtration. When this thick extract was directly chromatographed on Sephadex, resolution was poor unless small batches of glands were used (e.g. 100 glands for a column with dimensions of 8.5 x 90 cm). The addition of ethanol to the alkaline extract to 25% concentration proved to be a valuable step. It not only prevented the progressive disappearance of prolactin (Fig. 11b), but it also precipitated the bulk of the proteins in the extract without precipitating much of the small molecular weight prolactin, (Fig.12, TableIX). The remaining supernatant contained relatively small amounts of protein which could be readily solubilised and chromatographed, making it possible to process 500-1000 glands at a time. It should be pointed out that ethanol fractionation at an alkaline pH has also been employed successfully by other investigators in the purification of animal as well as human prolactin (Bates and Riddle, 1935; Ellis et al, 1969; Lewis et al, 1972).

There appears to be little doubt that the material isolated from pituitary glands is human prolactin. The specificity of the radioimmunoassay employed in the purification procedure has been carefully evaluated (Section III). The electrophoretic mobility in polyacrylamide gel of our

material was very similar to that reported by Lewis et al, (1971). It is also clear that our preparations show full biological activity. Perhaps the most convincing piece of evidence that the purified protein is in fact human prolactin, is the amino acid sequence data of Dr. Niall (Niall et al, 1973; Fig. 18). The high degree of homology between the amino acid sequence of the protein isolated and that of sheep prolactin leaves little doubt that it is human prolactin. The same data also provide unequivocal evidence that human prolactin is distinct from human growth hormone.

The material purified from amniotic fluid has not yet been adequately examined. It cannot be distinguished from human pituitary prolactin immunologically and electrophoretically (see also Rodbard, 1973) and appears to have a similar molecular size (Lewis et al, 1971; Rodbard, 1973; and this study). In addition, its amino terminal amino acid is also leucine. Whether or not it is biologically active has not yet been determined, but it appears probable that it is identical to pituitary prolactin, although slight molecular modifications cannot be exlcuded.

There are reasons to believe that our pituitary prolactin preparation is very nearly homogeneous. The estimates of purity (Tables IX and X) may not be a true reflection of the state of homogeneity as they were based on a radioimmunoassay using an arbitrary standard. Dr. Niall found our preparation satisfactory for amino acid sequence analysis (Niall, personal communication, 1971). Growth hormone contamination was less than 0.5% by radioimmunoassay. The three components observed upon electrophoresis at an alkaline pH were all immunologically indistinguishable from one another, suggesting that the two more rapidly moving bands were probably deamidated forms of the hormone and not contaminating proteins. This conclusion is

supported by the observation that electrophoresis at an acid pH, a condition under which ionisation of carboxyl groups is suppressed, showed only one component. It remains to be demonstrated whether all three components observed electrophoretically at alkaline pH are biologically active.

The recovery of prolactin using the present purification scheme for frozen glands averages 40-50 μg per gland, which is a considerable improvement over previously published methods. In addition, the method permits the recovery of partially purified growth hormone from the side fractions. Indeed some of the fractions contained very high percentages of growth hormone and might be suitable for clinical use without further purification.

SECTION VI - SUMMARY

Prolactin has been purified from human pituitary glands with a yield of 50 μg/gland. The purified product was equipotent with highly purified sheep prolactin in bioassay (30.5 IU/mg) and was sufficiently pure for amino acid sequence analysis, which revealed a striking homology between human and sheep prolactin. Human prolactin has also been partially purified from amniotic fluid.

A specific and sensitive radioimmunoassay for human prolactin has been developed and applied to study variations of serum prolactin concentrations in man under different physiological and pathological conditions. In normal adults at rest serum prolactin level usually remained below 30 ng/ml, being slightly higher on the average in women than in man. No cyclic variation of of prolactin secretion was observed during the menstrual cycle. Serum levels of prolactin were elevated in the newborn, during pregnancy and the puerperium. Suckling was a powerful stimulus to prolactin secretion. Most patients with galactorrhea had elevated concentrations of circulating prolactin, but patients with breast cancer or acromegaly usually had normal levels. Chlorpromazine and thyrotropin releasing hormone increased while L-DOPA decreased serum prolactin; these agents may prove useful in evaluating hypothalamic-pituitary function or in treating conditions associated with abnormal prolactin secretion.

