STUDIES TO INVESTIGATE THE ROLE OF SUBCELLULAR ORGANELLES IN PITUITARY HORMONE SECRETION

A THESIS

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ABSTRACT

The work reported in this thesis examines the role of subcellular organelles in pituitary hormone secretion, particularly with respect to existing morphological data that lysosomes may dispose of excess intracellular prolactin (crinophagy) in situations where a chronic stimulus to prolactin secretion is removed.

As a chronic stimulus to prolactin secretion, the lactating rat was used and the suckling young removed to invoke lactotroph cell involution. Pituitaries were removed from the mothers at varying times and analysed for marker enzymes for the principal subcellular organelles, showing significant increases in lysosomal and plasma membrane enzyme activities. These changes were temporaly related to the rise in pituitary and fall in serum, of prolactin. Furthermore, restoration of intrapituitary prolactin to control levels was coincident with a decline in lysosomal enzyme activities, suggesting removal of excess prolactin by this organelle, providing a biochemical basis to the morphological concept of crinophagy.

To demonstrate prolactin proteolysis in rat pituitary, an assay was developed with radiolabelled prolactin which showed a characteristic pH optimum at 4.3. Density gradient fractionation showed the prolactin protease to be localised to the lysosomes and this was further confirmed with selective lysosomal inhibitors which demonstrated the involvement of both cathepsins B and D.

Since the dominant physiological control of prolactin secretion is dopaminergic inhibition, the effects of the dopamine agonist bromocriptine on pituitary enzymes were investigated in lactating rats. There was a marked increase in pituitary prolactin protease activity as well as plasma membrane marker enzymes, coincident with time suppression of secretion. In addition, there was a significant decrease in pituitary DNA but not protein.

The activity of lysosomal enzyme activities in human pituitary tissue was decreased in functioning secretory tumours and was increased in non-secreting tumour tissue compared to normal tissue. Density gradient fractionation studies showed the distribution of lysosomal enzymes not to be different in pituitary tumours compared to normal tissue apart from the prolactin-secreting adenomas which had a single rather than dual population.

It is concluded that lysosomes may have a regulatory role in pituitary hormone turnover and alterations of their enzyme activities have been shown in pituitary pathology.

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Chapter One

INTRODUCTION

1.1 The anterior pituitary

1.2 The hormones of the anterior pituitary and their regulation

1.3 Clinical expression of hormone hypersecretion

1.4 Methods of investigation of anterior pituitary hormone secretion

CHAPTER 1

INTRODUCTION

1.1 THE ANTERIOR PITUITARY GLAND

1.1.1 Discovery

The existence of the pituitary gland was first reported by Claudius Galen in the second century A.D. However, it was only in 1543 that Andreas Vesalius suggested that the gland had a secretory function and the first description of amenorrhoea in association with a pituitary tumour came as late as 1759 by Anton de Haen. The pituitary was held to be part of the brain until Rathke, in 1838, described its comparative anatomy and embryology. It first began to figure in the medical, mainly pathological literature when Pierre Marie (1866) noted the frequent occurrence of a tumourous condition of the pituitary gland in association with acromegaly. In summary, the history of research on the hormones of the anterior pituitary lobe prior to the beginning of this century was virtually non-existent.

As various functions came to be recorded and ascribed to the pituitary gland, a number of methods were used in an endeavour to establish a relationship between these functions and the individual cell. Benda (1900) explained the excessive growth in acromegaly and gigantism as a result of hyperplasia of the acidophils, and Aschheim and Zondek (1928) suggested an

association between basophils and gonadotrophin hormones. Following this evidence, Cushing (1932) expressed his belief that the sex-maturing principles were elaborated in the basophil elements of the anterior pituitary. Prolactin was discovered during experiments in which bovine pituitary extracts induced milk secretion in rabbits (Stricker and Grueter, 1928) and a variety of names, e.g. galactin, lactogen, mammotropin and prolactin, were employed to designate the hormone (Gardner and Turner, 1933; Lyons and Catchpole, 1933; Riddle, Bates and Dykshorn, 1933; Reece and Turner, 1936). In the rat, the histological picture of increased basophil cells and degranulation of the acidophil cells following thyroidectomy was described by Severinghaus, Smelser and Clark (1934). Heinbecker and Rolf (1944), working with dogs, produced hypoplasia of the adrenal cortex by hypophysectomy and concluded that adrenocorticotrophin was produced by the acidophils.

The landmark monograph of Harris (1955) signalled the onset of the modern era in our knowledge of the control of secretion of adenohypophyseal hormones. In studying the portal vessels located between the hypothalamus and the anterior pituitary he formulated the chemotransmitter hypothesis for the control of the anterior pituitary. That is, factors elaborated by the hypothalamus into the blood of the hypophyseal-portal vessels modulate, by inhibition or stimulation, synthesis and/or release of the various pituitary hormones.

1.1.2 Anatomy

The fundamental principles of the structure of the pituitary complex in animals are similar to those of humans, although in animals there are certain differences in the morphological relationship of the components of the pituitary to the hypothalamus that are associated with the lesser development of the forebrains. The pituitary gland lies in the hypophyseal fossa of the sphenoid bone (which is rudimentary in rats) where it is overlapped by the diaphragma sellae. On each side, the pituitary is related to the cavernous sinus and the structures which it contains. There is a basic division into two regions of different embryological, morphological and functional characteristics, the neurohypophysis or posterior pituitary and the adenohypophysis or anterior pituitary. The neurohypophysis is a down-growth from the diencephalon connected by neural pathways with the hypothalamus and comprised of the median eminence, the infundibular stem and the pars posterior. The anterior pituitary lobe is derived from the ectoderm of the stomatodeum and can be divided into a pars anterior (pars distalis) and a pars intermedia, which is rudimentary in man. A pars tuberalis or infundibularis is also an extension of the adenohypophysis although it surrounds most of the neural infundibular stem.

The anterior pituitary is a highly vascular structure consisting of epithelial cells of varying size and shape arranged in cords, irregular masses or follicles, separated by a thin-walled vascular sinusoids and supported by a network of reticular tissue. Pituitaries from mammalian species have

basically the same blood supply, with the arteries stemming from branches of the internal carotid artery. There is a primary plexus in the median eminence and venules, forming the hypophyseal-portal system, conveying blood from the capillary meshwork of the median eminence to the sinusoids of the pars distalis. The whole organ is drained by means of short veins that empty into sinuses in the dura mater or in the basi-sphenoid bone.

1.2 THE HORMONES OF THE ANTERIOR PITUITARY AND THEIR REGULATION

1.2.1 Growth Hormone (GH)

Human growth hormone is a polypeptide containing 191 amino acids with two intramolecular disulfide bonds between half-cysteines at positions 53 and 65 and between 182 and 189, and has a molecular weight around 22,000 daltons (Li and Dixon, 1971; Niall, Hogan, Tregear, Segre, Hwang and Friesen, 1973) whereas rat growth hormone contains 190 amino acids (Seeburg, Shine, Martial, Baxter and Goodman, 1977). The secretion of growth hormone by the anterior pituitary is under the control of the hypothalamus through the release of two substances: a growth hormone releasing factor, not yet isolated, and a growth hormone inhibiting factor. Regulation of the secretion of the inhibiting or releasing factors is not well understood but probably involves biogenic amines (Martin, 1973). In addition, other factors may be involved in GH regulation at the hypothalamic and pituitary

levels such as glucagon, steroid hormones and glucose (Podolsky and Sivaprasad, 1972; Krieger and Glick, 1974; Abrams, Parker, Blanco, Reichlin and Daughaday, 1966). Early evidence that the secretion of growth hormone was under the hypothalamic control derived from the observation that growth disturbances occurred in patients with tumours of the infundibulum or pituitary stalk (Frazier, 1936) or in experimental animals with large hypothalamic lesions (Hinton and Stevenson, 1959; Reichlin, 1959). The existence of a growth hormone inhibiting factor was established when Brazeau, Vale, Burgus, Ling, Butcher, Rivier and Guillemin (1973) isolated from sheep hypothalami a tetradecapeptide, which they named somatostatin, that inhibited the release of growth hormone in vivo and in vitro in rats. However, this peptide has wider actions and has subsequently been shown to inhibit the release of a number of other pituitary hormones (TSH and ACTH) as well as hormones from other endocrine and exocrine glands (Hall, Snow, Scanlon, Mora and Gomez-Pan, 1978; Gerich, 1978; Raptis, Schlegel, Lehman, Dollinger and Zoupas, 1978). Growth hormone was found to act indirectly on cartilage to cause growth, through a mediator, named sulfation factor or somatomedin, which was found to have a large number of biologic effects (Salmon and Daughaday, 1957; Daughaday, Hall, Raben, Van den Brande and Van Wyck, 1972). Somatomedin is now known to comprise a family of substances with growth-promoting activity, of which somatomedin A (Hall, Takano, Fryklund and Sievertsson, 1975), somatomedin C (Van Wyck, Underwood, Baseman, Hintz, Clemmons and Marshall, 1975) and NSILA-s (non-suppressible insulin-like activity) (Froesch, Schlumf, Heimann, Zapf, Humbel

and Ritschard, 1975) were purified from human sera. More recently, it has been suggested that somatomedin C mediates growth hormone negative feedback by an immediate effect on hypothalamic somatostatin and a delayed effect on the anterior pituitary (Berelowitz, Szabo, Frohman, Firestone, Chu and Hintz, 1981).

1.2.2 Adrenocorticotrophin Hormone (ACTH)

ACTH is synthesised as a portion of a single large pro-hormone called proopiomelanocortin (Crine, Gossard, Seidah, Blanchette, Lis and Chrètien, 1979) with a molecular weight around 31,000 daltons (Roberts and Herbert, 1977) and stimulates the adrenal cortex to produce corticosteroids. The human ACTH contains 39 amino acids (Riniker, Sieber, Rittel and Zuber, 1972) as does the ACTH from bovine and ovine pituitaries (Li, 1972; Johl, Riniker and Schenkel-Hulliger, 1974). The secretion of ACTH is controlled by the hypothalamus as well as by steroid feedback inhibition at the pituitary. A factor responsible for its control was the first hypothalamic-pituitary regulatory peptide detected and named corticotrophin-releasing factor or CRF (Saffram and Schally, 1955; Guillemin and Rosenberg, 1955). Only recently, however, has a 41 residue peptide been isolated from ovine hypothalami and shown to stimulate the secretion of corticotrophin and β-endorphin by the anterior pituitary (Vale, Spiess, Rivier and Rivier, 1981). Arginine-vasopressin has also been shown to stimulate ACTH release and may act as a potentiating factor to CRF (Gillies and Lowry, 1979).

1.2.3 Gonadotrophin Hormones

The pituitary gland also contains two glycoprotein hormones- luteinising hormone (LH) and follicle-stimulating hormone (FSH) which exert their action primarily on the gonads. They are glycoproteins composed of two subunits; an α-subunit, identical in amino acid sequence in both LH and FSH, and a specific β-subunit which has 70% of the antigenic determinants (Midgley, Niswender, Gay and Reichert, 1972). The isolated α-chain does not have biologic activity and the β-chain alone has a slight biologic activity which is totally regained after addition of α-chain. The molecular weight of both human LH and FSH is around 28,000 daltons with the α-chain containing 89 amino acids and the β-chain containing 115 amino acids (Shome and Parlow, 1974). These two hormones are apparently secreted by the same pituitary cell under the influence of the hypothalamus which releases a gonadotrophin-releasing hormone (GnRH or LHRH). The decapeptide amino acid sequence for LHRH was described by Matsuo, Baba, Nair, Arimura and Schally (1971). Control of gonadotrophin secretion at the pituitary level is a complex interplay between hypothalamic regulatory factors and sex steroid feedback

1.2.4 Thyrotrophin-Stimulating Hormone (TSH)

Another of the well established pituitary hormones is thyrotrophin-stimulating hormone which is responsible for the maintenance of normal thyroid hormone synthesis and secretion. TSH is a glycoprotein with a molecular weight around 29,000

daltons, composed of two polypeptide subunits; an α-subunit of 89 amino acids and a β-subunit of 110 amino acids (Cornell and Pierce, 1973). The β-subunit of bovine TSH has also been isolated and was shown to have 112 amino acid residues (Liao and Pierce, 1970). The thyrotrophin-stimulating hormone is under the stimulatory control of a tripeptide, thyrotrophin-releasing hormone (TRH), synthesised by the hypothalamus. The structure of ovine and porcine TRH was determined by Schally, Redding, Bowers and Barrett (1969). Regulation of TSH secretion results from an interaction between hypothalamus, pituitary and thyroid, the function of the entire complex being modified in a negative feedback manner by the availability of the thyroid hormones (Reichlin, 1978; Larsen, 1982).

1.2.5 Prolactin (PRL)

Prolactin is a single chain polypeptide with 198 amino acid residues and a molecular weight around 23,000 daltons (Li, Dixon, Lo, Schmidt and Pankov, 1970; Parlow and Shome, 1976; Shome and Parlow, 1977). Human prolactin sequence has an identity of 60% residues with rat prolactin and 16% with human growth hormone. A coding sequence of rat pre-prolactin was recently described, consisting of 681 nucleotides (Gubbins, Maurer, Lagrimini, Erwin and Donelson, 1980). These authors also showed that only limited segments of prolactin mRNA were similar to that of growth hormone mRNA, thus suggesting that prolactin and growth hormone probably might not be derived from the same gene.

In mammals, prolactin has been shown to have a role in modulating fluid and electrolyte metabolism although such a role in humans remains unknown (Horrobin, 1980). Studies to correlate plasma prolactin and alterations in plasma osmolarity have been contradictory. A direct relationship between plasma osmolarity and plasma prolactin has been described (Buckman and Peake, 1973) although Baumann and Loriaux (1976) found no evidence for that. In rats, prolactin has an essential luteotrophic as well as antigonadotrophic actions (Smith, 1980). Careful consideration must be given to the possible role of endogenous prolactin in regulating the function of the human corpus luteum as the studies demonstrated conflicting results (Tyson and Friesen, 1973; McNatty and Sawers, 1975). In addition, it has been shown that hyperprolactinaemia can inhibit luteal phase progesterone secretion and shorten the menstrual cycle (Fredricsson, Bjork and Carlstrom, 1977). Wass, Thorner, Morris, Rees, Mason, Jones and Besser (1977) showed a tendency of males with hyperprolactinaemia to have reduced libido and potency, which was reversed by the use of bromocriptine, a dopamine agonist. It has also been suggested that prolactin regulates ACTH-independent adrenal androgen production (Vermeulen, Suy and Rubens, 1977) and that prolactin is one of the factors determining testosterone-oestradiol binding globulin, at least in patients with prolactin-secreting adenomas (Vermeulen, Ando and Verdonck, 1982). The only well documented role of prolactin in humans is the initiation and maintenance of lactation (Tyson, Khojandi, Huth and Andreassen, 1975) but accurate studies of the actions of this hormone upon the human organism remain to be elucidated.

After nearly three decades of research, the specific neural mechanisms that control prolactin secretion have not been entirely elucidated. The secretion of prolactin was demonstrated to be under inhibitory control when hypothalamic extracts added to the culture medium were found to reduce prolactin secretion *in vitro* (Pasteels, 1962; Talwalker, Ratner and Meites, 1963). Following those reports, VanMaanen and Smelik (1968) proposed that dopamine was the prolactin-inhibiting factor, or PIF as it came to be known. In addition, *in vitro* incorporation of [^{3}H]leucine into rat pituitary cells in the presence of dopamine, epinephrine and norepinephrine caused a highly significant inhibition of prolactin release during 7 h incubation (MacLeod, 1969), although the concentrations used were considerably greater than the total catecholamine content of the hypothalamus. Shaar and Clemens (1974) showed that physiological amounts of dopamine were able to inhibit prolactin release from pituitaries incubated *in vitro*. It has been shown that dopamine receptors are present on anterior pituitary cells (Calabro and MacLeod, 1978; Cronin, Roberts and Weiner, 1978; Caron, Beaulieu, Raymond, Gagné, Droiun, Lefkowitz and Labrie, 1978) and that dopamine, but not norepinephrine or epinephrine, is present in rat hypophyseal circulation (Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977; Gibbs and Neill, 1978), establishing dopamine as a secretory product of the median eminence. More recently, Nansel, Gudelsky and Porter (1979) characterised the subcellular compartmentalisation of dopamine in rat anterior pituitary, demonstrating an association between intracellular dopamine

and the prolactin secretory granule. There is also evidence that dopamine may not be the only PIF, as Enjalbert, Moos, Carbonell, Priam and Kordon (1977) reported some inhibiting activity in hypothalamic extracts subjected to alumina treatment. In addition, it has been shown that γ-aminobutyric acid (GABA) can inhibit prolactin release (Schally, Redding, Arimura, Dupont and Linthicum, 1977). Other accumulating lines of evidence support the hypothesis that prolactin release is also regulated by a prolactin-releasing factor (PRF). Thyrotrophin-releasing hormone (TRH) was one of the first peptides shown to stimulate prolactin release (Tashjian, Barowsky and Jensen, 1971) although recent findings suggest that this effect could be due to a non-TRH prolactin-releasing factor (Koch, Golhaber, Fireman, For and Tal, 1977; Boyd, Sanchez-Franco, Spencer, Patel, Jackson and Reichlin, 1978; Harris, Christa and Vagenaki, 1978). Many other substances have been reported to modify prolactin secretion but their mechanisms of action remain to be established.

1.3 CLINICAL EXPRESSION OF HORMONE SECRETION

1.3.1 Introduction

The hormones of the anterior pituitary play a vital part in the modulation of a wide variety of metabolic processes in the human organism. Abnormal patterns of secretion of the pituitary or of its hypothalamic control mechanisms can occur in several systemic diseases, e.g. reticuloendotheliosos and

sarcoidosis (Christy and Warren, 1979). The pathophysiology of events leading to hypersecretion of a hormone of the anterior pituitary remains to be established.

In this thesis we are concerned with the excessive secretion of some of the anterior pituitary hormones in association with pituitary tumours, these being the prolactin-secreting adenomas, growth hormone-secreting adenomas and functionless tumours, and some clinical data of each will be discussed.

1.3.2 Prolactin-Secreting Adenomata (Prolactinomas)

Progress in the elucidation of the pathophysiology of prolactin hypersecretion derives mainly from the establishment of the first satisfactory radioimmunoassay for human prolactin (Hwang, Guyda and Friesen, 1971).

Clinically, most of the patients with hyperprolactinaemia give a history of secondary amenorrhoea and galactorrhoea. The incidence of hyperprolactinaemia in patients with amenorrhoea is reported to be between 13 and 30% (Franks, Murray, Jequier, Steele, Nabarro and Jacobs, 1975; Bohnet, Dahlen, Wuttke and Schneider, 1976; Bergh, Nillius and Wide, 1977; Reichlin, 1979). The great majority of patients harbouring a prolactin-secreting tumour are women (Kleinberg, Noel and Frantz, 1977) and of the 10% male incidence, virtually all present with impotence (Thorner, Edwards, Hanker, Abraham and Besser, 1977).

The basal serum prolactin level is the most useful index of prolactin secretion and normal values for children, adult men, pregnant and non-pregnant women have been established (Jacobs, Mariz and Daughaday, 1972), with 25 µg/l considered the upper

normal limit for all except the pregnant women. There has been an enormous controversy concerning the definition of levels of diagnostic value in a pituitary tumour, mainly because in some cases there is no radiological evidence of a tumour and/or serum prolactin levels are not very high. In addition, several dynamic tests have been described but none has proved to discriminate between a functional and organic disease (Schaison and Cesselin, 1980).

The management of patients with prolactin-secreting adenomas is also somewhat controversial. Several approaches to the disease have been described with variable efficacy and these tumours are now classified as microadenomas, i.e. with less than 10 mm, and macroadenomas.

Transsphenoidal surgery has been advocated as a treatment of choice with an overall success rate of between 60 and 75% (Fahlbusch, 1981; Hardy, 1981). Treatment with local implantation of radioactive ^{90}Yttrium or proton beam external irradiation has also been shown to be as effective as surgery in reducing prolactin levels to normal (Kelly, Mashiter, Doyle, Banks and Joplin, 1978; Kelly, Doyle, Mashiter, Banks, Gordon and Joplin, 1979; Kjellberg, Kliman and Swisher, 1980). In addition to these forms of treatment, medical management of prolactin-secreting adenomas has been shown to be highly effective in lowering prolactin secretion by the anterior pituitary. Various investigators have demonstrated not only lowering of serum prolactin but also regression of the tumour mass after treatment with the dopamine agonist 2-bromo-α-ergocryptine, or simply bromocriptine (Besser, Parker, Edwards,

Forsyth and McNeilly, 1972; McGregor, Scanlon, Hall and Hall, 1979; Sobrinho, Nunes, Santos and Mauricio, 1981; Corenblum and Hanley, 1981) although its mechanism of action has not been established.

1.3.3 Growth Hormone-Secreting Tumours (Somatotrophinomas)

Disturbances of growth hormone secretion begin at any age but are most frequently recognised between the third and fifth decades of life. If the derangement occurs before puberty, i.e. before epiphyseal closure, excess growth hormone secretion results in generalised overgrowth of the skeleton and soft tissues with resulting marked changes in height and size, a syndrome called gigantism. The excessive secretion which develops after puberty causes overgrowth of all responsive tissues, producing a characteristic clinical syndrome of acromegaly. In these patients, an enlargement of facial bones as well as a soft-tissue swelling of the feet and hands gradually takes place. The cause of this hypersecretion of growth hormone is a pituitary tumour, although its pathogenesis has not been elucidated.

In the patient with fully developed acromegaly, diagnosis is made by the typical changes in body configuration. In many patients, however, diagnosis is made by roentgenographic evidence of a pituitary tumour and by demonstrating elevated serum growth hormone. Human growth hormone is secreted intermittently during a 24 h period and secretory spurs of growth hormone are best correlated with the onset of sleep (Takahashi, Kipnis and Daughaday, 1968), exercise (Roth, Glick, Yalow and

Berson, 1963) and hypoglycaemia (Frantz and Rabkin, 1964; Greenwood, Landon and Stamp, 1966). Oestrogens have also been shown to augment basal growth hormone secretion (Frantz and Rabkin, 1965; Vela and Yen, 1969).

Acromegaly is characterised by elevated basal growth hormone levels (Lawrence, Goldfine and Kirsteins, 1970). Moderate correlations have been established between tumour size and circulating growth hormone (Wright, McLachlan, Doyle and Fraser, 1969), although in some cases, serum levels are within the normal range (Ewer and Kotheimer, 1970; Mims and Bethune, 1974). The absence of the suppression of serum growth hormone levels which normally follows the administration of glucose load is most often used in the diagnosis of acromegaly (Earll, Sparks and Forsham, 1967; Daughaday, 1968) although some patients have substantial elevation in serum growth hormone following glucose administration rather than the expected decrease, a phenomenon called paradoxical response (Beck, Parker and Daughaday, 1966). Normal levels before and after glucose administration are less than 5 mIU/l in our laboratory.

Acromegaly has been shown to reduce life expectancy (Wright, Hill, Lowy and Fraser, 1970); therefore, this chronically progressive disease warrants treatment. Surgical treatment has been advocated by some investigators; Hardy (1980) reported 82% success rate cure using transsphenoidal microsurgery in patients harbouring a microadenoma (less than 10 mm). However, analysis of his data shows that only 25% of the cases are microadenomas. Giovanelli, Gaini, Tomei, Motti and Villani (1980),

employing the transsphenoidal route, showed 86% cure for micro-adenomas and 50% cure for infra and supra sella tumours. Conventional radiotherapy has been used alone or in conjunction with surgery, showing more than 60% remission of the disease after 5 years (Eastman, Gorden and Roth, 1979; Bataini and Glinski, 1980). Use of Bragg peak proton beam in 456 acromegalic patients induced 80% remission (GH less than 10 ng/ml) after 2 years of treatment (Kjellberg, Kliman and Swisher, 1980). Interstitial irradiation with ^{90}Yttrium has also been used for the treatment of growth hormone-secreting tumours (Joplin, Cassar, Doyle, Kelly, Mashiter and White, 1980). In their series, 87% had a satisfactory clinical remission after one year of treatment. Several groups have used bromocriptine, a dopamine agonist, as the treatment for acromegaly and reported that approximately 30% of the patients would benefit from long-term treatment with the drug (Liuzzi, Chiodini, Batalla, Gumascoli, Muller and Silvestrini, 1974; Besser, Wass and Thorner, 1978; Cassar, Mashiter and Joplin, 1977; Pelkonen, Vlikahri and Karonen, 1980; Moses, Molitch, Saverin, Jackson, Biller, Furnalleto and Reichlin, 1981). It has also been shown that lisuride, a semi-synthetic ergot derivative, is effective in reducing plasma growth hormone levels after acute administration (Liuzzi, Chiodini, Oppizzi, Botalla, Verde, De Stefano, Colussi, Graf and Horowitz, 1978).

1.3.4 Chromophobe 'Functionless' Tumours

Earlier microscopic studies classified the pituitary cells by the use of tinctorial methods and the term chromophobe was given to those pituitary cells which were not stainable by acid or basic dye (Purves, 1966). These tumours were considered to be the most common among the pituitary tumours (Kohler and Ross, 1973), but new findings demonstrated that, when examined by immunocytochemistry and/or electron microscopy, they contained a variable number of uncharacteristic and small secretory granules and could be associated with prolactin (PRL), growth hormone (GH), adrenocorticotrophic hormone (ACTH), luteinising hormone (LH), follicle-stimulating hormone (FSH) and thyrotrophin-stimulating hormone (TSH) (McCormick and Halmi, 1971; Lewis and Van Noorden, 1974; Snyder and Sterling, 1976; Capella, Usellini, Frigerio, Buffa, Fontana and Solcia, 1979; Kovacs, Horvath, Ryan and Ezrin, 1980).

Despite the advances in techniques to detect hormone secretion, there remains a group of these adenomas that is clinically and biochemically silent and invariably shows negative immunostaining (Landolt, 1979; Kovacs, Horvath, Ryan and Ezrin, 1980; Kovacs, Ryan, Horvath, Singer and Ezrin, 1980). Recent studies suggest that the majority of these tumours could have defined, low levels, of hormone secretory activity. Pituitary tumours associated with increased levels of FSH as well as α-subunits have been reported (Wide and Lundberg, 1981; Ridgway, Klibanski, Ladenson, Clemmons, Beitins, McArthur, Martorana and Zervas, 1981). In addition, an isolated LH-secreting pituitary tumour was also reported (Peterson,

Kourides, Horwitz, Vaughan, Saxena and Fraser, 1981). Recently, Mashiter, Adams and Van Noorden (1981), studying pituitary tissue from 10 patients with functionless tumours by a combination of cell culture, immunocytochemistry and electron microscopy, showed that 80% of these adenomas had detectable levels of LH/FSH secretion, often accompanied by prolactin.

In a retrospective study of 464 patients with chromophobe or functionless adenomas, Crichton, Christy and Damon (1981) found that two-thirds of them sought medical help because of visual disturbances and 25% because of endocrine symptoms of all kinds (e.g. change in libido, change in menses, sweatness) although it was shown that patients delayed reporting endocrine dysfunction, especially sex-related problems, even though these might long precede the neurologic symtpoms.

As for the other pituitary tumours, the pathogenesis of these so-called functionless tumours are not understood. The current available treatment is directed towards the removal of the tumour, usually because lack of clinical expression results in late detection with consequent expansion of the tumour and compression of the optic chiasma, often necessitating emergency admission.

1.4 METHODS OF INVESTIGATION OF ANTERIOR PITUITARY HORMONE SECRETION

1.4.1 Choice of Investigational Model

The advent of new techniques in neuroendocrine research and the elucidation of the nature of the hypothalamic polypeptides are among the advances of the past decade that have greatly enhanced the understanding of the pituitary gland. However, the pathways and mechanisms in the processing of secretory products by the anterior pituitary cells during the various stages of the secretory cycle have not been well understood. Most information is probably available in relation to prolactin, where the sequence of intracellular events are based largely on the morphological studies of Smith and Farquhar (1966) and are virtually identical in all exocrine (Palade, 1975) and endocrine cells (Howell, Kostianovsky and Lacy, 1969; Hopkins and Farquhar, 1973). In a more recent work, Farquhar, Reid and Daniell (1978) employed a dissociated cell system in connection with electron microscopic autoradiography to validate and obtain new information on the route and kinetics of prolactin transport from one cell compartment to another in the lactotroph. These authors showed that, in anterior pituitary cells from oestrogenised rats, the [^{3}H]leucine pulse label is initially distributed randomly over the rough endoplasmic reticulum but moves in 5 to 15 min of chase to the stacked Golgi cisternae where concentration into secretory granules takes place. They also showed that the concentration of small granules in larger forms also occurs rapidly but goes on over a prolonged period (up to 3 h).

Lactotrophs from lactating animals show the morphological features associated with cells active in protein secretion - a highly developed rough endoplasmic reticulum, a large population of attached polyribosomes and a large Golgi complex with many forming secretory granules. Synthesis of prolactin apparently occurs randomly within the rough endoplasmic reticulum and is rapidly transported to the Golgi system where concentration takes place. Several of the small granules merge to form immature granules of increasing size and shape. These immature granules eventually form mature granules which move out of the Golgi to the cytoplasm and remain confined to the secretory compartment until continuity is established with the extracellular space by fusion of secretory granules with the plasma membrane during exocytosis (Smith and Farquhar, 1966; Smith and Farquhar, 1970; Shiino, William and Rennels, 1972). After exocytosis has occurred, the dynamics of intracellular membranes are affected, particularly membrane retrieval. It has been shown that anionic ferritin did not bind to the pituitary cell surface and it was confined to endocytic vesicles and lysosomes, whereas incoming vesicles charged positively with cationic ferritin fused with compartments of the secretory pathway (Farquhar, 1978). The continual insertion of a considerable amount of Golgi-derived membranes into the plasmalemma at the time of exocytosis has also been shown to occur in plasma cells (Ottosen, Courtoy and Farquhar, 1980). However, granule discharge can occur not only to the extracellular space but also to the lysosomal compartment by a process designated as crinophagy. Cells that have stopped

secretion accumulate in their cytoplasm the material they have elaborated. In the rat, when lactation is interrupted, the lysosomes of the lactotrophs coalesce with secretory granules that they progressively digest. They can also phagocytose ribosomes and fragments of endoplasmic reticulum membranes (Smith and Farquhar, 1966). This intracellular disposal mechanism that exists for the turnover of secretory protein can occasionally be found in normal animals but is greatly enhanced in the post-lactating animals. A diagrammatic representation of these proposed events in the secretory process of lactotrophs in the anterior pituitary gland is illustrated in Fig. 1.1.

The secretory granules are disposed of primarily by direct fusion with pre-existing lysosomes (dense bodies and multivesicular bodies) rather than by the usual form of autophagy which involves de novo membranous sequestration or envelopment by a Golgi or endoplasmic reticulum cisternae (de Duve and Wattiaux, 1966). These authors provided a functional classification for lysosomes in which they are divided in primary lysosomes, or those which have not yet been involved in digestive events; and secondary lysosomes, which are or have been sites of digestive activity. Table 1.1 shows this classification.

In the case of the post-lactating rats as studied by Smith and Farquhar (1966), 24 h after the removal of the suckling young, secretory granules could be recognised within the reactive dense and multivesicular bodies. Although few

Fig 1.1 CONTROL OF PITUITARY PROLACTIN SECRETION

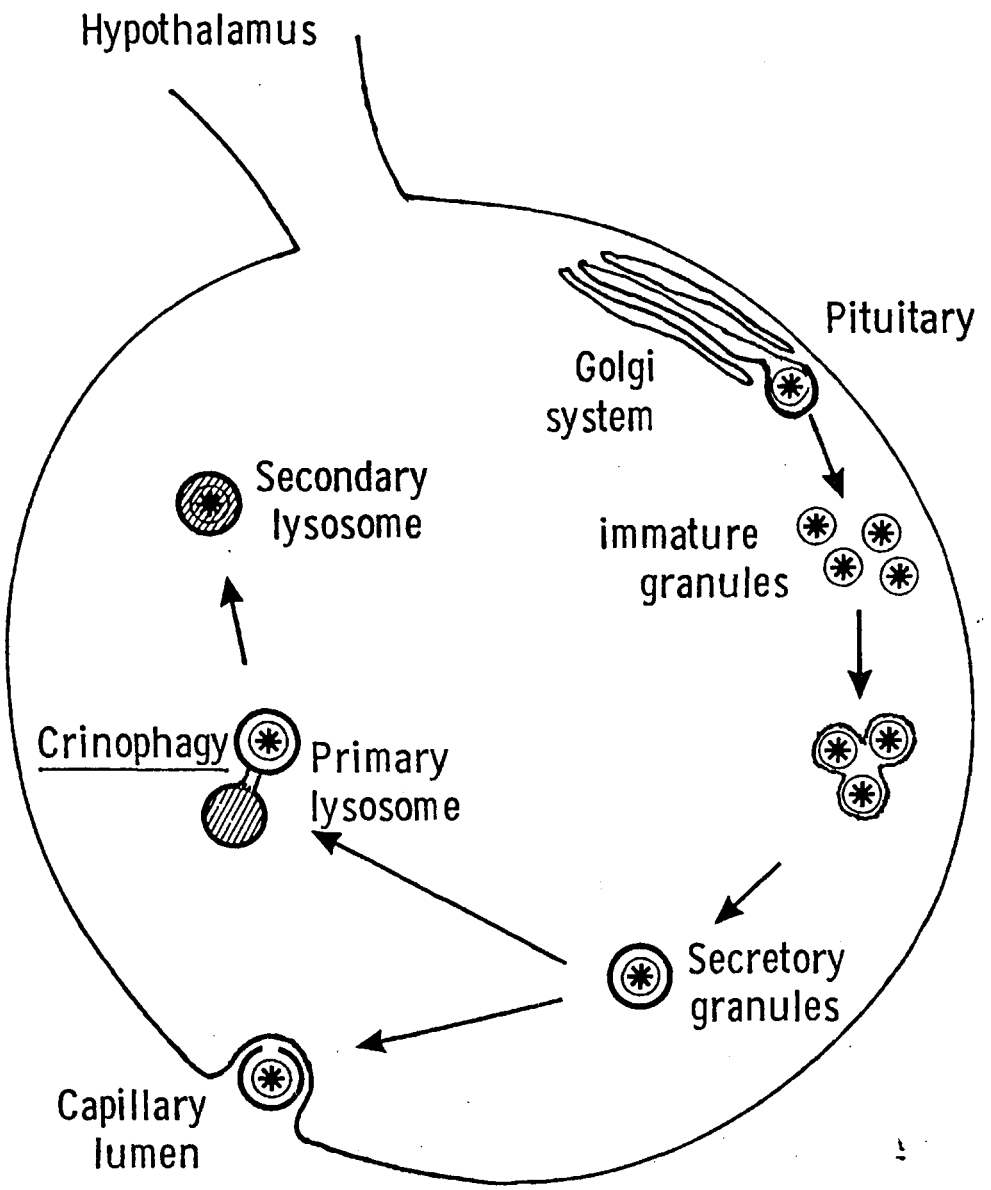


Table 1.1

FUNCTIONAL CLASSIFICATION OF LYSOSOMES*

Nomenclature	Functions of lysosomal particles		Formation of lysosomes
	Heterophagy	Autophagy	
Pre-lysosomes	Heterophagosomes	Autophagosomes	Rough-endoplasmic reticulum
Lysosomes	Heterolysosomes 2^{o} Lysosomes Telolysosomes	Autolysosomes	Smooth-endoplasmic reticulum Golgi complex 1^{o} Lysosomes proteins for export
Post-lysosomes	Residual bodies		

* From de Duve and Wattiaux (1966)

autophagic vacuoles were seen, these were comprised mainly of rough-surfaced endoplasmic reticulum and ribosomes. In addition, it has recently been demonstrated that, during the oestrous cycle, there is a marked variation in the capacity of dopamine to stimulate the activity of the lysosomal enzyme ß-glucuronidase in the anterior rat pituitary; pituitaries from diestrous rats were the most sensitive to the action of dopamine (Nansel, Gudelsky, Reymond and Porter, 1981). In addition, morphometric analysis of rat anterior pituitaries throughout the oestrous cycle showed that the volume of secondary crinophagic lysosomes per lactotroph increased during late oestrous and remained elevated throughout early diestrous 1. An inverse relationship between the volume of mature secretory granules per cell and of the crinophagic system has also been demonstrated recently (Poole, Mahesh and Costoff, 1981).

Thus the lactotroph cell has proved to be a favourable cell type for studies on secretory processes of the anterior pituitary because its secretory activity can be easily manipulated under a variety of physiological conditions such as lactation, oestrogen treatment or removal of suckling young. In addition, pharmacological substances for suppression of secretion, such as the dopamine agonist bromocriptine, are readily available. In the case of pregnancy, and consequent lactation, it has been shown that lactotrophs hypertrophy and are highly active (Everett and Baker, 1945; Goluboff and Ezrin, 1969) becoming the predominant cell type, thereby reducing difficulties associated with cell heterogeneity, making it an ideal model for the study of secretion in pituitary cells.

1.4.2 Cell Biology

The investigation of prolactin cell secretory mechanisms, prior to this study, had relied largely on morphological techniques. Our interest was to determine the mechanisms and pathways for processing the secretion within anterior pituitary cells by the application of more refined biochemical procedures.

Tissues are composed of cells held within a fibrous framework. A degree of organisation is contained in these cells in which membrane systems and structural entities or organelles have been observed by electron microscopy. Characterisation of subcellular organelles by tissue fractionation (Bensley and Hoerr, 1934) and electron microscopy (Porter, Claude and Fullam, 1945) introduced the concept of subcellular compartmentation. Histochemical methods for staining were introduced in the nineteenth century and microtechniques were developed, capable of measuring enzyme activities in small pieces of tissue, single cells or fragments of cells. In this way, the distribution of a number of enzymes such as catalase, urease, peptidase (Linderstrom-Lang, 1938), and other enzymes could be demonstrated by specific staining procedures (Pearse, 1960). These methods for revealing enzyme activities in the cell acquired validity at the intracellular level after the work on liver cells by Novikoff and Essner (1960). The initial approach was a preparative fractionation in which individual organelles were isolated to high purity, often using electron microscopy to characterise the organelles. A relationship

between specific fractions and marker enzymes was then determined. These marker enzymes could subsequently be used for analytical subcellular fractionation. By quantifying the distribution of enzyme activities in each tissue fraction after analytical fractionation (Schneider and Hogeboom, 1951), it was possible to use enzymes as markers for intracellular particles and thus, tissue fractionation experiments could be effected as chemical fractionations.

The fate of almost every distinct cytoplasmic entity that has since come to be recognised derives from the data of Novikoff, Podber, Ryan and Noe (1953) working with liver homogenates. Tissue fractionation was originally developed as a cytological method, with the microscope as a guide and chemical analysis as an adjacent tool. The discovery that certain enzymes were largely concentrated in one fraction resulted in enzymes prevailing over the morphological observations with the consequent new interpretation of biochemical heterogeneity.

The use of an enzyme as a marker for its host-particle was based on the assumption that an enzyme belonging to a single class of particle (single location) and its specific activity being the same in the different particle subclasses separated by the fractionation procedure (biochemical homogeneity). Biochemical homogeneity is defined as the fixed association of an enzyme with a specific organelle. In addition, heterogeneity within a given population of subcellular organelles is almost bound to emerge at some stage and this may be difficult to assess, since factors such as partial inactivation of the enzyme, injury

to the particles or cellular heterogeneity can account for it (Leighton, Poole, Beaufay, Baudhuin, Coffey, Fowler and de Duve, 1968).

Early tissue fractionation techniques only allowed the discrimination of four main fractions: nuclear, mitochondrial, microsomal and cytosol. With the development of new procedures it became obvious that each of these fractions could be further subdivided, resulting in better resolution of subcellular organelles.

Centrifugation of rat liver homogenates separated the hydrolytic enzyme acid phosphatase from other respiratory enzymes, indicating the existence of an acid phosphatase-containing particle (de Duve, 1969).

These particles were given the name lysosome by Appelmans, Wattiaux and de Duve (1955) and were described by these authors as a sac-like structure containing a variety of acid hydrolases surrounded by a membrane. The discovery of structure-linked latency for an enzyme arose when apparently less acid phosphatase activity was found in the liver tissue homogenised in isotonic sucrose assayed immediately, compared with assays performed after freezing and thawing the tissue. Particulate fractions separated by differential centrifugation from rat liver showed low acid phosphatase activity (Wattiaux and de Duve, 1956), and increasing acid phosphatase activity was found in the particulate fraction after standing at room temperature for a few days or in aged preparations. Later, other methods for activation and solubilisation of acid phosphatase (Berthet, Berthet, Appelmans and de Duve, 1951) further defined the concept of particles

containing acid hydrolases within a membrane which restricted access of internal enzyme to external substrate. The cytochemical definition of lysosomes (Novikoff, 1961), although reported later than the biochemical one, is essentially similar and based on the presence of a single unit membrane and positive staining reaction for acid phosphatase.

The anterior pituitary contains several types of cells and the great majority of subcellular fractionation studies have been directed towards the isolation of each hormone granule.

Attempts to separate and characterise cytoplasmic hormone granules have been done with pituitary glands from different species, using different homogenising media, times and speeds of centrifugation. These studies were mainly related to the isolation of gonadotrophs (Catchpole, 1948; McShan and Meyer, 1952). The hormonal and enzymatic activities of the granules, especially gonadotrophs, and other particulate fractions were studied by Perdue and Meyer (1962) who showed that a fraction containing predominantly gonadotrophs could be isolated although with some contamination with thyrotrophin and alkaline phosphatase. These authors also measured protein, glucose-6-phosphatase, acid protease and succinic dehydrogenase in the various fractions. In more recent studies, bovine anterior pituitary was employed in fractionation procedures aimed at isolating prolactin, growth hormone, thyrotrophin stimulating hormone, luteinising hormone and follicle-stimulating hormone containing granules (Tesar, Koenig and Hughes, 1969; LaBella, Krass, Fitz, Vivian, Sin and Queen, 1971).

Differential centrifugation was used to isolate fractions from normal and castrated adult female rats (McShan, Rozich and Meyer, 1953). Amino transferase was located in the supernatant fraction and succinoxidase was distributed between the nuclear and granule fractions. Further studies by LaBella and Brown (1958) demonstrated that, in pigs, an acid proteinase activity predominated in the mitochondrial fraction, whereas alkaline phosphatase was found in the microsomal fraction. McDonald, Reilly, Zeitman and Ellis (1968) purified an hydrolase, dipeptidyl arylamidase II, and showed by sucrose density gradient centrifugation, that it was localised along with acid phosphatase in the pituitary lysosomes.

The study of the human anterior pituitary has relied, in the past, mainly on morphological data. There have been few studies on the levels, intracellular localisation and dynamics of hormones in the human pituitary. Ultrastructural studies have demonstrated a bewildering array of abnormalities in human pituitary tumours (Olivier, Vila-Porcile, Racadot, Peillon and Racadot, 1975). These include marked heterogeneity of hormone granules, prominent lysosomes and conglomerate mitochondria but it has not been possible with this technique to study functional changes in these glands as there appears to be little correlation between ultrastructural alterations and functional disorders of the adenomas. Microscopic identification of secretory cells depends primarily on the capacity of these cells to store the hormones they synthesise. The hormone accumulates in the form of cytoplasmic granules and their affinity for certain dyes and

probably their ultrastructure reflect the chemical composition of the hormone. These tinctorial characteristics provided the basis for the classification of anterior pituitary cells (Pearse, 1952; Wilson and Ezrin, 1954). The utilisation of the electron microscope by Farquhar and Rinehart (1954) provided additional data about the identification of the pituitary cells. The utilisation of immunocytochemistry in electron microscopy, introduced by Nakane (1970), for determining the cellular origin of pituitary hormones has been of great value. More recently, in order to investigate the control of anterior pituitary secretion, several authors have described in vitro culture systems (Vale, Grant, Amoss, Blackwell and Guillemin, 1972; Zimmerman, Defendini and Frantz, 1974; Gillies, Ratter, Grossman, Gaillard, Lowry, Besser and Rees, 1980; Adams, Brajkovich and Mashiter, 1981) that would be suitable for combined morphological and biochemical studies.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Rat pituitary tissue

2.2 Human pituitary tissue

2.3 Enzymic analysis

2.4 Subcellular fractionation

2.5 Radioimmunoassay

2.6 Electron microscopy

2.7 Cell culture

CHAPTER 2

MATERIALS AND METHODS

2.1 RAT PITUITARY TISSUE

The anterior pituitary is an heterogeneous endocrine gland, containing five or more cell types capable of producing numerous different hormones. The prolactin-secreting cell (lactotroph or mammotroph) has been identified as a distinct entity in all mammalian species, the original description of its appearance at the electron microscope level being based on the examination of pituitaries from lactating rats (Farquhar and Rinehart, 1954). Lactotrophs are present in both male and female pituitaries but are particularly prominent in pituitaries from pregnant and especially lactating females where secretory activity is maximal (Pasteels, 1961). Oestrogens are known to be potent stimulators of prolactin secretion in man and rat (Jacobs, Snyder, Utiger and Daughaday, 1973; De Lean, Garon, Kelly and Labrie, 1977), and are probably responsible for the marked increase of lactotroph cells that occur during pregnancy and lactation.

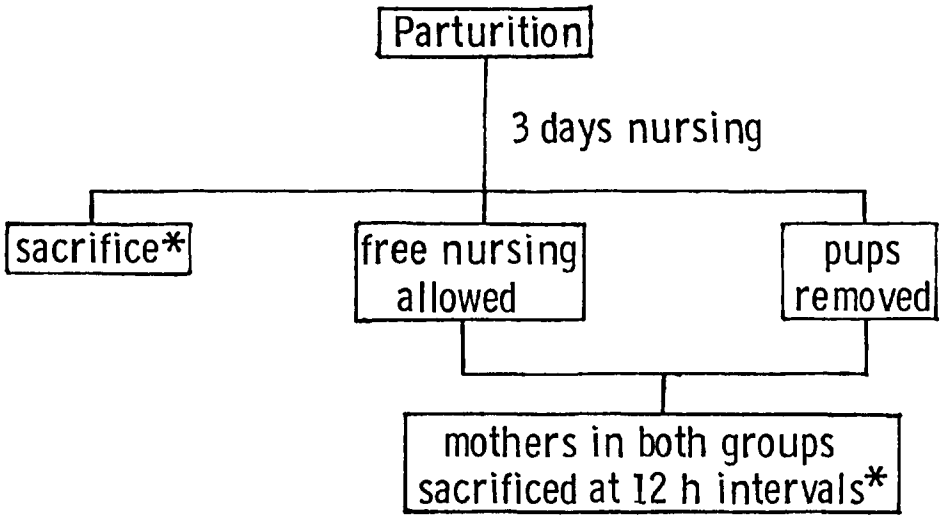
Smith and Farquhar (1966) in their early studies of the morphology of the lactotroph cell chose the lactating rat, as the majority of pituitary cells (70%) are lactotrophs. Furthermore, following removal of the suckling young they had demonstrated the presence of lysosome - hormone granule fusion. Although methods of separation of the various types of pituitary cells have

subsequently been reported (Hymer, Evans, Kraicer, Mastro, Davis and Griswold, 1973; Lloyd and McShan, 1973) they were designed for use with large amounts of bovine tissue. Hence, for this study the procedure adopted was based on that of Smith and Farquhar (1966) using the lactating-suckling rat with suckling withdrawal being applied as the stimulus to lactotroph involution.

The experimental procedure is shown in Fig. 2.1. To determine pituitary enzyme levels in lactating and post-lactating rats, adult primiparous Sprague-Dawley rats, weighing 150-250 g, were housed in individual cages with food and water ad libitum and exposed to 14-10 h light-dark cycles. After parturition the rats were allowed to freely nurse 8-10 pups each (this number being adjusted as necessary) for 3 days. This period of lactation was used because it has been shown that between 3 and 5 days after parturition the secretion of prolactin is maximal (Grosvenor and Turner, 1958).