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SECTION VIII: APPENDIX I

AFFINITY CHROMATOGRAPHY

General Principles.-

Affinity chromatography is a recently developed technique of adsorption chromatography which has been widely employed for the purification of many biologically important macromolecules. It exploits the unique biological property of these molecules to bind ligands specifically and reversibly. The basic principle is to covalently link an appropriate ligand, usually a protein, to an insoluble porous matrix which is then packed into a column. A solution containing the macromolecule to be purified is passed through the column. Proteins not having affinity for the ligand will pass through the column unretarded, whereas those molecules which bind the ligand will be retained. The specifically adsorbed macromolecule can be eluted from the column by altering the experimental conditions so that dissociation occurs. A batch procedure, instead of column chromatography, can also be employed in purification of the macromolecule.

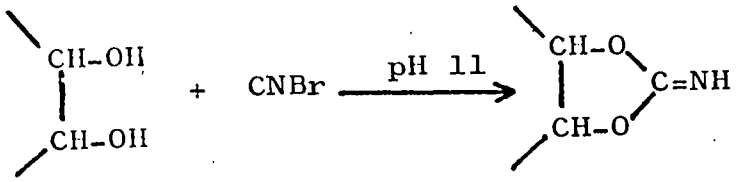
Affinity chromatography differs from conventional methods of separating macromolecules which depend on physico-chemical differences between the molecules to be separated. These chemical methods are often tedious and the separation achieved may be incomplete, frequently with large losses. The great advantage of affinity chromatography over physico-chemical methods is that the material of interest can often be purified in high yield by a single step operation. The procedure has the additional advantage that the macromolecule to be purified is rapidly removed from any destructive contaminants such as proteases which are often present in tissue extracts or biological fluids.

In principle, affinity chromatography can be used to purify antigens, antibodies, enzymes, hormone receptors, and many other macromolecules. Its actual application has been adequately reviewed (1, 2). The specific conditions employed for the purification of a given molecule are highly individualized since they depend on the unique biological interaction between the molecule and its specific ligand.

Insoluble Matrices : Among the numerous insoluble matrices which have been employed in affinity chromatography, it appears that beaded agarose comes close to being ideal in that it is very stable, shows little non-specific adorption effects, has good flow properties, and its open pore structure permits extensive attachment of the protein to be coupled and gives easy access to the molecules to be adsorbed subsequently. Chemical modification of agarose has made it possible to couple a large variety of compounds to it, including those not containing amino groups (1), thus increasing the versatility of affinity chromatography.

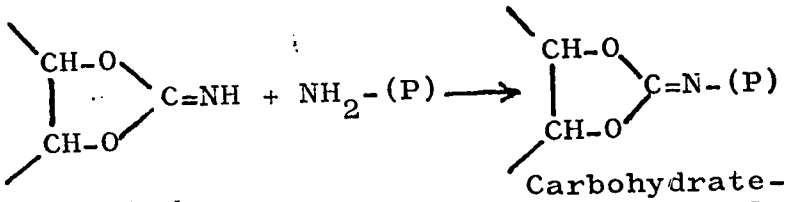
Coupling : Many methods have been proposed for the coupling of proteins or other macromolecules to insoluble matrices, and a large number of insoluble carriers have been employed for coupling. The details of these aspects of affinity chromatography have been adequately reviewed (1,2, 3). Porath and his associates (4,5) have developed a gentle general method for coupling compounds with primary aliphatic or aromatic amino groups to insoluble carbohydrate derivatives. In this procedure, cyanogen bromide reacts with the hydroxyl groups on the carbohydrate derivative to form carbamate and imidocarbonate groups. During the subsequent coupling of a protein to the 'activated' matrix, the imidocarbonate groups react with the amino groups of the protein, forming stable covalent linkages (6) :

(1) Activation



Carbohydrate derivative — Activated matrix

(2) Coupling



Activated matrix — Protein — Carbohydrate-Protein Complex (Insoluble)

By means of such a coupling procedure under relatively mild conditions, antigens, antibodies, enzymes, and other biologically active molecules can be fixed to insoluble support with substantial retention of biological activity.

Elution : Unbound substances are simply washed away with starting buffer, whereas elution of adsorbed material usually requires a change of the experimental conditions. In many cases, an increase in ionic strength or a change of pH is sufficient to dissociate the complex. Ideal elution consists of using a solution which causes sufficient alteration of the conformation of the specifically adsorbed macromolecule to decrease appreciably its affinity for the ligand but which is not sufficient to cause complete denaturation of the macromolecule. The more commonly used eluting agents belonging to this category include 4M sodium thiocyanate, glycine-HCl at pH 2.8, 6M guanidine-HCl at pH 1.5, and others. More specific eluting agents include haptens, enzyme inhibitors, and other competing molecules.

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