The number of animals used in each individual period studied ranged from 7 to 14 rats. After the initial period of 3 days lactation, 14 rats were sacrificed, in a separate room, by decapitation. Trunk blood was collected in heparinised vials which were centrifuged at 400 x g for 10 min in an MSE Coolspin centrifuge (Measuring and Scientific Equipment, Crawley, Sussex) and plasma stored at -20°C until measurement of prolactin by radioimmunoassay. The anterior pituitary was removed and disrupted by 12 strokes of a loose-fitting (type A) Dounce homogeniser (Kontes Glass Co., Uniscience, Cambridge) containing 3 ml ice-cold 0.25 mol/l sucrose, 1 mmol/l EDTA disodium salt, pH 7.2 and 20 mmol/l ethanol. Pituitary homogenates were stored at -20°C until assayed for enzyme activity, prolactin, protein and DNA content. In addition, anterior pituitaries removed from

Fig. 2.1 Experimental model for pituitary crinophagy using suckling rats



*

1) Plasma prolactin measured

2) Pituitary homogenates assayed for:

   a) Organelle marker enzymes

   b) prolactin content

   c) DNA content

   d) protein content.

lactating rats and from a lactating rat which had her suckling litters removed 24 h before, were subjected to analytical subcellular fractionation as described in Section 2.4.

To determine the effects of the dopamine agonist bromocriptine on prolactin secretion in lactating animals, 24 rats were given a single subcutaneous injection of bromocriptine mesylate (Sandoz Products, Feltham, Middlesex) with 0.1 ml doses containing from 500 µg to 500 µg dissolved in 150 mmol/l sodium chloride containing 5.5 mmol/l ethanol; a further 8 lactating rats were given 0.1 ml of the vehicle diluent. All rats were allowed to keep their pups suckling and were sacrificed by decapitation 12 and 24 h later. The procedure was similar to the one described for studying pituitary enzyme levels in lactating and post-lactating animals. A single anterior pituitary from a lactating rat treated with 500 µg of bromocriptine mesylate was subjected to analytical subcellular fractionation as described in Section 2.4.

To investigate the effects of bromocriptine on prolactin secretion in post-lactating rats, 8 lactating rats were given a subcutaneous injection of 500 µg of bromocriptine mesylate dissolved in 0.1 ml of 150 mmol/l sodium chloride containing 5.5 mmol/l ethanol. A further 8 rats were given 0.1 ml of the vehicle diluent.

2.2 HUMAN PITUITARY TISSUE

Human pituitary tumours and normal pituitary tissue were obtained from neurosurgical explorations and processed immediately. Pituitary tissue from other centres was transported to the laboratory in 10 ml Eagle's Minimum Essential Medium containing 10% fetal calf serum (Grand Island Biological Co., Glasgow), 100 U/ml penicillin, 100 µg/ml streptomycin (Glaxo Laboratories Ltd., Greenford, Middlesex) and 2.5 µg/ml fungizone (Squibb & Sons, Twickenham, Middlesex) buffered with 20 mmol/l Hepes (Hopkin & Williams, Chadwell Heath, Essex). Transport time varied from a few hours to 2 days. On receipt the tissue was divided into small pieces, portions being examined by histochemistry, electron microscopy and cell culture techniques to determine: that it was solely tumorous and not contaminated with adjacent normal tissue; and its type in terms of hormone content and secretory activity. Clinical diagnoses of individual patients were obtained from referring physicians and neurosurgeons and from analysis of pre-operative blood hormone levels.

The pieces used for enzymic studies were placed in 3 ml ice-cold 0.25 mol/l sucrose containing 1 mmol/l Na_2EDTA, pH 7.2 and 20 mmol/l ethanol. Tissue was disrupted by 12 strokes of a loose-fitting (type A) pestle in a small Dounce homogeniser as described previously in Section 2.1. The procedure described for analytical subcellular fractionation of fresh pituitary tissue was similar to that described for rat pituitary tissue in Section 2.4. The homogenates or subcellular fractions were stored at -20°C until assayed for enzyme activities, protein, prolactin, growth hormone (GH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

2.3 ENZYMIC ANALYSIS

2.3.1 Introduction

An enzyme is a protein with specific catalytic properties. It is not indiscriminate in its catalytic action but is strictly limited to one substance or, at most, a small group of closely related substances. The first general theory of chemical catalysis was published by Berzelius in 1835 but the name enzyme, or 'in yeast' was suggested some years later by Kuhne, in 1878. Nearly 2,000 different enzymes are now known and they are widely distributed in living cells. However, relatively little is known about the concentration of enzymes or their localisation in the anterior pituitary gland. Because of the small amount of tissue (less than 15 mg) that was obtained from either the rat or human pituitary, it was necessary to adopt microanalytical techniques for this study and some of the properties and characteristics of the enzymes assayed will be described.

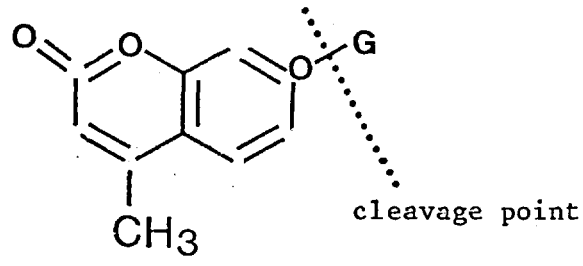
Enzyme activities measured in this thesis, except for catalase, are expressed as milliunits of activity per milligram protein (mU/mg). where 1 milliunit corresponds to the hydrolysis of 1 nmol of substrate per minute at 37°C. All results are expressed as a mean ± standard error of the mean (SEM). Since many of these assays had not been applied previously to pituitary tissue it was necessary to determine the optimum conditions for each enzyme, including pH, time of incubation and amount of tissue homogenate.

The majority of assays employed fluorogenic substrates. The ability of natural substances to fluoresce, either directly or after chemical reactions, has widespread use in enzymic analysis, especially

enzymes involving NAD, NADP or FAD as cofactors. In addition, hydrolases involving synthetic substrates which yield fluorescent products are readily assayed by this approach. Most hydrolases (glucosidases and phosphatases) show relatively little specificity towards the aglycone moiety of the substrate molecule (Walker, 1964) and have been termed group specific hydrolases. The reaction catalysed may be represented:

$$G\text{---}O\text{---}R + H_2O \rightarrow G\text{---}OH + R.OH$$

There is low specificity for group R (4-methylumbelliferyl group). The enzyme is specific for the G group, which when cleaved leaves the highly fluorescent compound 4-methylumbelliferone.



2.3.2 Enzyme Assays

(i) Acid hydrolases

A modification of the method of Leaback and Walker (1961) was used for these assays. Artificial substrates of 4-methyl-umbelliferyl derivatives (Koch Light Laboratories, Colnbrook, Kent) were prepared as 10 nmol/l stock solutions of moisture-free 2-methoxyethanol (BDH Chemicals Ltd., Poole, Dorset). The substrate (0.21 nmol/l) was freshly diluted in the appropriate buffer containing 1 g/l Triton X-100 (Sigma Chemical Co. Ltd., London).

The assays were carried out with 0.1 ml of a suitably diluted sample incubated for 5 - 60 min at 37°C with 0.25 ml of the appropriate buffered substrate in 0.1 mol/l acetic acid-sodium acetate buffer, over a pH range 3.5 - 6.5. The reaction was stopped with 1 ml ice-cold 50 mmol/l glycine-NaOH buffer, pH 10.4 and the liberated maximal fluorescence of 4-methylumbelliferone measured in a Model 204 Fluorescence Spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, Bucks.). The exciting wavelength was 365 nm and the emission setting was 460 nm. Appropriate substrate blanks were performed in all assays. A standard fluorescence block was calibrated with a solution of 4-methylumbelliferone and the activity of the samples calculated, 0.1 nmol 4-methylumbelliferone being equivalent to 23 fluorescent divisions.

The pH, time course and concentration-activity graphs of N-acetyl-β-glucosaminidase (EC 3.2.1.30) in rat anterior pituitary homogenates are shown in Fig. 2.2. A pH optimum of 5.7 was found and linear kinetics were obtained with incubation periods up to 60 min. Fig. 2.3 shows time course and concentration-activity graphs for β-glucuronidase (EC 3.2.1.31) and acid phosphatase (EC 3.1.3.2), linear kinetics being obtained for both enzymes.

Latent N-acetyl-β-glucosaminidase, a measure of lysosomal integrity (Peters, Heath, Wansbrough-Jones and Doe, 1975), was determined by assaying the enzyme activity in sucrose medium with the appropriate buffered substrate in the absence (free activity) or presence (total activity) of Triton X-100 (1 mg/ml) and was expressed as a percentage:

$$\frac{\text{Total - Free}}{\text{Total}} \times 100\%$$

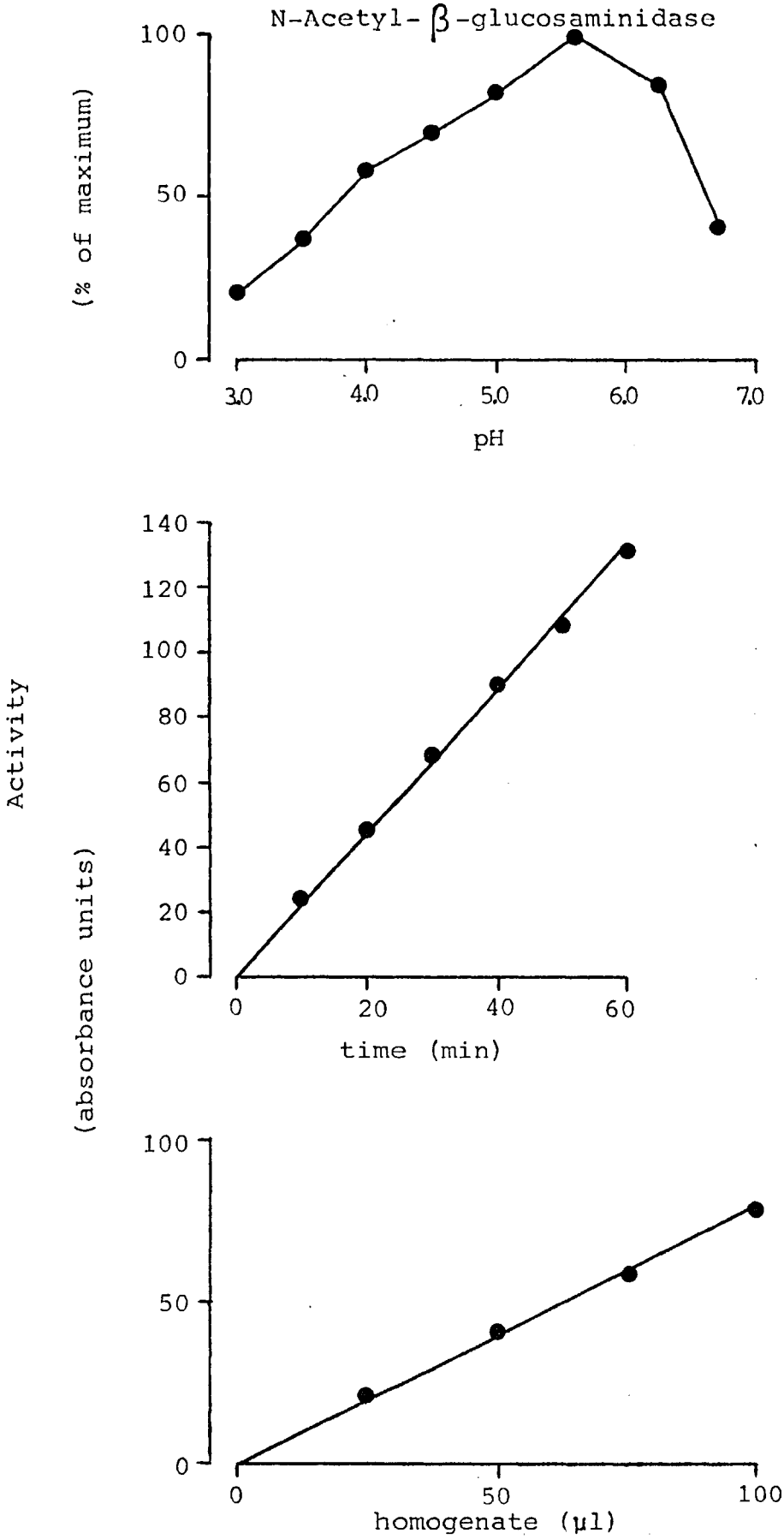


Fig. 2.2 pH optimum, time course and concentration-activity graphs for N-acetyl-β-glucosaminidase

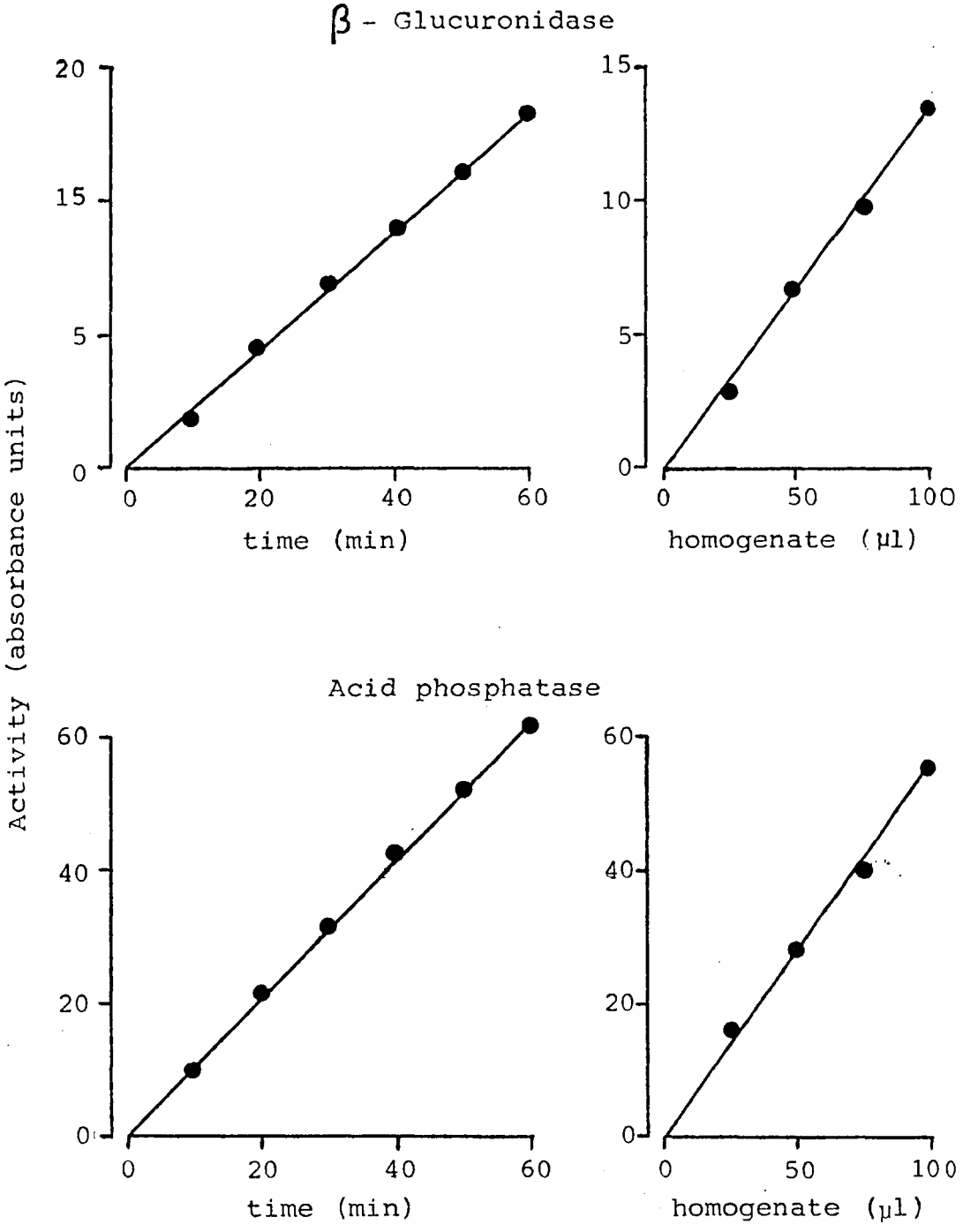


Fig. 2.3 Time course and concentration-activity graphs for β-glucuronidase and acid phosphatase

(ii) 5' Nucleotidase (EC 3.1.3.5)

Typical substrates are adenosine-5' phosphate and inosine-5' phosphate which are also hydrolysed by the non-specific phosphatases. It was previously reported that non-specific alkaline phosphatase activity could be suppressed by incorporating sufficient 2-glycero-phosphate into the reaction mixture to saturate the activity of the interfering enzyme (Belfield and Goldberg, 1968).

The enzyme was assayed by a modification of the methods of Avruch and Wallach (1971) and Douglas, Kerley and Isselbacher (1972), where a fresh substrate containing 24 mmol/l magnesium chloride, 12 mmol/l 2-glycerophosphate, 0.12 mmol/l adenosine-5' monophosphoric acid (Type III, Sigma Co.), 1 g/l Triton X-100 (Sigma Co.) and 250 µl tritiated adenosine 5' monophosphate (diammonium salt, specific activity 15 Ci/mmol; New England Nuclear, Winchester, Hants.) in 50 ml of 60 mmol/l piperazine-HCl buffer at a pH optimum of 9.0.

A suitably diluted sample or blank, 0.1 ml, was incubated with 0.5 ml substrate for periods up to 60 min at 37°C. The reaction was terminated with 0.25 ml of 0.25 mol/l zinc sulphate, and the tubes were immediately vortexed. Protein and unhydrolysed adenosine monophosphate were precipitated following the addition of 0.25 ml of 0.25 mol/l filtered barium hydroxide. The mixture was left on ice-bath for 30 min and then centrifuged at 1500 x g for 30 min at 4°C in an MSE Coolspin centrifuge (Measuring and Scientific Equipment). To 0.25 ml of supernatant was added 5.0 ml PCS scintillant (Amersham International, Amersham, Bucks.) and the mixture counted for 5 min in a Beckman LS 250 scintillation counter (Beckman-RIIC Ltd., High Wycombe, Bucks.). The standard was prepared by mixing 1.0 ml of the buffered substrate with 1.2 ml of deionised water; 0.25 ml of

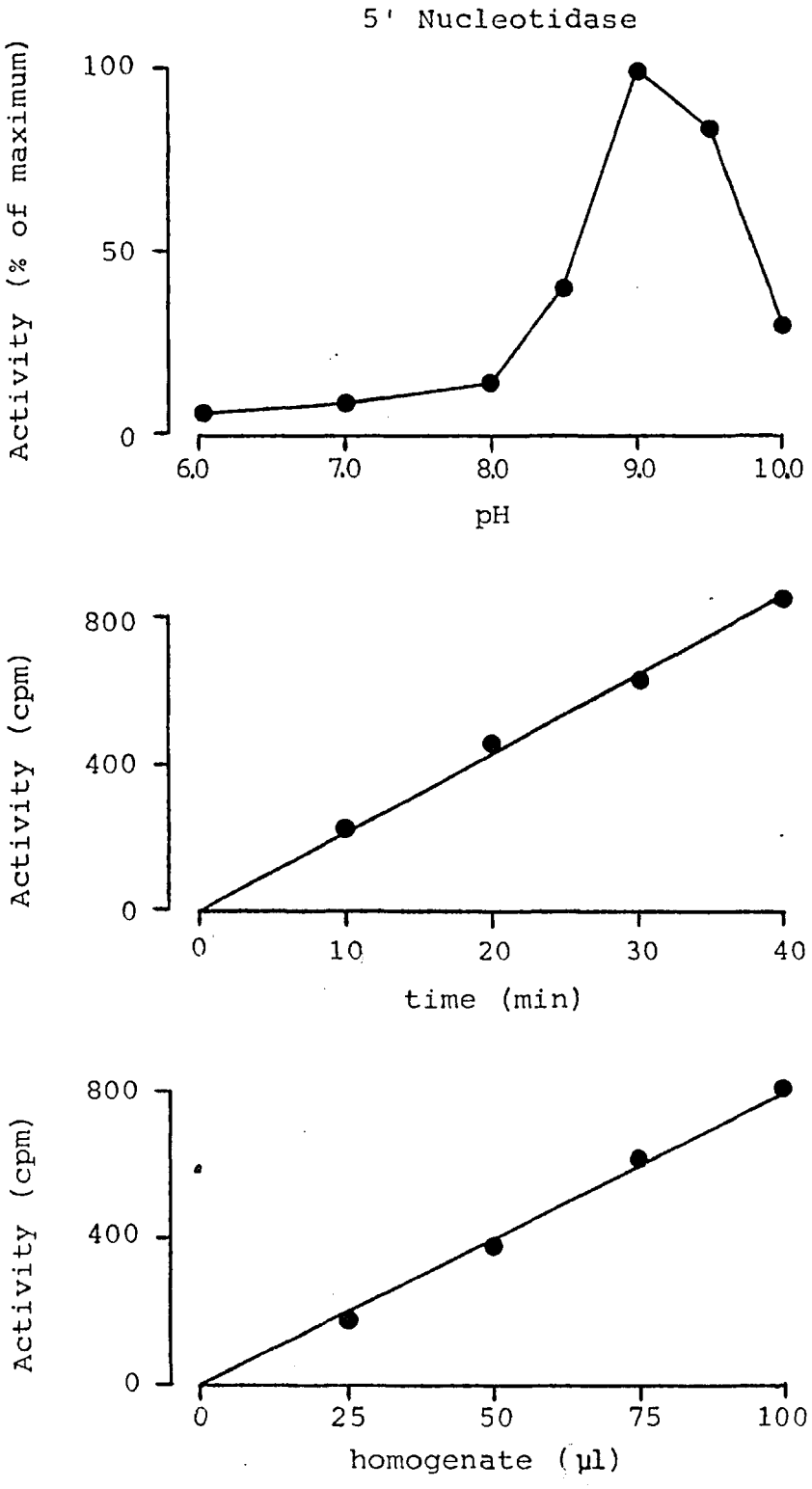


Fig. 2.4 pH optimum, time course and concentration-activity graphs for 5'nucleotidase

this solution plus 5.0 ml of PCS was counted as described for the samples. The enzyme activity was calculated according to the formula:

$$\text{Activity (mU/ml)} = \frac{\text{cpm sample} - \text{cpm blank}}{\text{cpm standard} - \text{cpm}} \times \frac{600}{\text{t(min)}}$$

Fig. 2.4 shows the effects of pH, time course and concentration-activity graphs of the enzyme. A pH optimum of 9.0 was obtained and showed linear kinetics with incubation periods up to 40 min.

(iii) Alkaline phosphatase (EC 3.1.3.1)

Alkaline phosphatases are a group of relatively non-specific enzymes which hydrolase almost all orthophosphoric monoesters under alkaline conditions. They may act either as hydrolases (a) or as transferases (b).

(a) $RO.P(O^-)(=O)(O^-) + H_2O \rightleftharpoons R.OH + HO.P(O^-)(=O)(O^-)$

(b) $RO.P(O^-)(=O)(O^-) + R'.OH \rightleftharpoons R.OH + R'O.P(O^-)(=O)(O^-)$

This enzyme was assayed according to the method of Fernley and Walker (1965). Suitably diluted samples or blank, 0.1 ml, were incubated with 0.25 ml buffer containing 0.21 mmol/l 4-methylumbelliferyl phosphate in 0.1 mol/l diethanolamine-HCl, pH 9.25 for up to 30 min at 37°C. The addition of 2 ml 50 mmol/l glycine-NaOH buffer, pH 10.4 stopped the reaction and the liberated 4-methylumbelliferone was measured as described for the acid hydrolases.

The effects of pH, time, pituitary homogenate concentration and Mg^{2+} are shown in Fig. 2.5. A pH optimum of 9.25 was found with an optimum $MgCl_2$ concentration of 1.4 mmol/l, and activity was linear for up to 30 min.

(iv) Neutral α-glucosidase (EC 3.2.1.20)

This enzyme, which is thought to hydrolyse maltose and maltotriose, was assayed according to Peters, Müller and de Duve (1972). Appropriately diluted sample or blank, 0.1 ml, were incubated for up to 60 min at 37°C with 0.25 ml of 0.21 mmol/l 4-methylumbelliferyl-α-D-glucopyranoside (Koch Light Laboratories) in 0.1 mol/l cacodylate-HCl buffer, pH 7.5, containing 1 g/l Triton X-100 (Sigma Co.). The reaction was stopped with 2 ml buffered glycine-NaOH (50 mmol/l), pH 10.4 and the liberated 4-methylumbelliferone calculated as described for the acid hydrolases. Time course and concentration-activity graphs are shown in Fig. 2.6, linear kinetics being obtained.

(v) Cathepsin C (EC 3.4.14.1)

This enzyme is of lysosomal origin and cleaves amide, arylamide, ester or peptide substrates. The assay was carried out according to Peters, Müller and de Duve (1972). Suitably diluted samples, 0.1 ml, were incubated for up to 30 min at 37°C with 0.25 ml of 0.21 mmol/l glycyl-L-phenylalanyl-2-naphthylamide (Sigma Co.), in 0.1 mol/l acetic acid-sodium acetate buffer, pH 5.0 containing 1 g/l Triton X-100 (Sigma Co.), 1 mmol/l disodium EDTA, 10 mmol/l NaCl and 1 mmol/l dithiothreitol (Sigma Co.). The reaction was stopped

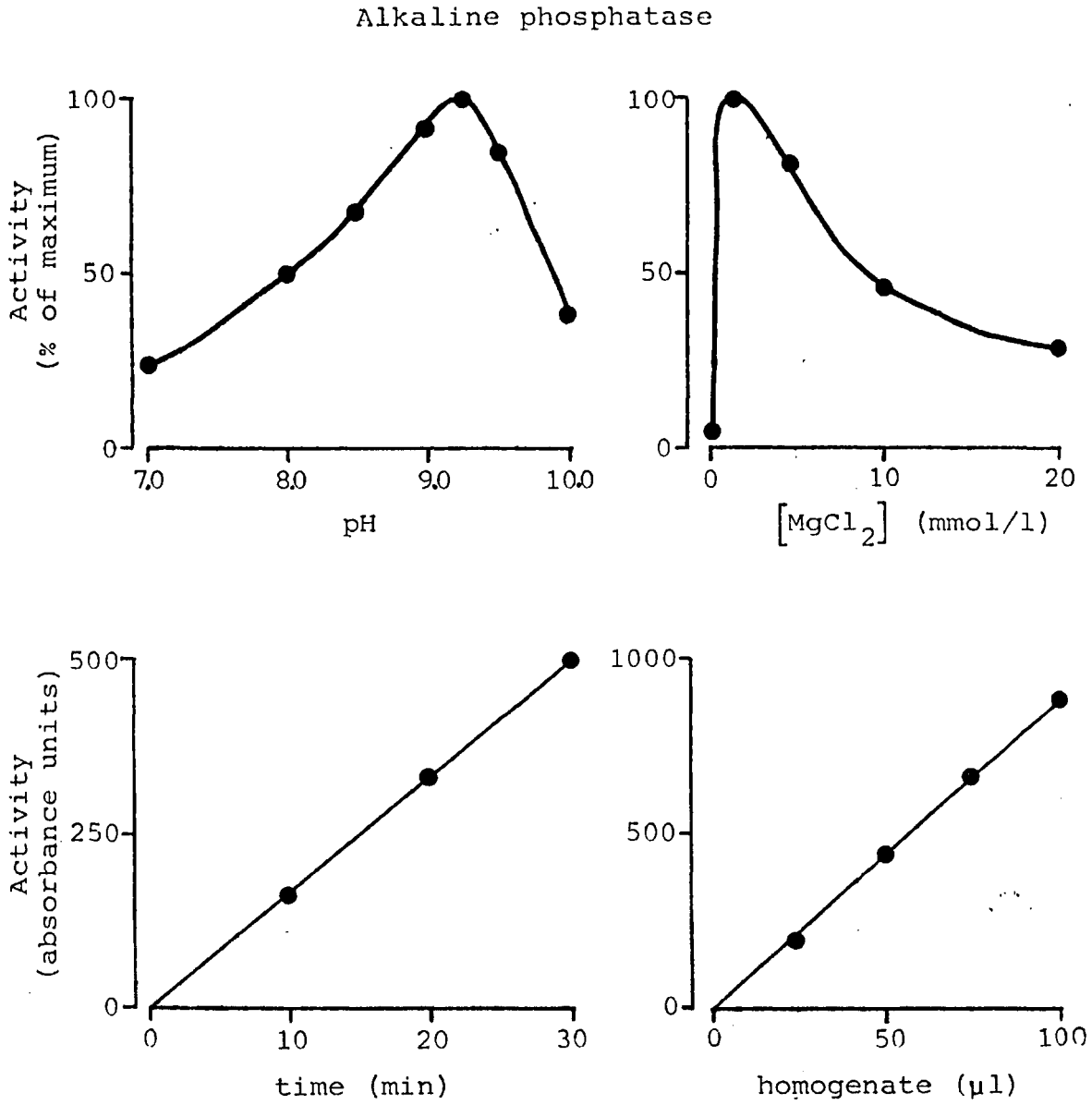


Fig. 2.5 pH optimum, effect of cofactor (magnesium chloride), time course and concentration-activity graphs for alkaline phosphatase

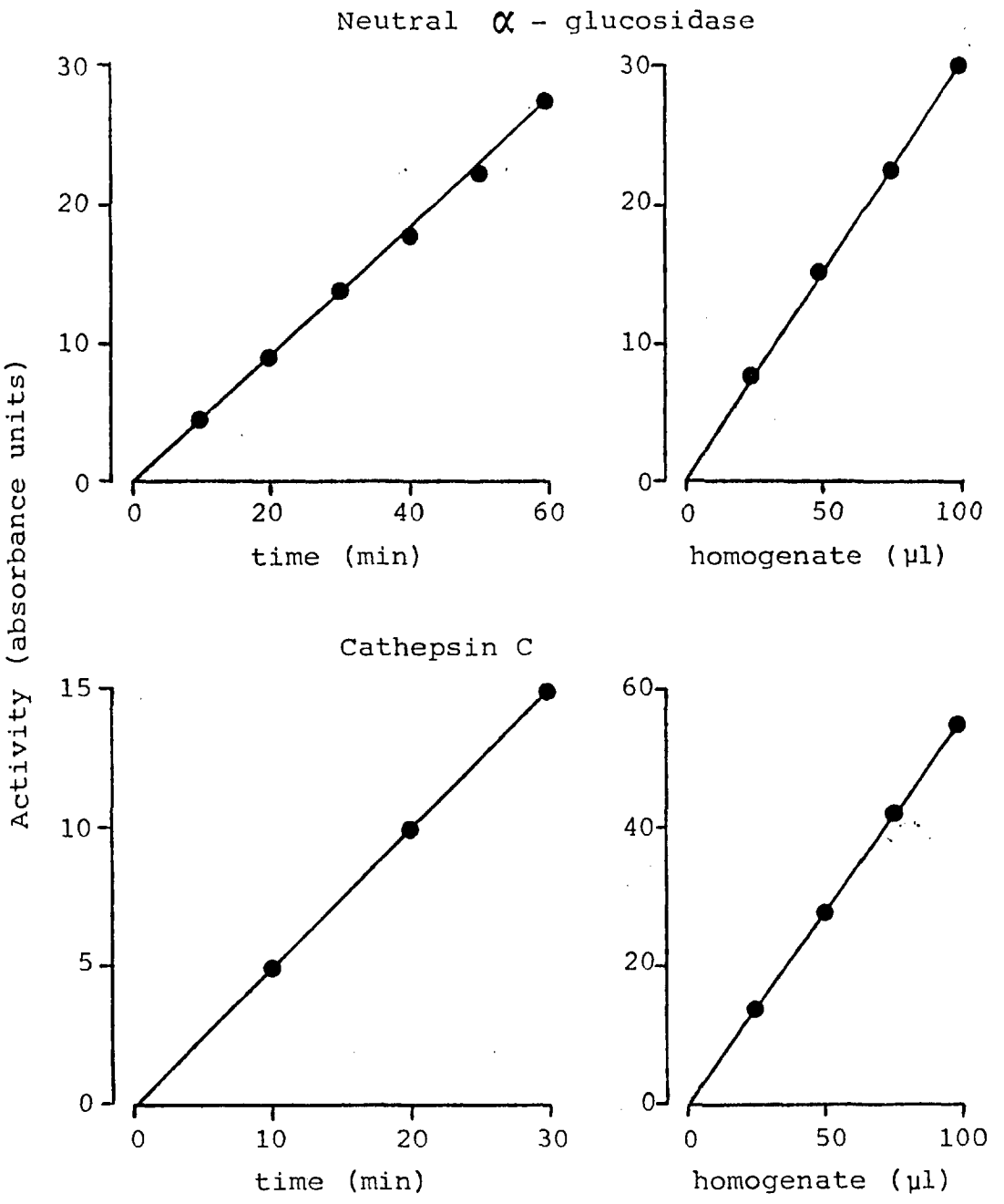


Fig. 2.6 Time course and concentration-activity graphs for neutral α-glucosidase and cathepsin C

by the addition of 2 ml ice-cold 50 mmol/l glycine-NaOH buffer, pH 10.4 and the liberated 2-naphthylamine estimated fluorometrically in a Model 204 Perkin-Elmer Fluorescence Spectrophotometer (Perkin-Elmer Ltd.). The exciting wavelength was 370 nm and the emission setting was 410 nm. Suitable enzyme and substrate blank assays were performed. Standardisation was performed by mixing 2-naphthylamine in 0.1 mol/l acetic acid-sodium acetate buffer, pH 5.0 with 2 ml of 50 mmol/l glycine-NaOH buffer and measuring the fluorescence as described above. The time course and concentration-activity graphs are shown in Fig. 2.6, with linear kinetics obtained with incubation periods up to 30 min.

(vi) Catalase (EC 1.11.1.6)

This enzyme is a haem protein with an iron protoporphyrin as a prosthetic group and it catalyses a very rapid decomposition of hydrogen peroxide to water and oxygen.

The enzyme was assayed according to Peters, Müller and de Duve (1972). Suitably diluted tissue sample, 0.1 ml, was incubated for 30 min at 25°C with 0.25 ml of freshly prepared buffered substrate. This was prepared by dissolving 50 mg of bovine serum albumin, fraction V (Sigma Co.) in 5 ml of 0.2 mol/l imidazole-HCl buffer, pH 7.0; 1.25 g/l Triton X-100 (Sigma Co.) and 0.075 ml of 30% hydrogen peroxide (BDH Chemicals Ltd.) were added and the solution was made up to 50 ml with distilled water. The reaction was stopped by adding 2 ml titanium sulphate reagent. The titanium sulphate solution was prepared by suspending 1.7 g in 500 ml sulphuric acid (1 mol/l) and boiling for 2 h. After an overnight cooling, this

was filtered through no. 42 paper (Whatman Ltd., Maidstone, Kent) and a further 250 ml sulphuric acid (1 mol/l) added to the filtrate.

Absorbance was read in a Gilford 300N (Gilford Instrument Laboratories Inc., Oberlin, Ohio, U.S.A.) spectrophotometer at a wavelength of 405 nm against a water blank. Activities are given in terms of the first order velocity constant of the reaction, in units defined by Bandhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques and de Duve (1964).

$$\text{Activity (milliUnits)} = \log_{10} \frac{\text{blank absorbance}}{\text{sample absorbance}} \times \frac{0.35}{50 \times \text{time(min)}} \times 10{,}000$$

Fig. 2.7 shows time course and concentration-activity graphs for the enzyme, with linear kinetics obtained with incubation periods of up to 15 min.

(vii) Malate dehydrogenase (EC 1.1.1.37)

Malate dehydrogenase reversibly catalyses the conversion of L-malate to oxaloacetate in the presence of NAD^+ but in the mitochondria this reaction normally favours the formation of oxaloacetate.

The assay was performed using the method of Lowry, Roberts and Kapphahn (1957) to determine NAD formation. A suitably diluted sample, 0.1 ml, was added to 0.25 ml freshly prepared incubation medium (0.1 mol/l sodium phosphate-phosphoric acid buffer, pH 7.4 and 0.1 g/l Triton X-100) containing the substrate and incubated at 37°C for up to 10 min. The substrate mixture consisted of 0.28 mmol/l oxaloacetate (grade I, Sigma Co.),

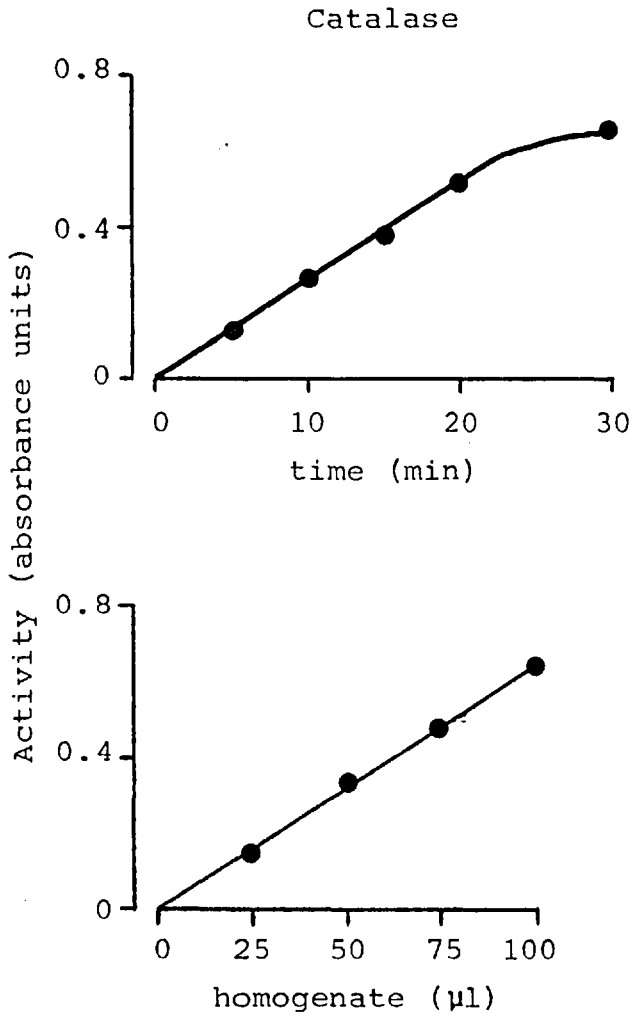


Fig. 2.7 Time course and concentration-activity graphs for catalase

0.14 mmol/l NADH (grade II, Sigma Co.), 1 mg/ml bovine serum albumin fraction V (Sigma Co.) and 1 mmol/l dithiothreitol (Sigma Co.). The reaction was stopped by adding 0.05 ml of 1.2 mol/l hydrochloric acid to destroy any remaining NADH. Then 0.2 ml of 11 mol/l sodium hydroxide was added to amplify the fluorescence of the NAD. The mixture was left at room temperature in the dark for 60 min and 2.5 ml deionised water added. The fluorescence was read in a Perkin-Elmer Model 204 spectrophotometer at an exciting wavelength of 365 nm and fluorescing wavelength of 460 nm. The amount of NAD produced was calculated by reference to a standard block which had previously been calibrated to a standard solution of NAD. The production of 1 mmol of NAD per minute corresponded to 1 unit of activity and was equivalent to 0.117 fluorescent divisions. The time course and concentration-activity plot is shown in Fig. 2.8. Linear kinetics were obtained with incubation periods of up to 10 min.

(viii) Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase catalyses the reduction of pyruvate to lactate.

This enzyme was assayed using the technique of Lowry, Roberts and Kapphahn (1957) to assay NAD. The assay was carried out by incubating 0.1 ml of a suitably diluted sample for up to 60 min at 37^{o}C with 0.25 ml of the appropriate fresh buffer, 0.1 mol/l sodium phosphate, pH 7.4, 0.1 g/l Triton X-100 containing 0.75 mmol/l pyruvate (Type III, Sigma Co.), 0.14 mmol/l NADH (grade III, Sigma Co.), 1 mmol/l dithiothreitol and 1 mg/ml bovine serum albumin (fraction V, Sigma Co.). The reaction was stopped with 0.05 ml of 1.2 mol/l hydrochloric acid to destroy any remaining NADH;

0.2 ml of 11 mol/l sodium hydroxide was added to increase the fluorescence of the NAD formed. After vortexing, the mixture was left at room temperature in the dark for 60 min, 2.5 ml deionised water was added and the fluorescence was read as for malate dehydrogenase. The enzyme activity was calculated in a similar way. The time course and concentration-activity graphs are shown in Fig. 2.8 with linear kinetics obtained with incubation periods of up to 60 min.

(ix) Protein

This was measured according to Schacterle and Pollack (1973) by a micromodification of the Lowry technique (Lowry, Rosebrough, Farr and Randall, 1951).

Appropriately diluted samples, 0.5 ml, were mixed with 1 ml alkaline-copper reagent (0.19 mol/l sodium carbonate, 0.87 mmol/l sodium tartrate, 0.4 mmol/l copper sulphate in 0.1 mol/l sodium hydroxide) and the mixture left standing at room temperature for 10 min; 2 ml of freshly diluted Folin-Ciocalteu reagent (2/3 v/v, BDH Chemicals Ltd.) was added and the mixture heated at 55°C for 5 min. After cooling in ice-water, the absorbance was read on a Gilford 300N spectrophotometer (Gilford Instrument Laboratories Inc.) at a wavelength of 650 nm. The protein concentration was calculated from a standard curve using bovine serum albumin, fraction V (Sigma Co.) in the range 5 - 150 µg. A typical standard curve is shown in Fig. 2.9.

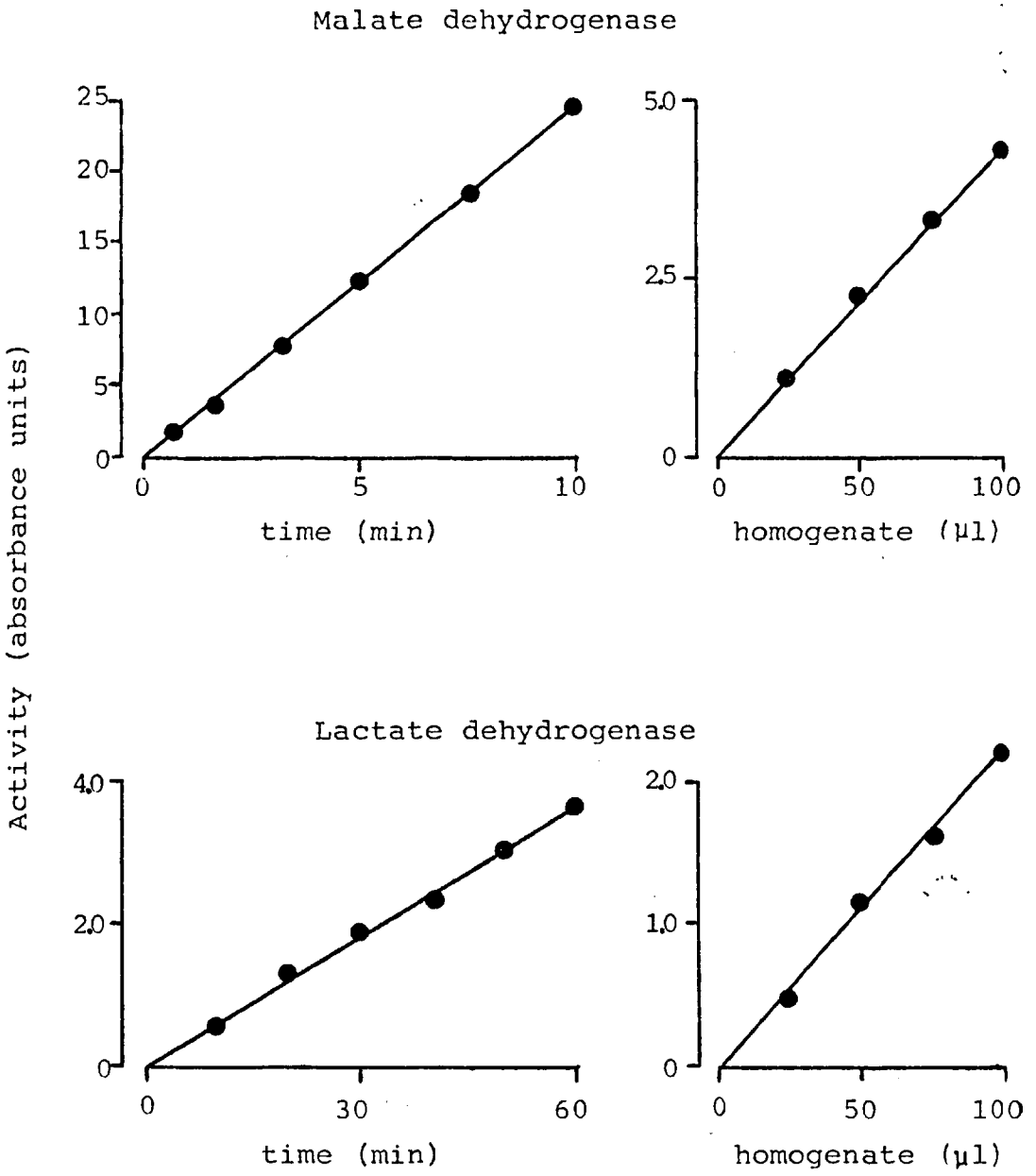


Fig. 2.8 Time course and concentration-activity graphs for malate dehydrogenase and lactate dehydrogenase

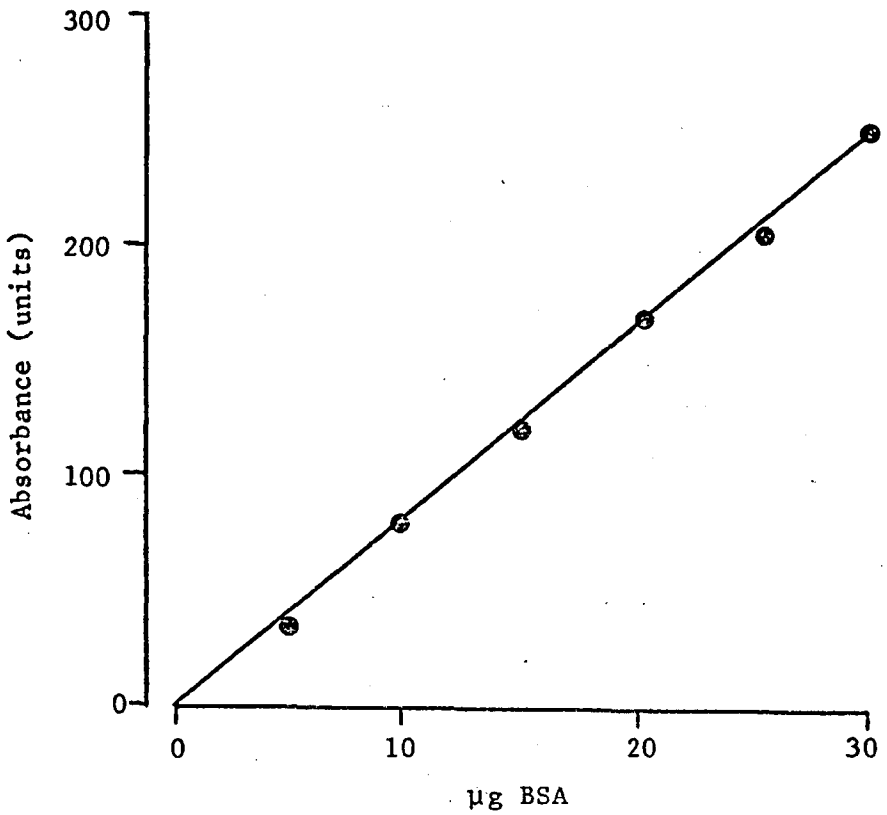


Fig. 2.9 Protein standard curve

(x) DNA

DNA was measured by the fluorimetric microassay technique of Kapuscinsky and Skoczylas (1977). The basis of the assay is that 4',6-diamidino-2-phenylindole · 2HCl (DAPI) reacts with DNA to form a fluorescent complex (Russel, Newman and Williamson, 1975). A stock DAPI (Sigma Co.) solution containing 2 mg DAPI in 50 ml 12 mmol/l sodium chloride, 5 mmol/l Hepes-buffer, pH 7.0 was prepared and stored at 4°C. A working solution, diluted 1:1000 in Hepes-buffer was prepared immediately before use. To 0.5 ml suitably diluted sample or standard was added 0.5 ml of the working solution. A standard curve was constructed with calf thymus DNA (Sigma Co.) in the range 10 - 200 ng/ml with the volume being adjusted to 0.5 ml with Hepes-buffer. The mixture was vortexed and the fluorescence read in a Perkin-Elmer spectrophotometer, Model 204, with an exciting wavelength of 372 nm and an emission wavelength of 454 nm. The unknown samples were read directly from the curve obtained from the standars. A typical standard curve is illustrated in Fig. 2.10.

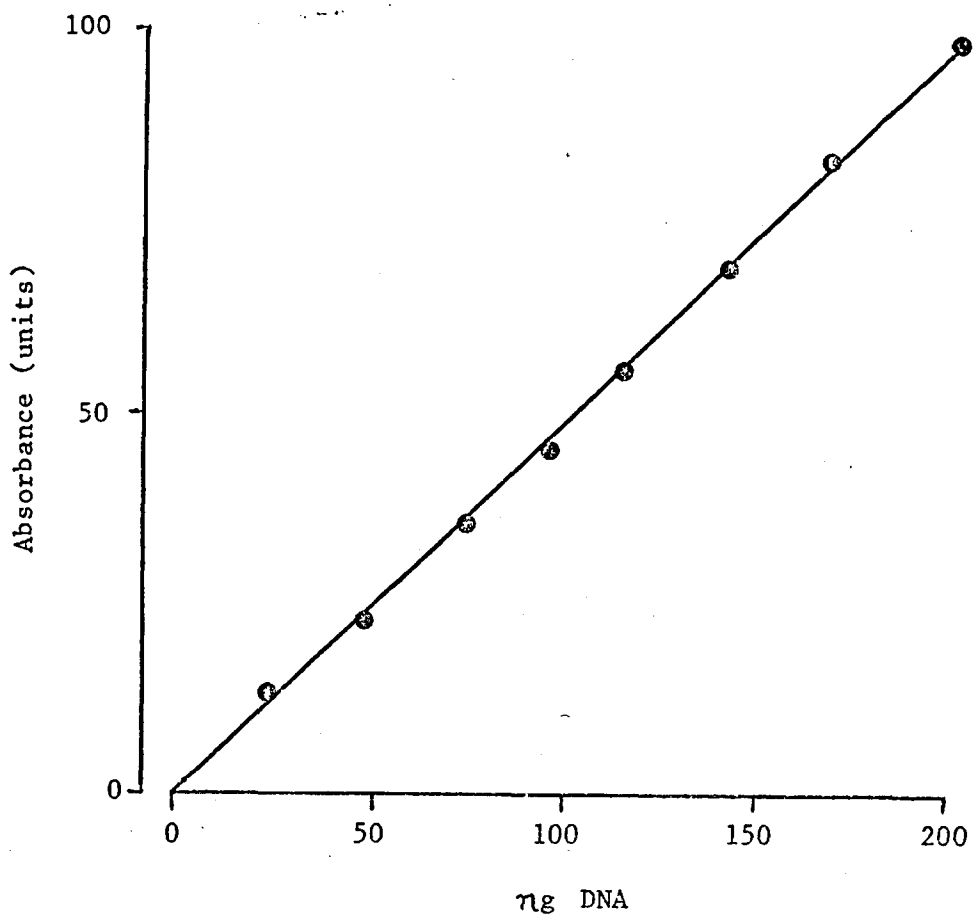


Fig. 2.10 DNA standard curve

2.4 SUBCELLULAR FRACTIONATION

2.4.1 Introduction

A goal of subcellular fractionation is the isolation of pure samples of particular subcellular organelles, in order to make possible studies of their chemical composition, metabolism, transport processes and other properties. The concept of breaking up cells and using a centrifuge to separate the different particles was first described by Miescher (1871) who isolated nuclei from pus cells by centrifugation and then extracted nucleic acids.

Centrifugation methods are used to separate particles either on the basis of their density or on the basis of their size. If a particle is placed in a fluid less dense than itself, then the particle will tend to sediment. Newton's laws of motion show that it will accelerate in the direction of gravity or of an applied centrifugal field until the viscous drag on the particle is the same as the apparent weight of the particle in the liquid medium. The forces acting on a particle suspended in a liquid medium in a centrifuge tube can be analysed and an equation can be derived which gives the rate of sedimentation in terms of the speed of centrifugation, the factors dependent on the properties of the liquid medium and of the density and sedimentation coefficient of the particle.

The simplest method of separation is differential pelleting whereby particles differing greatly in size are separated sequentially although this method is not very effective in detecting small differences in size. As demonstrated by Harvey (1931), subcellular particles differ not only in size but also in density. The technique

of isopycnic zonal centrifugation was first extensively applied by de Duve (1959). A suspension of the particles to be separated is either layered over or beneath a density gradient. Alternatively, the particles are actually suspended in the solutions used to make the gradient. During centrifugation, the particles either rise or sediment until they reach a liquid of their own density, and they may be recovered as a series of zones, each particle at its own equilibrium density.

Beaufay (1966) and Anderson (1966) introduced the zonal rotor for the isopycnic centrifugation. Because of its small size (35 ml volume), short path length (circa 0.5 cm) and minimal hydrostatic pressure on organelle structures at higher speeds, the Beaufay automatic rotor is preferred and has been used in these studies.

The first stage of a fractionation procedure is homogenisation of the tissue sample with minimal damage to the organelles themselves. Liver homogenisation using a gentle grinding technique with mortar and pestle and 'physiological' saline as the medium was employed by Claude (1946) but his first pellet obtained by centrifugation consisted not only of many intact cells but also agglutinated cytoplasmic particles. In order to have complete breakage and avoid agglutination, Hogeboom, Schneider and Palade (1948) used a Potter-Elvehjem homogeniser in hypertonic sucrose (0.88 mol/l). However, hypertonic sucrose damaged organelles and was replaced by isotonic sucrose (0.25 mol/l) (Schneider, 1948) and has since then been extensively used.

Claude (1946) introduced the quantitative assessment of enzyme activity in each fraction obtained after centrifugation in terms of the whole homogenate. This concept was further developed by Schneider and Hogeboom (1951) who quantified the distribution of enzyme activities in each tissue fraction after performing analytical centrifugation. The two major assumptions of this approach were, firstly, that of biochemical homogeneity of the organelles (de Duve, Pressman, Gianetto, Wattiaux and Appelmans, 1955) and secondly, that each enzyme was restricted to a single site within the cell (de Duve and Berthet, 1954). Enzymes could therefore be used as markers for intracellular particles and thus, tissue fractionation experiments could be effected as chemical fractionations.

(i) Analytical subcellular fractionation

The tissue was disrupted in a small Dounce homogeniser (Kontes Glass Co.) with 12 strokes of a loose-fitting (type A) pestle containing 3 ml ice-cold 0.25 mol/l sucrose, 1 mmol/l EDTA disodium salt, pH 7.2, and 20 mmol/l ethanol (sucrose medium) and the homogenate centrifuged at 600 x g for 10 min in a 4 x 50 ml swing-out rotor (r_{av} = 16.0 cm) in an MSE Coolspin centrifuge. The supernatant was decanted and stored on ice. The pellet was resuspended in a further 2 ml of isotonic sucrose medium and homogenised with 3 strokes of the type A pestle, recentrifuged and the post-nuclear supernatants were combined (PNS fraction). The low speed pellet was resuspended in 2 ml sucrose medium with 5 strokes of a tight-fitting (type B) pestle (N fraction).

A portion (3 - 4 ml) of PNS fraction was layered onto a 28 ml sucrose density gradient, extending linearly with respect to volume, from a density of 1.05 g/ml to one of 1.28 g/ml and resting on a cushion of 6 ml sucrose, density 1.32 g/ml in a Beaufay small volume, type E50 (Beaufay, 1966) automatic zonal rotor. All density gradient solutions contained disodium EDTA (1 mmol/l, pH 7.2) and ethanol (20 mmol/l). The rotor was accelerated to 35,000 rev/min and run for 35 min with an integrated angular velocity ($W = \int_0^t \omega^2 \cdot dt$) of $3.3 \times 10^{10} \cdot rad^2 \cdot sec^{-1}$. The rotor was then slowed to 8000 rev/min for automatic unloading and collection of the gradient fractions. Some 16 fractions were collected into tared tubes. After reweighing and mixing, the density of the fractions was determined indirectly by refractometry in an Abbé refractometer (Bellingham & Stanley Ltd., London) and by reference to conversion tables (de Duve, Berthet and Beaufay, 1959). The use of a zonal rotor and ancillary equipment has been described in detail by Leighton, Poole, Beaufay, Baudhuin, Coffey, Fowler and de Duve (1968).

Results were expressed in the form of frequency-density histograms; calculation and plots were performed on a CDC 1600 line computer (Beaufay, Jacques, Baudhuin, Sellinger, Berthet and de Duve, 1964; Leighton et al., 1968). The diagrams were plotted on a density scale (ρ) expressed in g/cm^3 and divided into sections of density increments ($\Delta\rho$). The ordinate is frequency which is therefore defined as $\Delta Q/\Sigma Q.\Delta\rho$ where ΔQ is the amount of constituent present within the density span and ΣQ is the sum of the amounts found in all subfractions. The

dimension of the frequency scale was cm^3/g. The area of each section of the diagram which was the product of the frequency-density increment, was equal to $\Delta Q/\Sigma Q$ and gave the fractional amount of constituent present within the section. The results in the lightest fractions, with density less than 1.10 g/ml related mostly to soluble activity remaining in the starting or lightest layers and were plotted over the arbitrary density interval of 1.05 - 1.10 g/ml.

(ii) Counter-current distribution

Partition experiments were performed by preparing 80 g of polymer phase mixture containing dextran (Dextran T500, batch no. 3447, Pharmacia, Uppsala, Sweden), polyethylene glycol (PEG 6000, batch no. 24577, BDH Chemicals Ltd.), 10 mmol/l sodium phosphate-phosphoric acid buffer, pH 7.4, 0.25 mol/l sucrose, 0.2 mol/l EDTA disodium salt, pH 7.4, and 1.1 mmol/l ethanol. The concentration of dextran used varied from 5.8 to 6.8% (w/w) and that of polyethylene glycol was 4.0% (w/w). The phase mixture was allowed to stand overnight in the cold room and the two phases separated.

Fresh rat pituitary tissue was disrupted in a Dounce homogeniser in 3 ml of ice-cold polyethylene glycol-rich upper phase with 12 strokes of a loose-fitting (type A) pestle. The homogenate was then centrifuged for 10 min at 800 x g at 4°C in an MSE Coolspin centrifuge. The supernatant, 0.65 ml, was loaded onto 0.65 ml dextran-rich lower phase in the sample well of the counter-current device. Complete polymer mixture (1.3 ml) was added to the other 17 wells of the device. The phase volumes were adjusted so that

there was a stationary interface. All steps were conducted at 4°C. The apparatus and partitioning procedure are illustrated in Fig. 2.1k.

The contents of the device were mixed by 20 inversions, each well (6.35 mm i.d.) containing a 3 mm diameter nickel ball-bearing to facilitate mixing. Phase separation was achieved by centrifugation at 250 x g for 5 min in an MSE Coolspin centrifuge with a specially constructed swing-out rotor (r_{av} = 20.5 cm). After each centrifugation step, the lower section of the device was rotated in an anticlockwise direction until the index line was aligned with the adjacent well. This cycle was repeated until 17 transfers were effected. Materials remaining with the original interface or dextran-rich lower phase was recovered in well 18. Sucrose (0.25 mol/l), 0.4 ml, was then added to each well and, after mixing to form a homogenous suspension, the contents of each well were removed and stored at -20°C for subsequent enzymic and hormonal analysis.

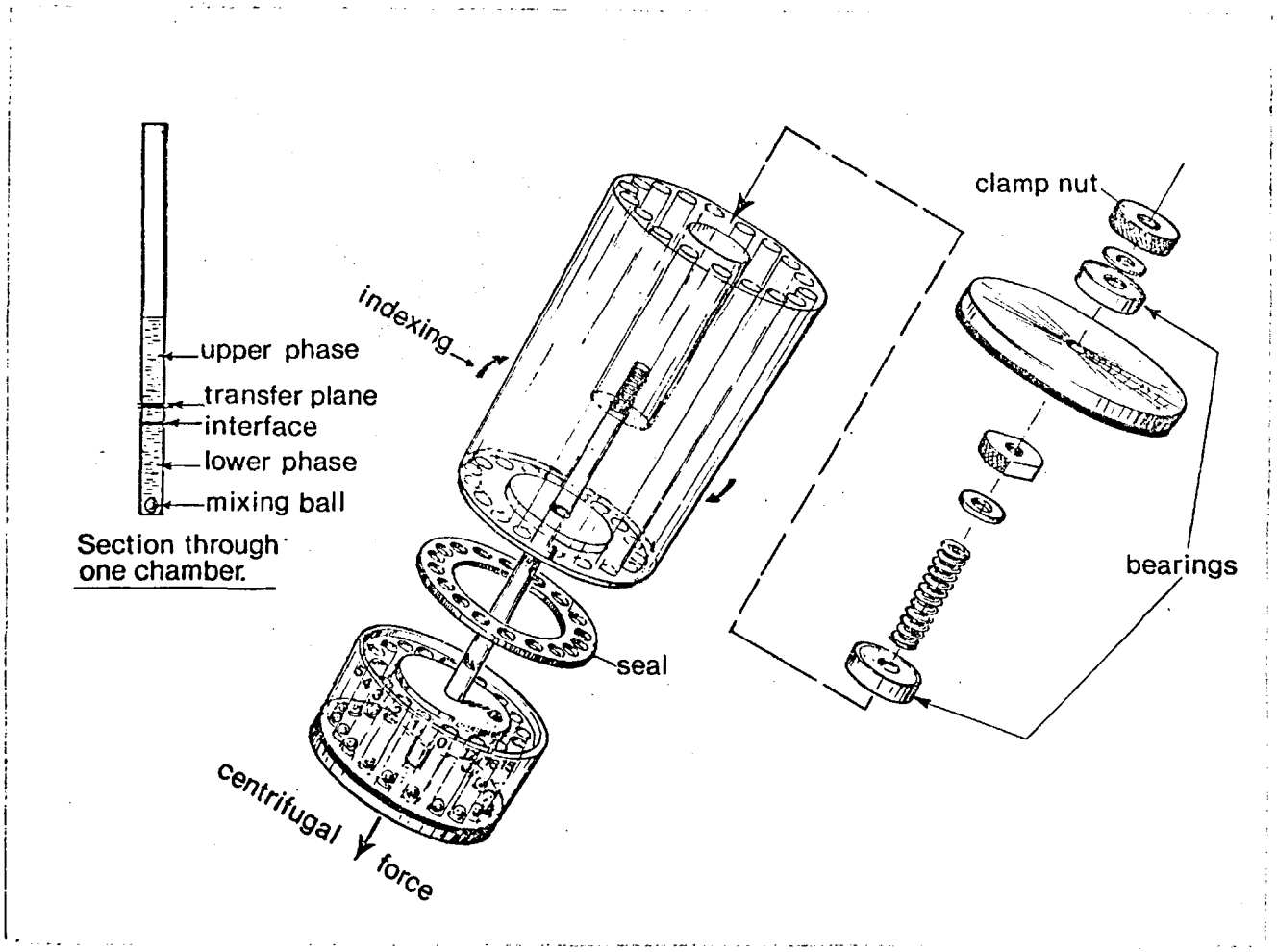


Fig. 2.11 Exploded diagram of the centrifugal counter-current device. Numbering of chambers and direction of indexing are illustrated. Apparatus aligned for the start of an experiment. Insert shows a section through one of the chambers. Note that the interface remains, after tranfer, with the lower dextran-rich phase.

2.5 RADIOIMMUNOASSAY

Radioimmunoassay is widely used to measure minute concentrations of peptide and non-peptide hormones, drugs, enzymes and other organic substances of biological interest in blood, tissue and other biological fluids. This well-established technique has revolutionised endocrinology since Berson, Yalow, Bauman, Rothschild and Newerly (1956) first reported an interaction between a radioactive labelled insulin and the gamma-globulin fraction from the serum of insulin-treated patients suffering from diabetes and schizophrenia. A sensitive and specific assay for measuring plasma insulin levels was subsequently reported by Yalow and Berson (1960) using specific antibodies to insulin raised in guinea-pigs. At the same time, Ekins (1960) published a method for assaying thyroxine using hormone binding proteins found in serum and expounded the principle of saturation analysis for competitive protein binding assays.

In the radioimmunoassay method, a fixed amount of labelled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limiting. When unlabelled antigen is added, there is competition between labelled tracer and unlabelled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabelled antigen increases. When equilibrium is reached and since the total antigen concentration is greater than the antibody binding site concentration, all specific antibody should be occupied by antigen and a proportion of this is labelled.

Separation of free antigen from antibody-bound antigen can be achieved by exploiting differences in molecular size, charge, adsorption or solubility properties of the two moieties or by precipitation of the bound antigen using a second antibody raised to the gamma-globulin fraction of this first antibody.

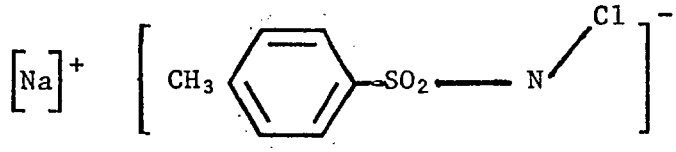
Hormone labelled with radioactive isotope has been used as tracer because its distribution between free and reagent-bound moieties can be readily measured, reflecting the distribution of the endogenous (unlabelled) hormone in the system. The labelled hormone need not be structurally identical to the endogenous hormone, nevertheless, it must closely simulate the unlabelled material and participate sufficiently in the binding reaction to ensure that its distribution varies in response to changes in concentrations of the unlabelled hormone present.

The ability to measure picogram quantities can be achieved only if the antigen can be labelled to high specific activity. Although tritium ^{3}H and radiocarbon ^{14}C have been employed in hormone labelling, they have a relatively low specific activity and necessitate the use of liquid scintillation counters. The technique of hormone labelling with isotopes of iodine was introduced by Hunter and Greenwood (1962) and was applicable to any protein containing a tyrosine residue among its aminoacid constituents.

Initially the iodine isotope ^{131}I was employed in labelling hormones but because of a short half-life of only 8 days and low isotopic abundance (20%), ^{127}I being a major contaminant, it has become less widely used. Hence, ^{125}I, which has a longer half-life of 60 days and an isotopic abundance approaching 100% has been the preferred isotope for radioiodination.

Under some circumstances, iodine may react with the sulphydryl groups of histidine or tryptophan but in general the chemistry of radioiodination is essentially the chemistry of substitution of iodine into tyrosine groups (Hughes, 1957). Iodine so substituted constitutes a chemically relatively stable tracer and the conditions of iodination are arranged so as to favour this reaction. This is achieved by using a pH just on the alkaline side of neutrality.

Various oxidising agents including persulphate, iodate and nitrite have been used but the chloramine T method (Hunter and Greenwood, 1962) has been widely favoured and was used for the present work. Chloramine T is the sodium salt of the N-mono-chloro-derivative of p-toluene sulphonamide.



In aqueous solution, chloramine T slowly yields hypochlorous acid and is consequently a mild oxidising agent. On addition of chloramine T to a slightly alkaline aqueous solution of protein and iodide, a quantitative incorporation of iodine into the protein is obtained. The reaction is optimal at pH 7.5 (Freedlender, 1969). Above pH 8.5 there is a predominant substitution into groups other than tyrosine (Chisholm, Young and Lazarus, 1969). To inactivate the excess chloramine T, sodium metabisulphate, a reducing agent, is added. Finally, separation of the iodinated protein from unreacted iodide and other low molecular weight reactants is performed. One of the simplest methods is to adsorb the hormone onto a solid phase such as silica. After centrifugation, the

supernatant, containing low molecular weight reactants, is discarded and the hormone eluted from the silica with an acid-alcohol wash. A similar procedure involves ion-exchange gels instead of silica.

Initial purification of the iodinated hormone is usually carried out on gel chromatography (Sephadex; Pharmacia, Uppsala, Sweden) or ion-exchange columns. The grade of Sephadex used varies according to the molecular size of the protein to be separated from the unreacted iodine and other iodination reagents which are of low molecular size. The eluting gradient must contain carrier protein in order to minimise adsorption of the hormone onto the glassware.

It is often necessary to undertake a further purification of the iodinated protein to remove damaged or aggregated hormone and this purification procedure can be achieved by employing longer columns of a Sephadex gel having a fractionation range encompassing the molecular size of the monomer hormone.

The greater the incorporation of radioiodine into a compound, the higher is the specific activity of the resulting labelled preparation (usually expressed as μCi/μg). When considering protein radioimmunoassay systems, it is widely accepted that an average incorporation of one iodine atom per protein molecule is most desirable. The introduction of multiple atoms into the molecule can significantly affect its binding to antibody, particularly if tyrosyl residues form part of the hormonal antigenic determinant, thus reducing the assay sensitivity (Ekins , 1976). Another reason to limit the incorporation of radioiodine into a peptide to one atom per molecule is to minimise primary radiation decomposition or decay catastrophe (Yalow and Berson, 1969). If the hormone molecule is labelled

with two or more atoms, disintegration of the first active atom results in chemical dislocation of the host molecule and the production of radioactively labelled hormone fragments whose chemical and immunological behaviour in the system are likely to be altered, giving rise to an increased background of totally or partially unreactive labelled material.

2.5.1 Rat prolactin

Iodination of rat prolactin was carried out employing the chloramine T method.

Reagents:

1) ^{125}I, carrier free as sodium iodide, with specific activity of approximately 400 mCi/ml, pH 9 - 11 (Amersham International, Amersham, Bucks.).
2) 0.5 mol/l sodium phosphate buffer, pH 7.6.
3) 5 mg chloramine T (BDH Chemicals Ltd.) dissolved immediately before use in 10 ml of 0.05 mol/l phosphate buffer, pH 7.6.
4) 24 mg sodium metabisulphate ($Na_2S_2O_5$) (BDH Chemicals Ltd.) dissolved immediately before use in 10 ml of 0.05 mol/l phosphate buffer, pH 7.6.
5) Rat prolactin (NIAMD I-2/I-3, Rat Pituitary Hormone Distribution Program, National Institutes of Health, Bethesda, Maryland, U.S.A.) 5 µg in 25 µl of 0.01 mol/l sodium bicarbonate buffer. The 5 µg aliquots were stored in 'V' vial auto analyser cups (Griffith & Neilson, Billingshurst, Sussex) at -20°C.

A 1 x 30 cm chromatography column plugged with glass wool at its bottom neck was used for the separation of iodinated prolactin from the other reactants. Sephadex G-50 (Pharmacia) was equilibrated in 0.01 mol/l borate buffer, pH 8.6, at room temperature and poured onto the column to a height of approximately 25 cm. Crystalline bovine serum albumin (Sigma Co.), 2 ml (1 mg/ml), was then added to the column and eluted with the borate buffer.

The labelling procedure was carried out in a fume cupboard with lead shielding in a special 'hot laboratory' with all precautions laid down by the Medical Physics Department for working with radioiodine.

To the 'V' vial containing 5 µg of rat prolactin I-2/I-3 was added:

1) 35 µl of 0.5 mol/l phosphate buffer, pH 7.6;

2) 10 µl of $Na^{125}I$ (1 mCi);

3) 15 µl of chloramine solution.

The reagents were mixed by gently bubbling air through and, after 15 - 20 sec, to the mixture was added:

4) 50 µl of sodium metabisulphate solution.

The entire mixture was applied to the Sephadex column. The reaction tube was rinsed with 0.1 ml of borate buffer, pH 8.6, and this was placed on the column, which was then eluted with the borate buffer. Fractions of approximately 1.5 ml were collected in 75 x 12 mm plastic tubes (W. Sarstedt, Leicester) each containing 0.5 ml of 1 mg/ml crystalline bovine serum albumin (Sigma Co.) in phosphosaline buffer, pH 7.6 (0.15 mol/l sodium chloride, 0.05 mol/l EDTA disodium salt, 0.25 mmol/l thiomersal, 0.01 mol/l phosphate buffer).

Two peaks of radioactivity were detected. The first peak, containing the iodinated prolactin was contained in tubes 3 - 6. A second peak, consisting of free ^{125}I was contained in tubes 7 - 12. An elution profile of a typical gel chromatographic procedure is shown in Fig. 2.12. The fractions containing the iodinated prolactin (3 - 6) were stored at 4°C after calculation of the specific activity as follows:

1) Na ^{125}I = 1 mCi;

2) Na ^{125}I = 964 µCi, when corrected for decay;

3) rat prolactin = 5 µg;

4) sum of counts in 10 µl of each eluted fraction after the iodination reaction (10 sec) = 68,306 counts;

5) sum of counts in 10 µl of tubes containing iodinated prolactin (first peak) = 23,377 counts;

6) % iodination yield = $\frac{\text{counts in (5)}}{\text{counts in (4)}} \times 100 = \frac{23,377}{68,306} \times 100 = 34.2\%$

7) radioactivity incorporated = $\frac{\%\text{ iodination yield}}{100\%} \times$ original radioactivity = $\frac{34.2\%}{100\%} \times 964\ \mu Ci = 330\ \mu Ci$

8) specific activity = $\frac{\text{protein radioactivity}}{\text{mass of protein}} = \frac{330\ \mu Ci}{5\ \mu g} = 66\ \mu Ci/\mu g$

Radioimmunoassay of rat prolactin was carried out using a double antibody technique first reported for the assay of insulin by Morgan and Lazarow (1962). Separation of bound and free hormone was attained by using a second antibody against the gamma-globulin species of the primary antibody. Thus, a donkey anti-rabbit gamma-globulin (Guildhay antisera, University of Surrey, Guildford, Surrey) was used to precipitate the hormone bound to the rabbit anti-rat prolactin.

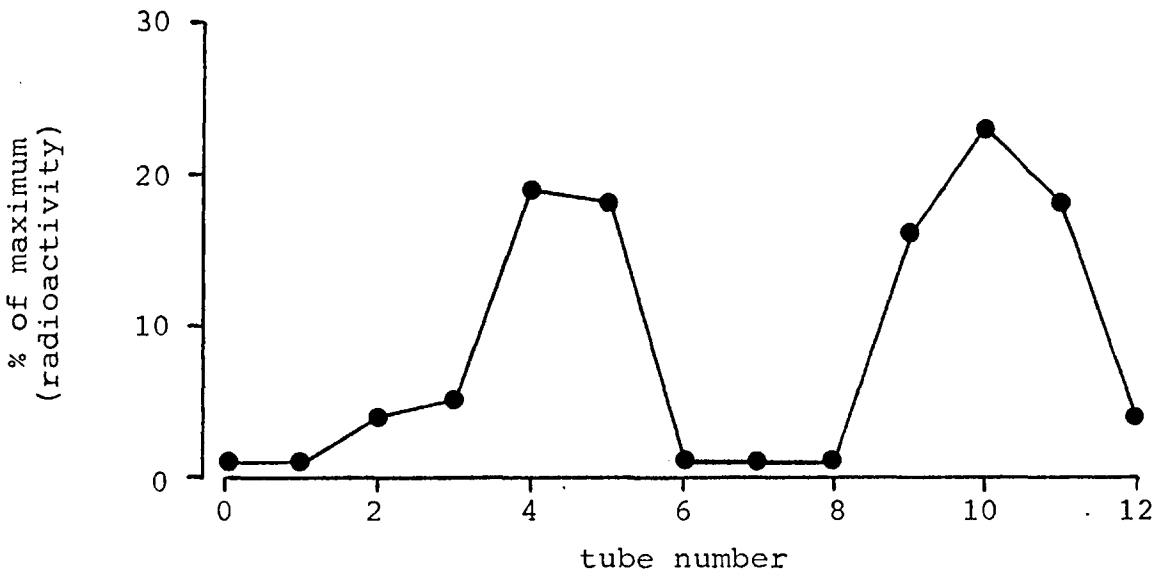


Fig. 2.12 Iodination gel chromatography of rat prolactin

The rat prolactin assay reagent used throughout was 10 mg/ml bovine serum albumin (Sigma Co.) in 0.15 mol/l sodium chloride, 0.05 mol/l EDTA disodium salt, 0.25 mmol/l thiomersal, 0.01 mol/l phosphate buffer, pH 7.6 (phosphosaline buffer). The reagents necessary for the assay were donated by the Rat Pituitary Hormone Distribution Program, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland, U.S.A.

1) Reference Preparation for RIA (NIAMD-Rat PRL-RP-1): approximately 1 mg of lyophilised material was provided. A small amount was weighted and dissolved in 0.01 mol/l phosphate buffer containing 10 mg/ml bovine serum albumin (Sigma Co.), 0.15 mol/l sodium chloride, 0.25 mmol/l thiomersal, pH 7.6, at a concentration of 0.1 mg/ml. Aliquots of 0.1 ml were stored at -20°C until used in the radioimmunoassay procedure. A vial was thawed immediately before use and diluted in the prolactin assay buffer to 500 ng/ml and subsequently serial double dilution was prepared down to a concentration of 1.9 ng/ml.

2) Antiserum to Rat Prolactin (NIAMD-rabbit anti-rat prolactin S-5 and S-6): 0.4 ml of each batch (S-5 and S-6) were provided undiluted. Immediately after receipt a 1:10 dilution in 0.01 mol/l phosphate buffer, pH 7.6, containing 0.15 mol/l sodium chloride, 0.05 mol/l Na_2 EDTA, 1% normal rabbit serum and 0.25 mol/l thiomersal was prepared. Aliquots of 0.1 ml were stored at -20°C until used in the assay.

All samples, standards and controls were assayed in duplicate at room temperature as shown in Table 2.1. The precipitate containing the bound hormone was counted in a NE 1600 gamma counter

(Nuclear Enterprises Ltd., Edinburgh). A standard curve was constructed on semilogarithmic paper by plotting the percentage of the total radioactivity bound ($\frac{B}{T}$ x 100%) or the amount bound compared to that in the absence of unlabelled prolactin ($\frac{B}{Bo}$ x 100%) against hormone concentration. The unknowns were read directly from the curve obtained with the reference preparation. Results were expressed as ng of RP-1 per ml sample.

To validate the assay, four control samples obtained by pooling previously assayed rat serum samples, with low, medium and high prolactin concentrations, were each included on at least four occasions (8 replicates) distributed throughout the assay. These values were examined for within (intra) assay standard deviation and coefficient of variation and the mean values from each assay was analysed to determine the between assay variation. These were expressed as:

$$\% \text{ coefficient of variation} = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

The results for a series of assays are shown in Table 2.2.

TABLE 2.1

Rat prolactin assay procedure flow chart

Day 1: Preparation of standard and sample tubes.

i) to each 0.1 ml standard or unknown, is added:
- 0.4 ml phosphosaline buffer, pH 7.6.
- 0.1 ml first antibody (NIAMD rabbit anti-rat PRL S5/S6, 1:5000) in a 1:200 solution of non-immune serum in phosphosaline buffer.

ii) to 'zero' tubes:
- 0.5 ml phosphosaline buffer.
- 0.1 ml first antibody solution.

iii) to non-specific binding tubes:
- 0.5 ml phosphosaline buffer.
- 0.1 ml 1:200 solution of non-immune rabbit serum.

iv) addition of labelled tracer:
- add 0.1 ml of ^{125}I-PRL in phosphosaline buffer to all tubes (approximately 15,000 cpm — 0.03 ng radiolabelled PRL). In addition, 4 tubes containing 0.1 ml were set aside for counting total radioactivity.

Incubate at room temperature for 24 h.

Day 2: Addition of second antibody.

i) add 0.2 ml donkey anti-rabbit gamma-globulin at 1:800 dilution to all tubes.

ii) incubate at room temperature overnight.

iii) separation of precipitate:
- centrifuge tubes at 800 x g for 30 min at room temperature.
- decant supernatant.
- count radioactivity in tubes containing precipitate.
- count total radioactivity in tubes.

TABLE 2.2

Radioimmunoassay of rat prolactin (ng/ml)

INTRA-ASSAY VARIATION

	Pool G	Pool B	Pool H	Pool K
	4.1	18.7	114.6	181.0
	3.7	18.0	125.2	207.4
	3.2	14.1	102.2	180.8
	3.6	15.3	113.0	188.2
	3.0	15.9	118.7	208.6
	3.3	14.7	99.2	228.8
	3.3	17.6	90.6	194.4
	3.7	16.6	96.0	203.3
$\bar{x}$±SE	3.5±0.12	16.4±0.58	107.4±4.3	199.1±5.8
CV(%)	10.1	10.0	12.5	8.2
n	8	8	8	8

INTER-ASSAY VARIATION

	Pool G	Pool B	Pool H	Pool K
	3.7	14.7	87.0	210.0
	3.2	17.5	116.9	191.4
	3.5	13.8	108.8	200.1
	3.6	16.4	107.4	199.1
	4.0	14.8	120.5	219.0
	4.2	14.5	84.4	196.0
	4.0	14.4	93.7	190.0
	4.1	18.6	-	-
	3.1	17.2	-	-
$\bar{x}$±SE	3.7±0.14	15.8±0.56	102.7±5.4	200.8±3.9
CV(%)	10.7	10.7	14.0	5.2
n	9	9	7	7

2.5.2 Human Prolactin Assay

The human prolactin was measured by a specific radioimmunoassay as described by Gwee and Mashiter (1978). Iodination of human prolactin was carried out by employing the lactoperoxidase technique (Merchalonis, 1969; Thorell and Johannson, 1971). The procedure was performed in the 'hot laboratory' by a member of the laboratory staff.

Reagents:

1) 2.5 µg human prolactin (VLS 3, National Pituitary Agency, NIAMDD, USA) in 10 µl, stored at -20°C in auto analyser 'V' vials (Griffith and Neilson).
2) ^{125}I, carrier free as sodium iodide, with specific activity of approximately 400 mCi/ml, pH 9-11 (IMS 30, Amersham International).
3) 0.05 mol/l sodium phosphate buffer, pH 7.4, containing 0.15 mol/l sodium chloride, 1 mg/ml bovine serum albumin (fraction V, Sigma Co.); hereafter referred to as sodium phosphate buffer.
4) 50 µg bovine lactoperoxidase (Sigma Co.) in 0.1 ml of 0.1 mol/l sodium acetate buffer, pH 5.6, stored at -20°C. Dissolved immediately before use in 3.9 ml of 0.05 mol/l sodium phosphate buffer.
5) Hydrogen peroxide (30% v/v, BDH Chemicals Ltd.) diluted 1:30 in distilled water immediately before use.

A 1.2 x 70 cm column of Sephadex G-100 was equilibrated in 0.01 mol/l sodium phosphate buffer at room temperature, with a flow rate of 10-15 ml/h.

To the 'V' vial containing 2.5 µg human prolactin were added:

5 µl of $Na^{125}I$ (0.5 mCi)

35 µl lactoperoxidase solution (0.625 µg)

2 µl hydrogen peroxide solution

The reagents were mixed by bubbling air through; after 10 min another 2 µl hydrogen peroxide solution were added to the reaction. A further period of 10 min was allowed for the reaction to continue after which the mixture was applied to the Sephadex column and eluted with 0.01 mol/l sodium phosphate buffer. Approximately 1 ml per tube was collected in plastic vials and the third peak of monomeric prolactin with a specific activity of about 80 µCi/µg was stored at -20°C until used for the assay.

A 24 h prolactin radioimmunoassay was carried out using the double antibody technique described previously (Gwee and Mashiter, 1978). The human prolactin assay buffer used throughout was 0.01 mol/l barbitone buffer, pH 8.6, containing 0.01 mol/l EDTA disodium salt and 25 mg/ml bovine serum albumin (fraction V, Sigma). The Reference Preparation (VLS 1) was donated by the National Pituitary Agency, NIAMDD, USA, and the rabbit anti-prolactin serum was obtained from our own laboratory (K. Mashiter).

A vial containing an aliquot of VLS 1 (standard) was diluted in prolactin assay buffer immediately before use to a concentration of 100 ng/ml and subsequent serial double dilutions to a concentration of 1.56 ng/ml. An aliquot (0.1 ml) of antiserum to human prolactin was diluted 1:5000 in 1% non-immune rabbit serum.

All samples, standards and controls were assayed in duplicate at room temperature; the procedure is shown in Table 2.3. A standard curve was constructed on semilogarithmic paper by plotting the percentage of the total radioactivity ($\frac{B}{T}$ x 100%) or the amount bound compared to that in the absence of unlabelled prolactin ($\frac{B}{BO}$ x 100%) against hormone concentration. To validate the assay, three control samples obtained by pooling previously assayed human serum, with low, medium and high prolactin concentrations, were included in each assay.

2.5.3 Growth Hormone Assay

Iodination of growth hormone was carried out by employing the chloramine T method (Hunter and Greenwood, 1962). The procedure was performed in the 'hot laboratory' by a member of the laboratory staff with all precautions laid down by the Medical Physics Department for working with radioiodine.

Reagents:

1) ^{125}I, carrier free as sodium iodide, with specific activity of approximately 400 mCi/ml, pH 9-11 (IMS 30, Amersham International).
2) 0.5 mol/l sodium phosphate buffer, pH 7.6.
3) 10 mg chloramine T (BDH Chemicals Ltd.) dissolved immediately before use in 10 ml of 0.05 mol/l phosphate buffer, pH 7.6.
4) 24 mg sodium metabisulphate ($Na_2S_2O_5$) (BDH Chemicals Ltd.) dissolved immediately before use in 10 ml of 0.05 mol/l phosphate buffer, pH 7.6.

TABLE 2.3

Human prolactin assay procedure flow chart

Preparation of standard and sample tubes:

1) To 0.1 ml each standard or unknown, is added:
   - 0.2 ml human prolactin assay buffer.
   - 0.1 ml first antibody solution diluted in 1:100 solution of non-immune rabbit serum.

2) To 'zero' tubes:
   - 0.3 ml human prolactin assay buffer.
   - 0.1 ml first antibody solution.

3) To non-specific binding tubes:
   - 0.3 ml human prolactin assay buffer.
   - 0.1 ml 1:100 solution of non-immune rabbit serum.

   The mixture is incubated at room temperature for 4 h.

Addition of labelled tracer:

4) Add 0.1 ml ^{125}I-prolactin (approximately 15,000 cpm and 0.03 ng iodinated prolactin) to all tubes. In addition, 4 tubes containing 0.1 ml were set aside for counting total radioactivity.

   Incubate at room temperature for 12 h.

Addition of second antibody:

5) Add 0.1 ml donkey anti-rabbit gamma-globulin diluted 1:50 to all tubes. Incubate at room temperature for 6 h.

6) Separation of precipitate:
   - centrifuge tubes at 800 x g for 20 min at room temperature.
   - decant supernatant.
   - count radioactivity in tubes containing precipitate.
   - count total radioactivity in tubes.

5) Human growth hormone (MRC 69/46, hGH for iodination) 5 µg in 10 µl of sodium phosphate buffer, pH 7.6. The 5 µg aliquots were stored in 'V' vials auto analyser cups (Griffith and Neilson) at -20°C.

A 1 x 18 cm chromatography column plugged with glass wool at its bottom neck was used for the separation of iodinated growth hormone from the other reactants. Sephadex G-50, medium, (Pharmacia) was equilibrated in 0.1 mol barbitone-HCl, pH 7.4, at room temperature and poured into the column to a height of approximately 15 cm. 2 ml, 1 mg/ml crystalline bovine serum albumin (Sigma Co.) was then added to the column and eluted with the barbitone-HCl buffer.

To the 'V' vial containing 5 µg of human growth hormone was added:

35 µl of 0.5 mol/l phosphate buffer, pH 7.6

10 µl of $Na^{125}I$ (1 mCi)

10 µl of chloramine T solution

The reagents were mixed by gently bubbling air through and after 10-15 sec, to the mixture was added:

100 µl of sodium metabisulphate solution.

The entire mixture was applied to the Sephadex column. The reaction tube was rinsed with 0.1 ml barbitone-HCl buffer, pH 7.4 and this wash placed on the column which was then eluted with barbitone-HCl buffer. After a void volume of approximately 6.5 ml, fractions of 0.5 ml were collected in 75 x 12 mm plastic tubes (W. Sarstedt, Leicester). Two peaks of radioactivity were detected. The first peak, containing the iodinated growth hormone was contained in tubes 4-5. A second peak, consisting of unreacted ^{125}I was

contained in tube 10 onwards. The fractions containing the iodinated growth hormone were stored at 4°C after calculation of the specific activity, as for rat prolactin.

Radioimmunoassay of human growth hormone was carried out using a double antibody technique (Morgan and Lazarow, 1962). Separation of bound and free hormone was attained by using a second antibody raised against the gamma-globulin species of the primary antibody.

The human growth hormone assay buffer used throughout was 7 mmol/l barbitone sodium, 10 mmol/l sodium acetate, 0.13 mol/l sodium chloride and 0.25 mmol/l thiomersal, pH 7.4.

The WHO First International Reference Preparation for human growth hormone (MRC 66/217) was used as standard, a vial containing 32 μIU dissolved immediately before use in growth hormone assay buffer to a concentration of 16 mIU/l and subsequent serial double dilutions were prepared down to a concentration of 0.5 mIU/l. Rabbit antiserum to human growth hormone was purchased from Wellcome Reagents, Beckenham, Kent.

All samples, standards and controls were assayed in duplicate at room temperature as shown in Table 2.4. The precipitate containing the bound hormone was counted in a similar way to that of rat prolactin. The results were expressed as mIU/l.

TABLE 2.4

Human growth hormone assay procedure flow chart

Day 1: Preparation of standard and sample tubes:

i) to 0.1 ml each standard or unknown tube, is added:
- 0.2 ml growth hormone assay buffer.
- 0.1 ml of first antibody (Wellcome Reagents) diluted 1:65,000 in a 1:200 solution of non-immune rabbit serum.

ii) to 'zero' tubes:
- 0.4 ml growth hormone assay buffer.
- 0.1 ml of first antibody.

iii) to non-specific binding tubes:
- 0.4 ml growth hormone assay buffer.
- 0.1 ml 1:200 solution of non-immune rabbit serum.

Incubate at room temperature for 4 h.

iv) addition of labelled tracer:
- 0.1 ml of ^{125}I growth hormone in assay buffer to all tubes (approximately 10,000 cpm and 0.1 ng iodinated growth hormone). In addition, 4 tubes containing 0.1 ml were set aside for counting total radioactivity.

Incubate at room temperature for 16 h.

Day 2: Addition of second antibody:

i) add 0.1 ml donkey anti-rabbit gamma-globulin at 1:40 dilution to all tubes.

Incubate at room temperature for 6 h.

ii) separation of precipitate.
- centrifuge tubes at 800 x g for 20 min at room temperature.
- decant supernatant.
- count radioactivity in tubes containing precipitate.
- count total radioactivity in tubes.

2.5.4 Human LH and FSH Radioimmunoassay

Radioimmunoassay of LH and FSH was carried out by colleagues within the laboratory, employing a double antibody technique, using human pituitary LH/FSH (MRC preparation 69/104) as standard.

2.6 ELECTRON MICROSCOPY

These studies were performed by Miss Susan Van Noorden in the Department of Histopathology, Royal Postgraduate Medical School.

For electron microscopy, rat and human pituitary tissue was fixed in 0.3% glutaraldehyde in 0.1 mol/l phosphate buffer followed by osmium tetroxide and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in an AEI 6B electron microscope.

For immunocytochemistry, human pituitary tissue was fixed in methanol-free formaldehyde in 0.01 mol/l phosphate buffer, pH 7.4, before being embedded in wax or araldite. The unlabelled antibody-enzyme (peroxidase-anti-peroxidase, PAP) technique (Sternberger, 1979) was carried out with rabbit antisera to hGH and ACTH (Wellcome Reagents) and to hPRL, hLH, hFSH, and hTSH β-subunits (NIAMDD, USA) at dilutions of 1/600 to 1/16,000 for 40 h at 4°C. The second layer was a 1/200 dilution of unconjugated goat antiserum to rabbit globulin (Miles) for 30 min at room temperature followed by 1/300 PAP (UCB Bioproducts) for 30 min at room temperature. The peroxidase was developed by the diaminobenzidine method of Graham and Karnovsky (1966) using 0.03% H_2O_2. Negative controls for immunostaining included use of non-immune rabbit serum in place

of primary antisera and absorbing the antisera to hGH, prior to staining, with 50 μg hGH per ml diluted antiserum. In order to ensure that the quantity of GH available would be sufficient to saturate the antibody, the maximum antibody dilution (1/48,000) giving positive staining was used for the absorption experiment.

2.7 CELL CULTURE

Cell culture experiments were performed by Mr. E.F. Adams.

Tissue from the patient's pituitary biopsy and another five adenomas from acromegalics obtained by selective transsphenoidal hypophysectomy was dissociated for the cell culture experiments with 0.1 or 0.2% trypsin in phosphate-buffered saline, as previously described (Adams, Brajkovich and Mashiter, 1979; Mashiter, Adams, Gillies, Van Noorden and Ratter, 1980; Adams, Brajkovich and Mashiter, 1981). Following washing, an aliquot was removed to determine cell number with a haemocytometer, and the viability was checked by trypan blue exclusion. This was greater than 95%. 2×10^5 cells were distributed into glass tubes (LABCO S100WC) containing 2 ml Hepes-buffered Minimum Essential Medium and allowed to attach. Incubation was at 37°C. Medium was changed every 1 to 3 days and samples stored at -20°C for hormone assay.

CHAPTER THREE

RESULTS

3.1 Characterisation of rat pituitary organelles

3.2 Rat prolactin protease assay

3.3 Enzyme activities and subcellular fractionation studies in lactating and post-lactating rats

3.4 Pituitary enzyme activities and subcellular fractionation in lactating and post-lactating rats treated with bromocriptine

3.5 Characterisation of human pituitary organelles in normal and adenomatous tissue

3.6 Enzyme activities and hormone content in human pituitary tissue homogenates

3.7 Human prolactin protease

3.8 Case report

CHAPTER 3

RESULTS

3.1 CHARACTERISATION OF RAT PITUITARY ORGANELLES

3.1.1 Introduction

In order to separate cell components into nuclear, mitochondrial, microsomal and supernatant fractions, differential centrifugation was used (Hogeboom, Schneider and Palade, 1948; Schneider, 1948). Enzyme activities in the different fractions were thought to have concentrated at certain particles as a result of their association with specific subcellular components. McShan, Rozich and Meyer (1953) determined gonadotrophic hormone activity in fractions obtained from anterior pituitary glands of adult, normal and castrate rats, as well as succinoxidase, glycolytic and transaminase enzyme activities and found both hormone and suceinoxidase to be in the granule fractions. Several studies have been performed on the isolation of the various hormone containing granules and cell types of the rat pituitary (Hymer, 1974; Snyder, Hymer and Snyder, 1977) but very few have been done to separate the various subcellular organelles.

In this section, a combination of single step analytical centrifugation in the small volume automatic zonal rotor and the highly sensitive marker enzyme assays described in Chapter 2 were used to resolve the principal organelles from the rat

anterior pituitary. In addition, the centrifugation method was compared with the alternative technique of counter-current partition. The techniques described permitted not only quantitative assessment of organelles but also revealed information on the subcellular location and distribution of these enzymes in lactating and non-lactating rats.

3.1.2 Results

(i) Subcellular fractionation

Analytical subcellular fractionation studies of pituitaries removed from 3-day-lactating rats showed that certain organelles as well as the prolactin granules could be resolved.

Figure 3.1 shows the mean distribution of several marker enzymes and of prolactin in the density gradients. N-Acetyl-β-glucosaminidase and β-glucuronidase, both lysosomal markers, were found to have a modal density of 1.19 g/ml. Prolactin proteolysis, assayed according to the protocol described in full detail in Section 3.2, showed a similar modal density but was more narrowly distributed. Prolactin granules were located at a higher density, 1.23 g/ml, whereas malate dehydrogenase, a mitochondrial marker, showed a modal density of 1.16 g/ml with a large soluble component. Neutral α-glucosidase (endoplasmic reticulum) also had a similar distribution to that of malate dehydrogenase but with little activity in the soluble fraction. 5' Nucleotidase, a plasma membrane marker, had a peak activity at a density of 1.13 g/ml whereas lactate dehydrogenase, a cytosol marker, was mainly localised to the soluble fraction.

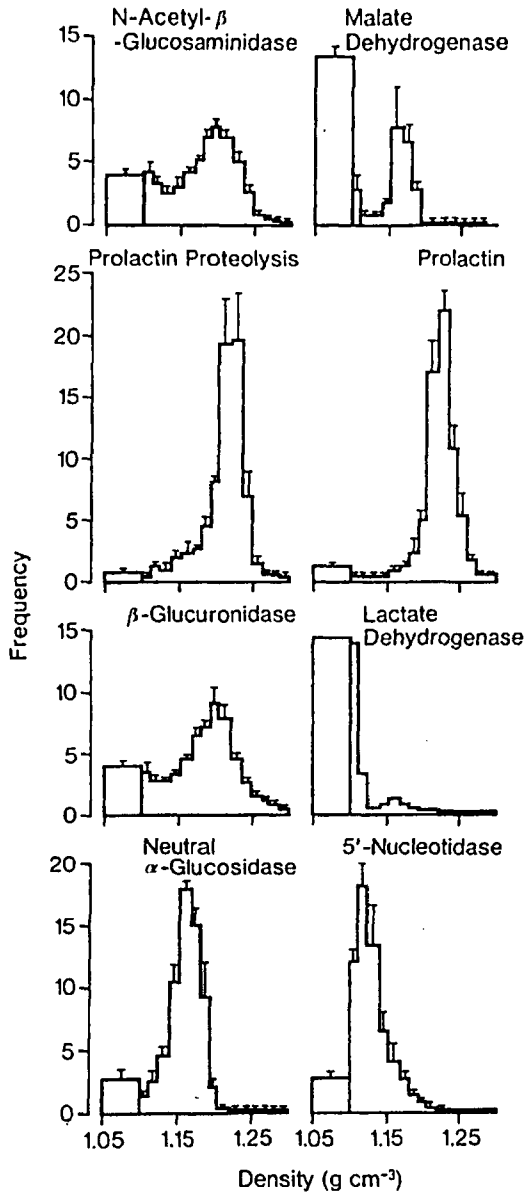


Fig. 3.1 Isopycnic centrifugation of 6000 x g-min supernatant homogenates of anterior pituitaries from 3 day lactating rats. Frequency (mean ± SD) is defined as the fraction of total recovered activity in the gradient fraction divided by the density span covered. The activity over the abcissa interval 1.05 to 1.10 represents enzyme remaining in the sample layer and is presumed to reflect soluble activity. The mean percentage of activity recovered, with number of specimens analysed in parentheses are: N-acetyl-β-glucosaminidase, 84(5); malate dehydrogenase, 90(3); prolactin proteolysis, 77(4); prolactin, 75(4); lactate dehydrogenase, 114(2); β-glucuronidase, 67(5); neutral-α-glucosidase, 98(4); 5'nucleotidase, 89(4).

Figure 3.2 shows the distribution profile of a further three marker enzymes and of alkaline phosphatase in the density gradient fractions. Alkaline phosphatase, a plasma membrane marker in other tissues (Solyom and Trams, 1972) showed a complex distribution pattern, with soluble and particulate components. Non-specific acid phosphatase, a dual marker for lysosomes and endoplasmic reticulum (Seymour and Peters, 1977), showed a broad distribution with peaks at densities 1.13 and 1.19 g/ml, the latter corresponding to the modal density of other lysosomal marker enzymes. Cathepsin C, also a lysosomal marker, had a peak activity at modal density 1.19 g/ml, again consistent with other lysosomal marker. Catalase, a peroxisomal marker enzyme, showed activity throughout the gradient with a broad peak of activity between densities 1.14 and 1.21 g/ml.

Lysosomal enzymes are normally contained within a membrane and therefore restricted from access to substrate in an in vitro system. Addition of a detergent perturbant allows enzyme and substrate to come into contact. A pituitary homogenate from a lactating rat was fractionated in sucrose medium containing 0.2 mmol/l digitonin. Digitonin has a dual action, binding to cholesterol in plasma membrane, increasing the density of this organelle and it also acts as detergent, releasing lysosomal enzymes into the cytosolic fraction (Amar-Costesec, Wibo, Thinès-Sempoux, Beaufay and Berthet, 1974; Peters and Seymour, 1978). This procedure resulted in a marked change in the distribution pattern of the lysosomal marker enzymes N-acetyl-β-glucosaminidase and β-glucuronidase, as well as of the prolactin degrading activity, which were now mainly located in the soluble fraction, not at the previous modal density of 1.19 g/ml.

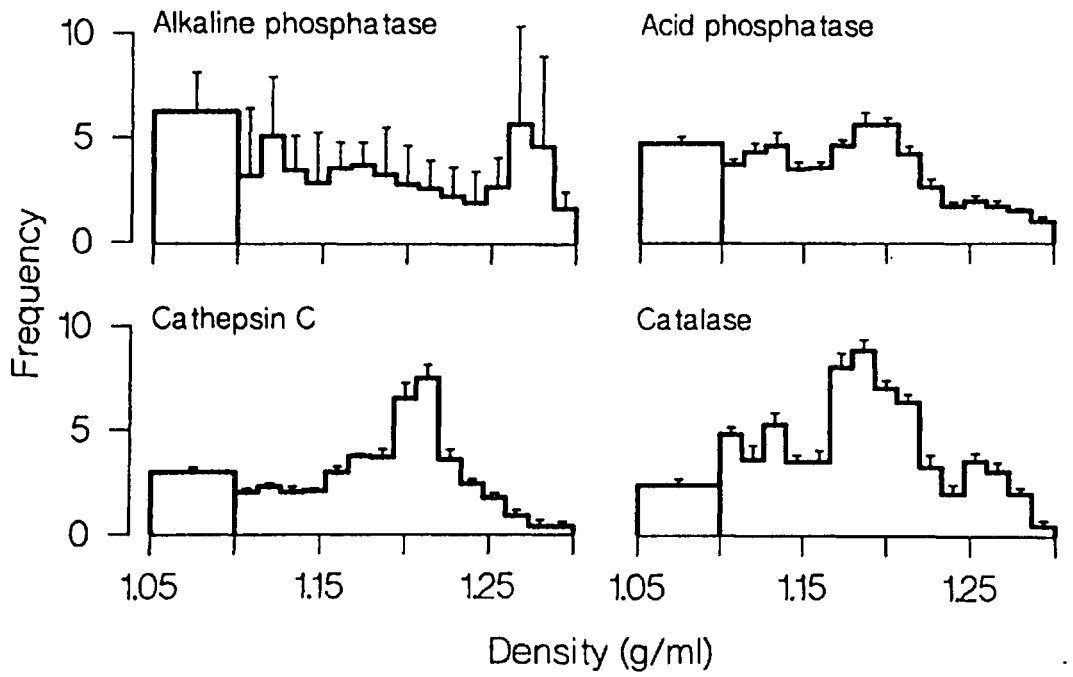


Fig. 3.2 Isopycnic centrifugation of 6000 x g-min supernatant homogenates of anterior pituitaries from 3 day lactating rats. Details as in Fig. 3.1. The mean percentage of recovered activities are: alkaline phosphatase, 66(4); acid phosphatase, 83(4); cathepsin C, 88(3); catalase, 92(3).

The prolactin granules and particulate malate dehydrogenase were not affected by the digitonin treatment, whereas 5'-nucleotidase showed a shift to a denser distribution.(Fig. 3.3).

(ii) Counter-current partition

In addition to the use of subcellular fractionation procedure, which separates particles on the basis of differences in density and sedimentation coefficients, fractionation of rat pituitary homogenates by counter-current partition between solutions of dextran and poly(ethylene) glycol were performed. Partition of biological materials in aqueous two-phase systems, which separate the various cell components, is determined by the interactions of membrane surface charge with the potentials of the phases used and also on the basis of membrane-lipid composition (Walter, Krob and Brooks, 1976; Albertsson, 1979). Three principal organelles and one hormone from lactating rat pituitary homogenates were investigated. Figure 3.4 shows the effect of the polymer composition on the partition of N-acetyl-β-glucosaminidase (lysosomes), neutral α-glucosidase (endoplasmic reticulum), 5' nucleotidase (plasma membrane) and prolactin. When the phasemixture contained 5.8% (w/w) dextran and 4.0% poly(ethylene)glycol, there was poor separation of mitochondria, plasma membrane and prolactin, although limited resolution was seen for neutral α-glucosidase. Increasing the concentration of dextran to 6.4% (w/w) changed the distribution of two of the enzymes, with 65% of neutral α-glucosidase partitioning to the

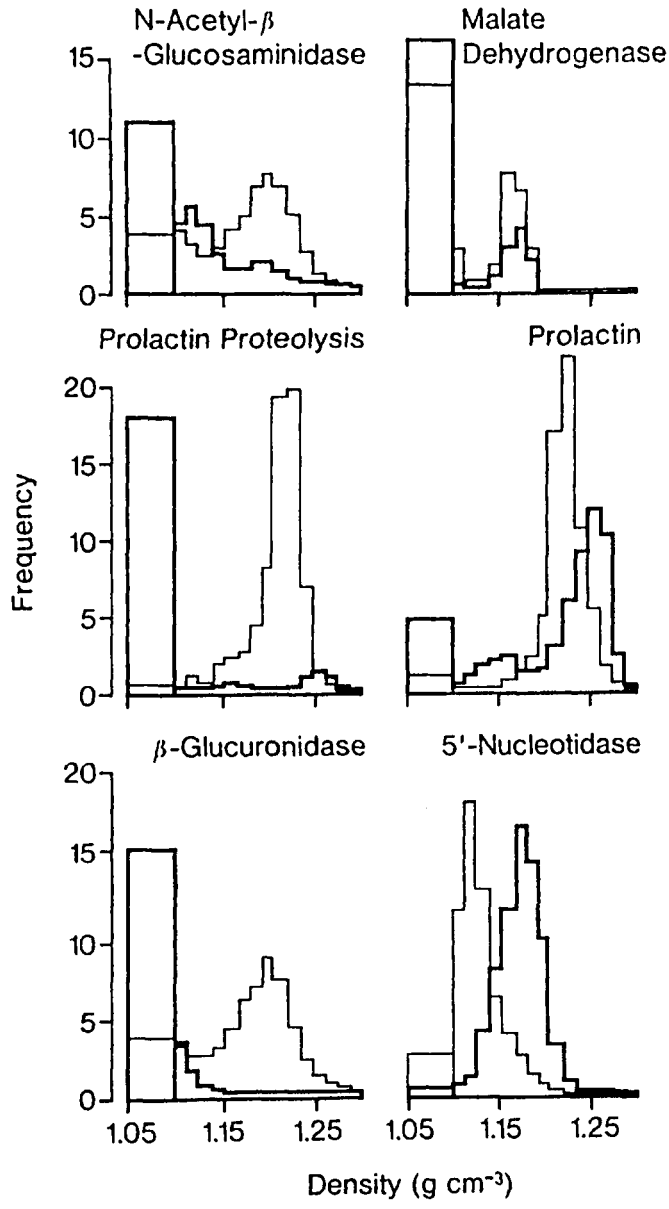


Fig. 3.3 Isopycnic centrifugation of 6000 x g-min supernatant from 3 day lactating rat anterior pituitary homogenized in iso-osmotic sucrose containing 0.2 mmol/l digitonin. Averaged controls from Fig. 3.1 are shown by the thin line. Details are as in Fig. 3.1. The percentage recoveries (digitonin-treated) are: N-acetyl-β-glucosaminidase, 96; malate dehydrogenase, 72; prolactin proteolysis, 102; prolactin, 132; β-glucuronidase, 83; 5'nucleotidase, 90.

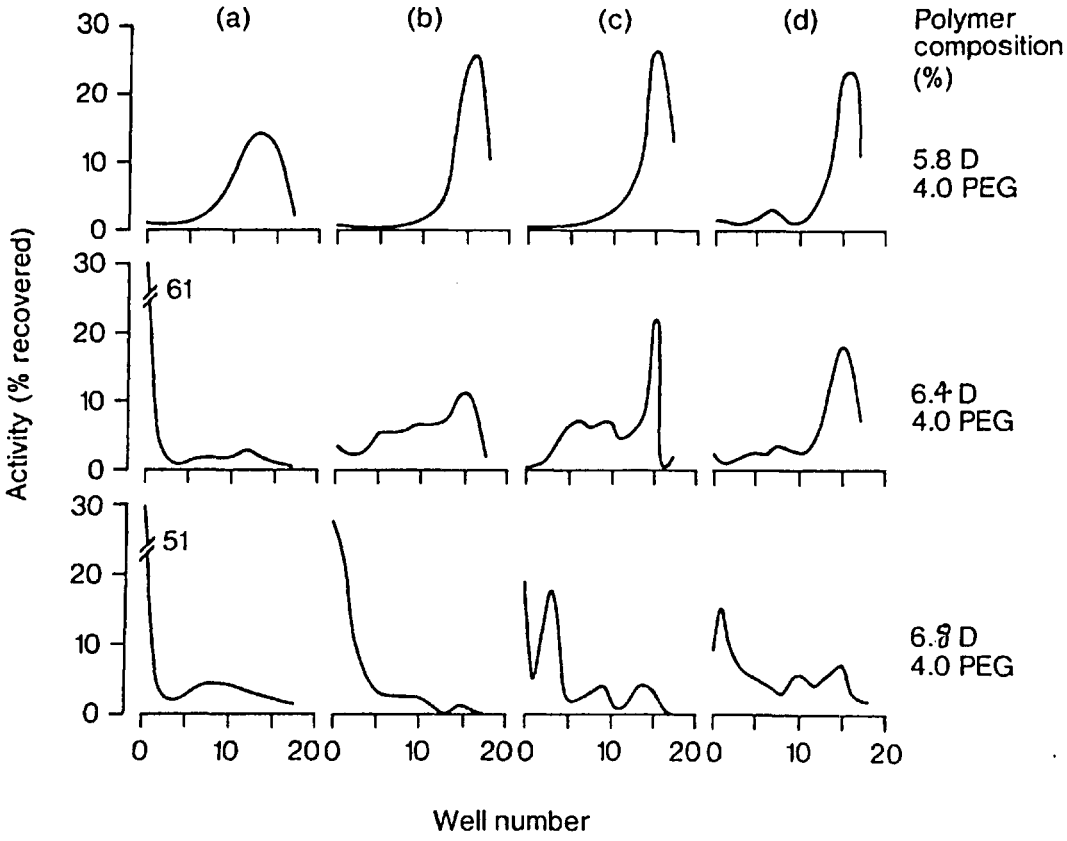


Fig. 3.4 Distribution of (a) neutral- α-glucosidase, (b) N-acetyl-β- glucosaminidase, (c) prolactin and (d) 5'nucleotidase with increasing polymer concentration after counter-current partition of homogenates from 3 day lactating rats. Low well numbers correspond to organelles with low partition coefficient. The interphase remained with lower dextran-rich phase in well no. 18 (stationery interface). (D= dextran; PEG= poly(ethylene)glycol).

lower phase, clearly separated from the other enzymes, with N-acetyl-β-glucosaminidase showing a broad distribution pattern. Prolactin showed a trimodal distribution, but 5'-nucleotidase was relatively unaffected by the higher concentration of dextran. A further increase in dextran to 6.8% (w/w) resulted in N-acetyl-β-glucosaminidase moving to the lower phase with neutral α-glucosidase. Prolactin showed a complex distribution pattern with hormone granule in the lower phase with three peaks of varying partition coefficient. The activity of 5'-nucleotidase was slightly altered by dextran addition, showing a trimodal profile and a shift to lower partition region of the extraction chain.

No clear resolution of lysosomes and prolactin granules was obtained but a useful separation of endoplasmic reticulum was achieved.

3.2 RAT PROLACTIN PROTEASE ASSAY

3.2.1 Introduction

Adam and Smith (1951) showed that aqueous extracts of hog anterior pituitary degraded haemoglobin and found two distinct pH optima of 3.8 and 8.3. Ellis (1960) further purified an acid proteinase I, pH 3.8, from sheep pituitary and showed that it degraded both prolactin and growth hormone; however, the subcellular localisation of this acid proteinase was not established. The presence of enzymic activity capable

of degrading prolactin granules in the rat pituitary is, however, implied by the morphological studies of Smith and Farquhar (1966) demonstrating crinophagy.

To investigate, characterise and localise the prolactin degrading activity in rat anterior pituitary cells, an assay for prolactin proteolysis was developed. Rat anterior pituitary glands were obtained as described in Section 2.1 and subcellular fractionation performed according to Section 2.4.

3.2.2 Procedure and Results

Suitably diluted homogenates or density gradient fractions, 0.1 ml, were incubated for up to 4 h at 37°C with 0.1 ml ^{125}I-labelled rat prolactin (iodination procedure described in Section 2.5) in 0.25 mol/l sodium acetate-acetic acid buffer, over a pH range 3-7 and 0.2 mol/l 2-amino-2-(hydroxymethyl)-propane-1,3-diol (Tris-HCl) over a pH range 7-10 (Figure 3.5). Both buffers contained 10 mmol/l dithiothreitol and 1 mmol/l EDTA, disodium salt. The reaction was stopped by the addition of 2.2 ml ice-cold trichloroacetic acid (150 g/l) and immediately vortexed. Bovine serum albumin, fraction V (100 mg/ml), 0.1 ml, was added and the tubes again vortexed. The tubes were kept in ice-water for 30 min and then centrifuged in an MSE Coolspin at 4°C for 30 min at 1500 x g. To 1.5 ml of the resulting supernatant were added 0.02 ml of 2.4 mmol/l potassium iodide followed by 0.1 ml hydrogen peroxide solution (30% w/w) to oxidise any free iodide to iodine. After standing exactly 5 min at room temperature, the free iodine was extracted from the supernatant

by two washes with 3.25 ml chloroform and 0.75 ml of the supernatant was then counted for radioactivity in an NE 1600 gamma-counter (Nuclear Enterprises Ltd., Edinburgh). Appropriate blanks (pituitary homogenates boiled for 10 min or sucrose medium alone) were performed in all experiments. A correction was made in the calculation of prolactin protease activity for the endogenous prolactin, determined by radioimmunoassay in the homogenates or appropriate fractions, according to the following formula:

$$\text{ng prolactin degraded/min/mg tissue protein} = \frac{\text{total prolactin/assay tube} \times \text{TCA soluble radioactivity/assay tube}}{\text{total radioactivity/tube} \times \text{incubation time(min)} \times \text{mg protein/tube}}$$

The results showed that a single peak of rat prolactin degrading activity occurred at pH 4.3 with low levels of activity at neutral or alkaline pH (Figure 3.5); therefore, all subsequent experiments were carried out at pH 4.3.

Prolactin degradation was found to be linear for at least 4 h (Figure 3.6) and apparent saturation was achieved with amounts of prolactin greater than 1 µg/assay tube (Figure 3.7). A linear relationship was observed between the amount of prolactin degraded and the concentration of pituitary homogenate (Figure 3.8).

Since application of this assay to the rat pituitary homogenate fractions described in the previous section (3.1) had shown this enzymic activity to have a subcellular distribution and peak modal density (1.19 g/ml) remarkably similar to that of the lysosomal marker enzymes, further evidence of its lysosomal

character was sought. Hence, the effects of specific lysosomal cathepsin inhibitors on prolactin protease were investigated. Addition of pepstatin A, 5 µmol/l (Sigma Co.), dissolved in distilled water containing 85 mmol/l methanol; leupeptin hemisulphate, 35 mmol/l (Sigma Co.); antipain dihydrochloride, 20 µmol/l (Sigma Co.) or iodoacetic acid, 10 mmol/l (Sigma Co.) dissolved in 0.25 mol/l sodium acetate-acetic acid buffer, pH 4.3, caused more than 90% inhibition of prolactin protease activity (Figure 3.9). These results were therefore entirely consistent with acid prolactin protease being lysosomal. In addition, the effects of two known inhibitors of prolactin secretion, 1-100 ng/ml dopamine hydrochloride (Arnar-Stone Laboratories, London) dissolved in 0.25 mol/l sodium acetate-acetic acid buffer, pH 4.3, containing 0.1 mmol/l ascorbic acid (Sigma Co.) and 1-100 µg/ml bromocriptine mesylate (Sandoz Products Ltd., Feltham) dissolved in 0.25 mol/l sodium acetate-acetic acid buffer, pH 4.3, containing 0.1 mmol/l ethanol, were investigated. Neither of these had any effect on prolactin protease in pituitary homogenates, these results being shown in Figure 3.9.

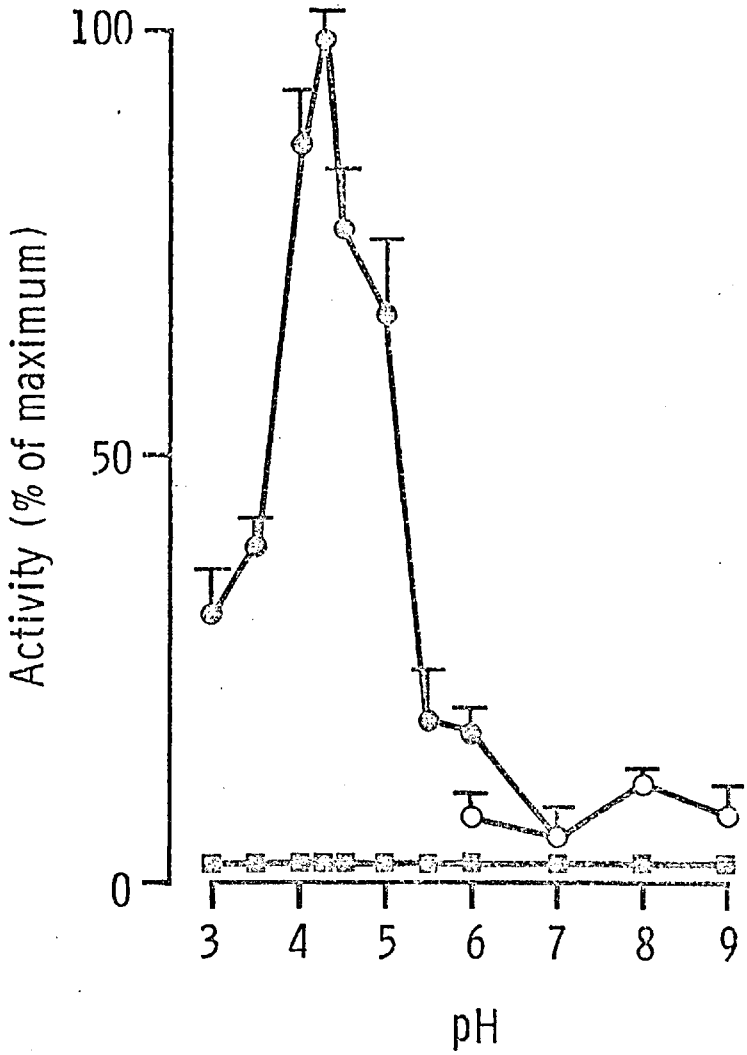


Fig. 3.5 pH dependence of rat prolactin degradation. ^{125}I-prolactin (100 µCi/µg) was incubated at various pH values for 90 min with either sucrose medium (blank) or pituitary homogenate. A sodium acetate-acetic acid buffer (final concentration 0.1 mol/l) was used between pH 3 and 6 and a Tris-HCl buffer between pH 6 and 9. Results are expressed as mean ± SE for between 3 and 5 duplicate determinations.

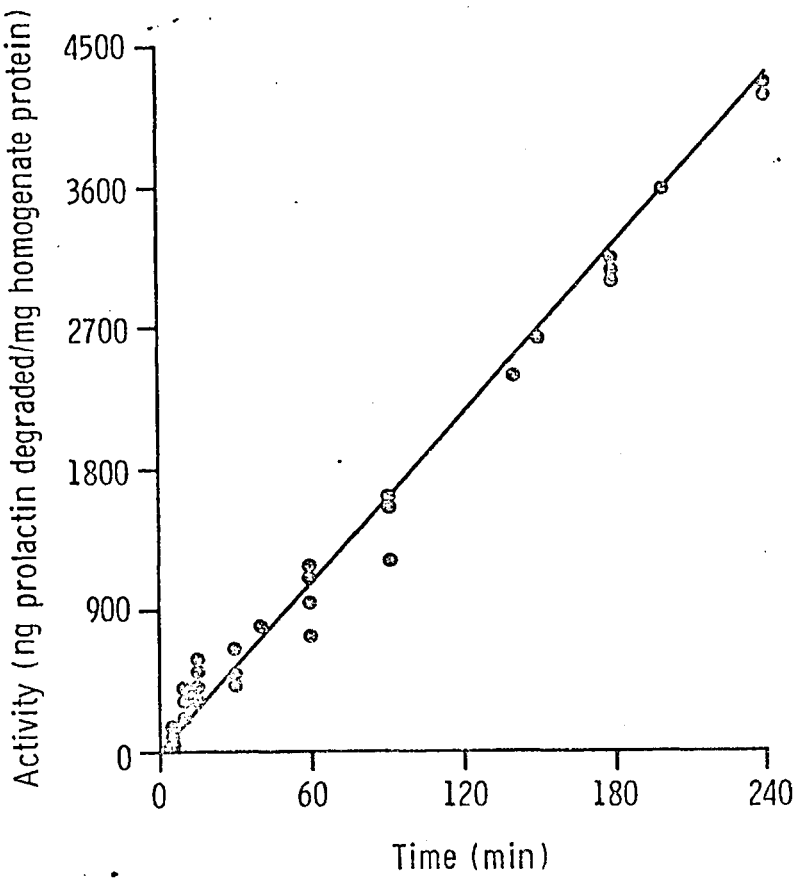


Fig. 3.6 Variation with time of the rate of degradation of rat prolactin.

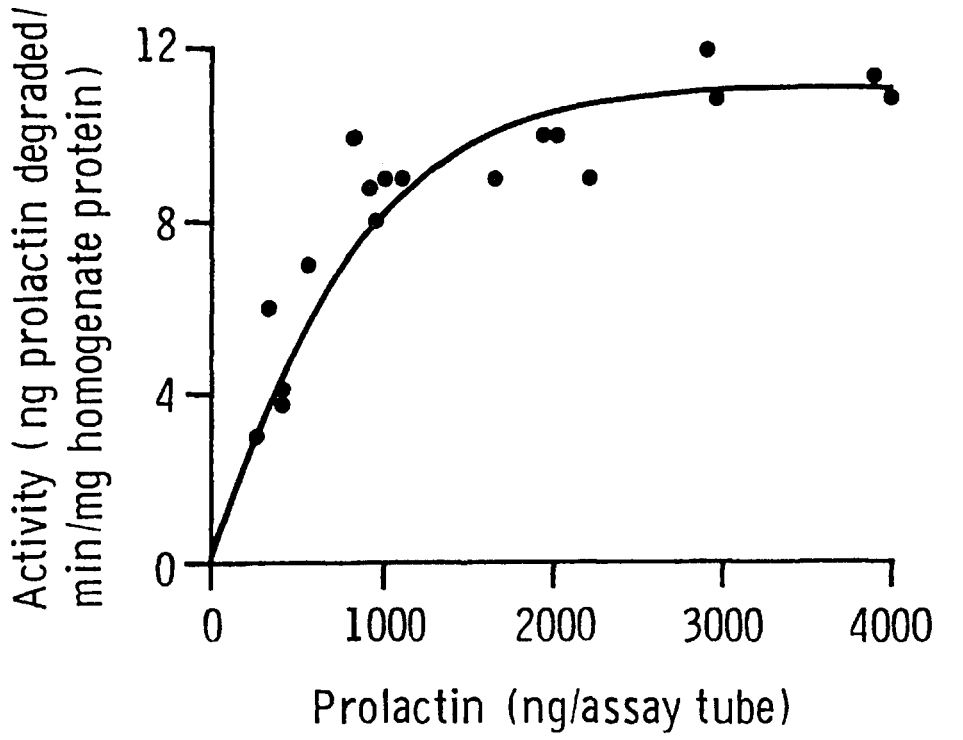


Fig. 3.7 Effect of varying prolactin concentration on the rate of degradation of rat prolactin.

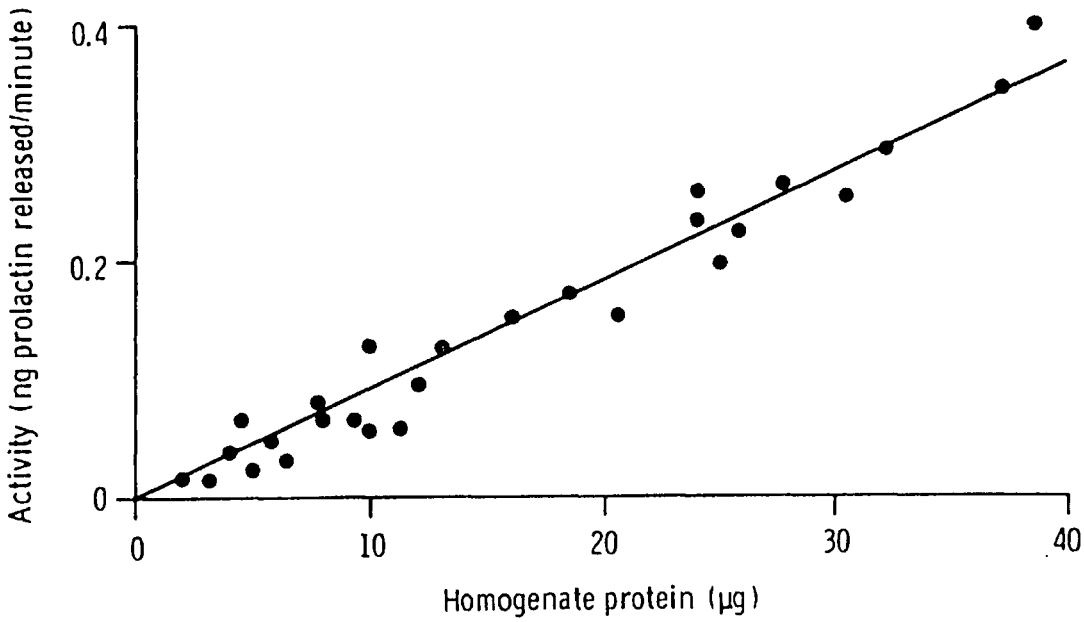


Fig. 3.8 Variation of activity with amount of pituitary homogenate protein.

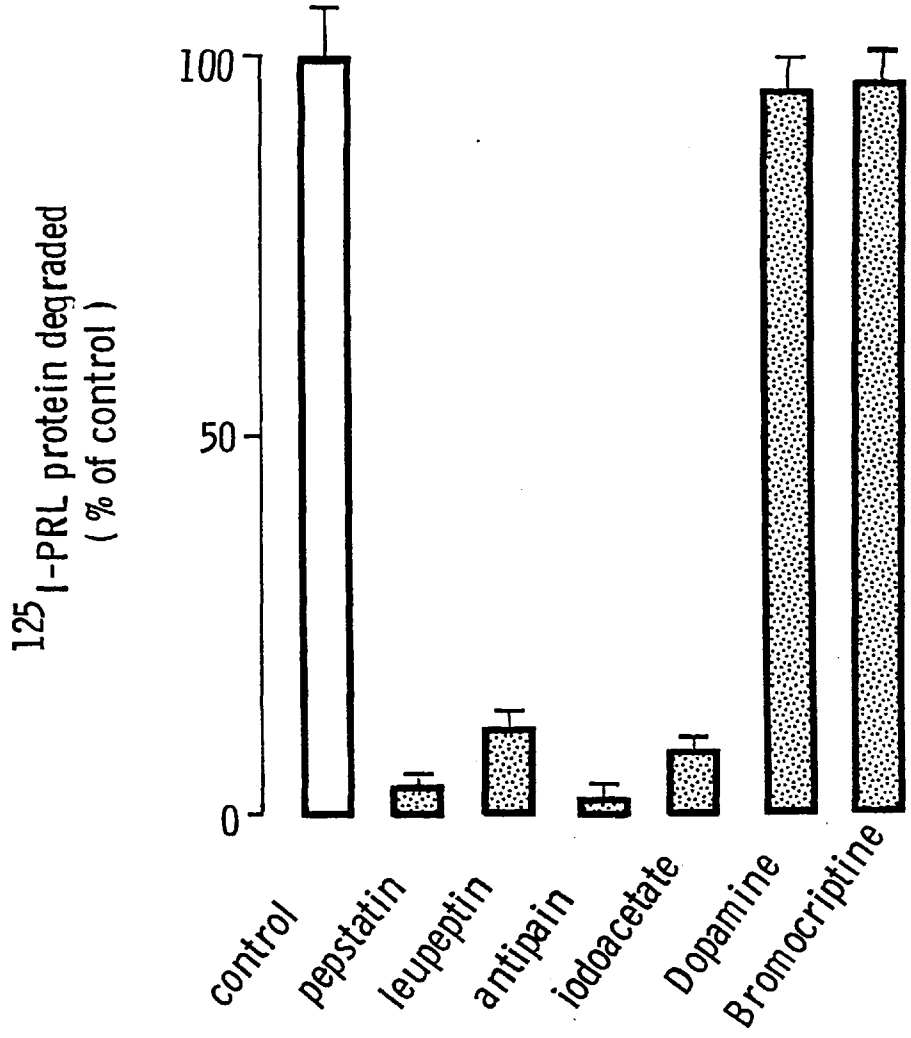


Fig. 3.9 Effect of catheptic inhibitors, dopamine and bromocriptine on the proteolytic degradation of ^{125}I-labelled prolactin. The mean ± SE of four observations is shown.

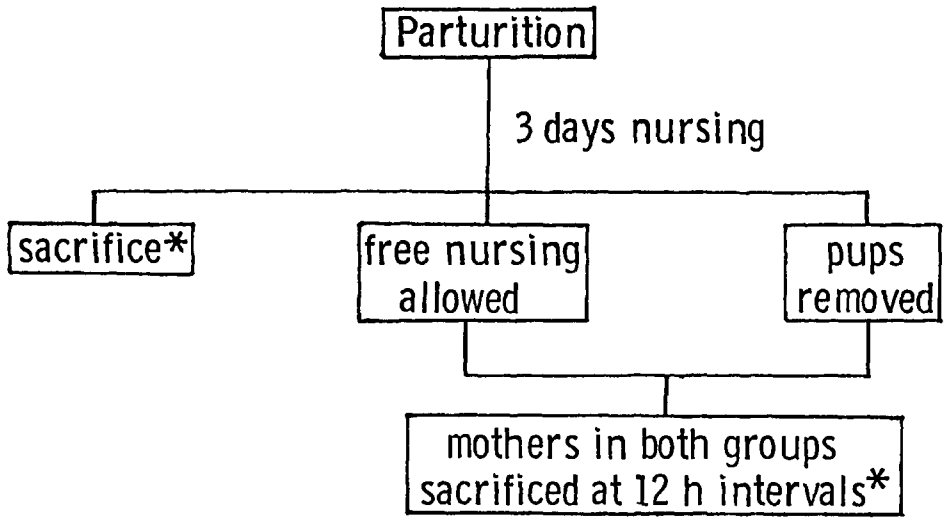
3.3 ENZYME ACTIVITIES AND SUBCELLULAR FRACTIONATION STUDIES IN LACTATING AND POST-LACTATING RATS

3.3.1 Introduction

Lysosomes were defined as sedimentable intracellular vesicles surrounded by a single membrane and containing hydrolases with optimal activity at acid pH (de Duve, 1959). Their role in many types of cells has been elucidated and they have been shown to have a variety of complex functions (de Duve and Wattiaux, 1966). Extensive cytochemical and electron microscopic studies on the rat anterior pituitary during lactation and after removal of suckling young have demonstrated that in the cells of the anterior pituitary gland, lysosomes may have a role in the intracellular homeostasis of prolactin by incorporating and degrading undischarged secretory granules, a process referred to as crinophagy (Smith and Farquhar, 1966).

Having developed methods that allowed characterisation of the subcellular organelles in rat pituitary, the objective of this aspect of the study was to seek biochemical evidence for the involvement of lysosomal enzymes in the degradation of excess intra-pituitary prolactin granules. In addition, to study changes in other organelle marker enzymes during this process. The animal model used was that of Smith and Farquhar (1966). The experimental plan was given in the Methods Section (2.1) but the summary outline is repeated in Figure 3.10 for clarity.

Fig. 3.10 Experimental model for pituitary crinophagy using suckling rats



*

1) Plasma prolactin measured

2) Pituitary homogenates assayed for:

   a) Organelle marker enzymes

   b) prolactin content

   c) DNA content

   d) protein content.

3.3.2 Results

(i) Whole homogenates

Measurement of plasma prolactin in the continuously lactating rats showed high levels ranging from 80 to 500 ng/ml throughout the experimental period with a significant decrease from 36 to 48 h (5 days total lactation). In contrast, the rats which had their litters removed after 3 days of suckling showed a striking fall in plasma prolactin levels to a mean of 66 ng/ml by 12 h with a further decline at 24 h to 12 ng/ml, a level comparable to that of a virgin rat, remaining at these levels thereafter (Fig. 3.11). Associated with this fall in plasma prolactin there was a marked increase in pituitary prolactin content from 65 to 225 µg/mg protein (Figure 3.11). This was maximal 12 h after removal of the litters and returned to control levels by 48 h.

The anterior pituitary homogenates were assayed for their total protein and DNA contents (Figure 3.12) but these did not show any significant difference in either group of rats during the experimental period. Removal of the litters caused highly significant changes in the specific activities of certain organelle marker enzymes, particularly those associated with the lysosomes. N-Acetyl-β-glucosaminidase (Figure 3.12) showed a two-fold significant increase ($p < 0.01$) in activity 24 h after the removal of the litters with a lesser, but still significant, degree of increment at 36 and 48 h. Acid prolactin protease showed a striking 18-fold rise in activity at 24 h which became apparent 12 h after pup removal and was still present at 36 h

but not different to controls at 48 h (Figure 3.12). β-Glucuronidase, a further lysosomal marker, was similarly elevated at 24 and 36 h (Figure 3.12). However, cathepsin C, also a lysosomal marker enzyme, did not show any significant change (Figure 3.13). Acid phosphatase, assayed with the fluorogenic substrate, is a dual marker for lysosomes and endoplasmic reticulum and showed a significant increase only at 24 h (Figure 3.13). Neutral α-glucosidase, an endoplasmic reticulum marker enzyme, did not show any difference between the two groups (Figure 3.13).

The latent N-acetyl-β-glucosaminidase, a measure of lysosomal integrity, was similar in both groups at all experimental times, as shown in Figure 3.14.

The activities of marker enzymes for the plasma membrane, mitochondria and cytosol are shown in Figure 3.15. The plasma membrane marker 5' nucleotidase showed a significant increase in activity between 24 and 36 h after removal of the litters, with a return to control levels by 48 h. Alkaline phosphatase, which has previously been shown to have a complex localisation in the gradient fractions, was significantly elevated between 12 and 36 h. Malate dehydrogenase showed a small increase only at 36 h and lactate dehydrogenase showed an increase at 12 h.

(ii) Electron microscopy

To determine whether these changes in organelle marker enzyme activities were represented by structural changes, electron micrographs of pituitaries from lactating and post-lactating rats were made. A lactotroph from a lactating rat

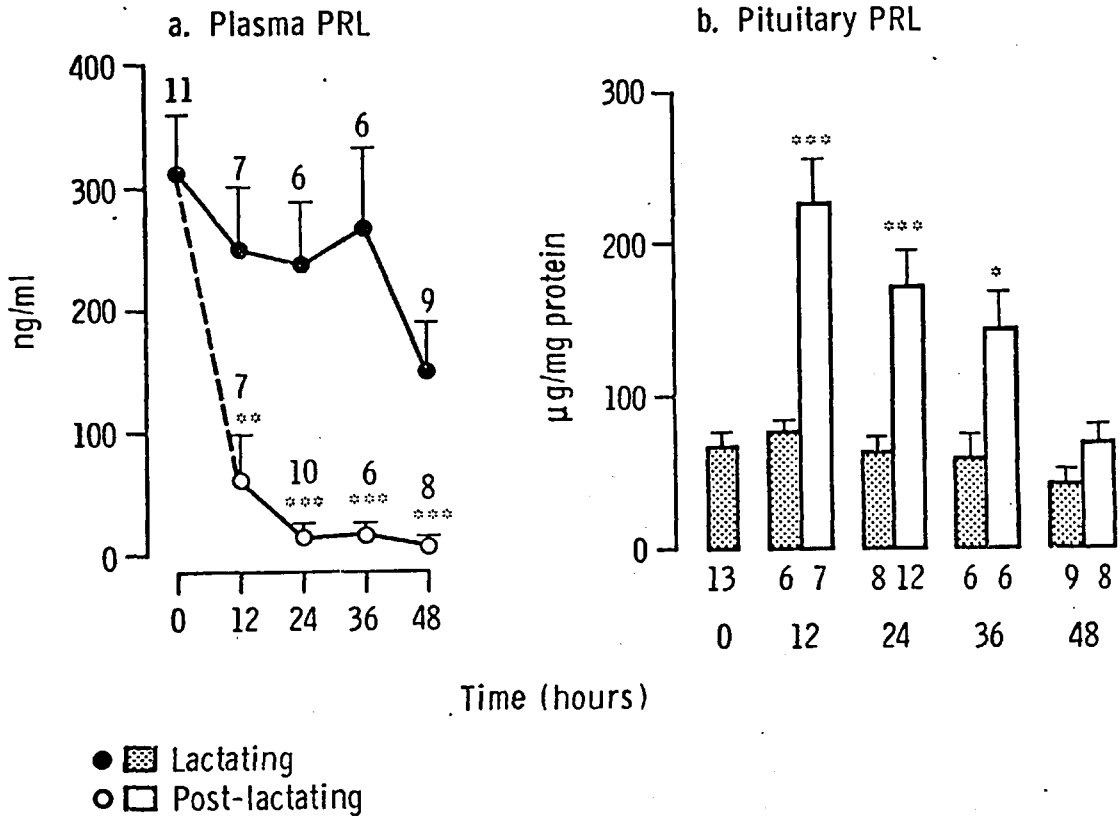


Fig. 3.11 Prolactin (PRL) concentration in the (a) plasma (ng/ml) and (b) pituitaries (µg/mg protein) of post-lactating rats at various times after removal of the pups compared to lactating rats in which suckling was uninhibited. The number of rats at each time point is indicated , above points in (a), below bars in (b). Results are expressed as the mean ± SE. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$.

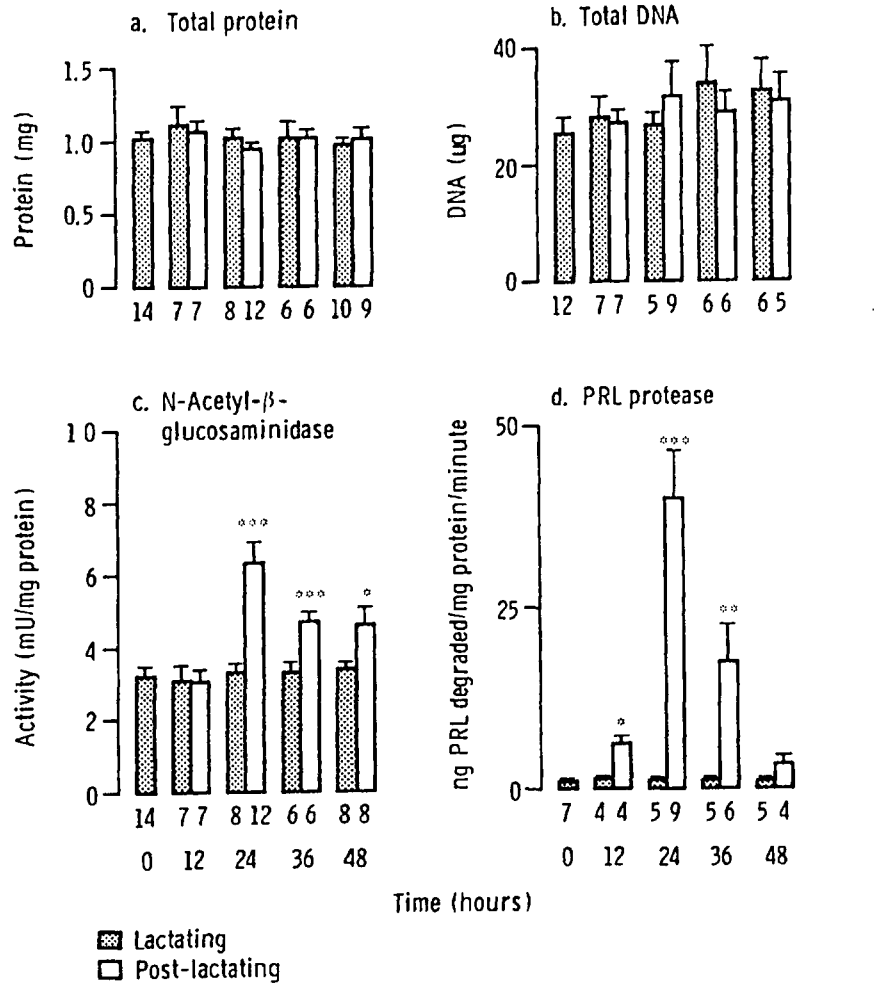


Fig. 3.12 Total protein (milligrams), total DNA (micrograms), and activities of the two lysosomal marker enzymes N-acetyl-β-glucosaminidase (milliunits per mg protein) and prolactin protease (nanograms PRL degraded per mg protein/min) in the pituitaries of post-lactating rats at various times after removal of the pups compared to lactating rats in which suckling was uninhibited. The number of pituitaries analyzed at each time point is indicated immediately below each bar. Results expressed as the mean ± SE. *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$.

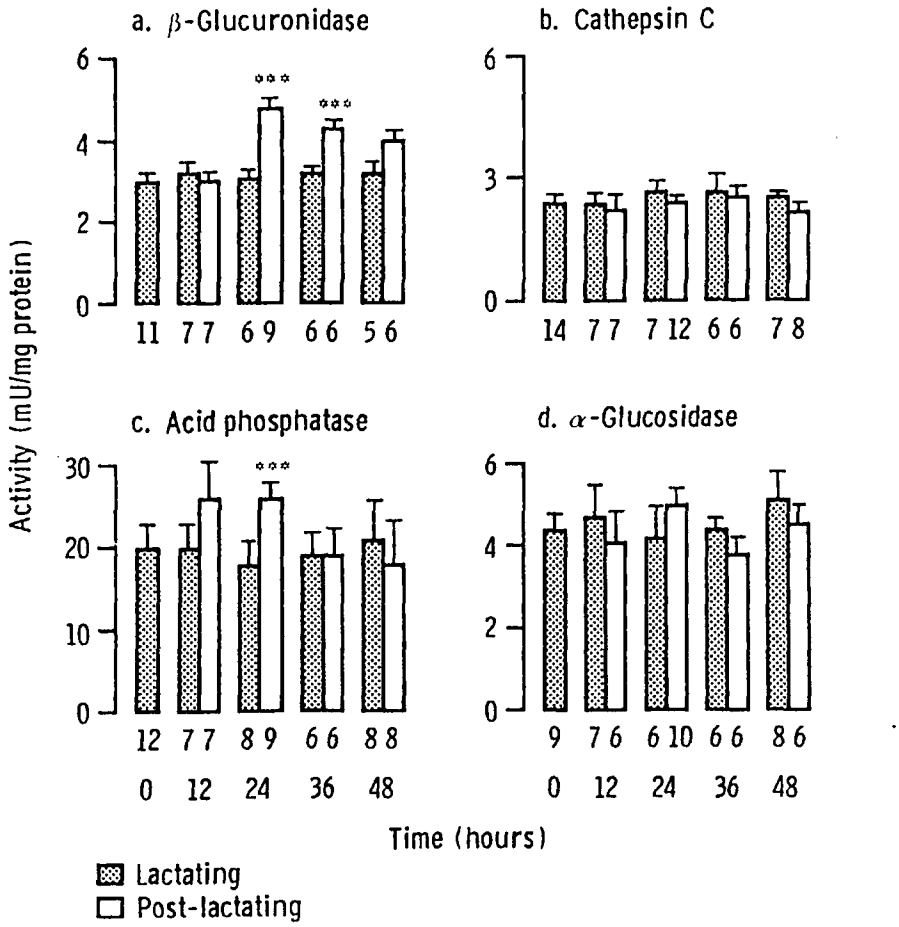


Fig. 3.13 Enzymic activities (milliunits per mg protein) of β-glucuronidase (lysosomal), cathepsin C (lysosomal), acid phosphatase (lysosomal/endoplasmic reticulum), and neutral-α-glucosidase in the pituitaries of post-lactating rats at various times after removal of the pups compared to lactating rats in which suckling was uninhibited. Details as in Fig. 3.11; ***, $p < 0.01$.

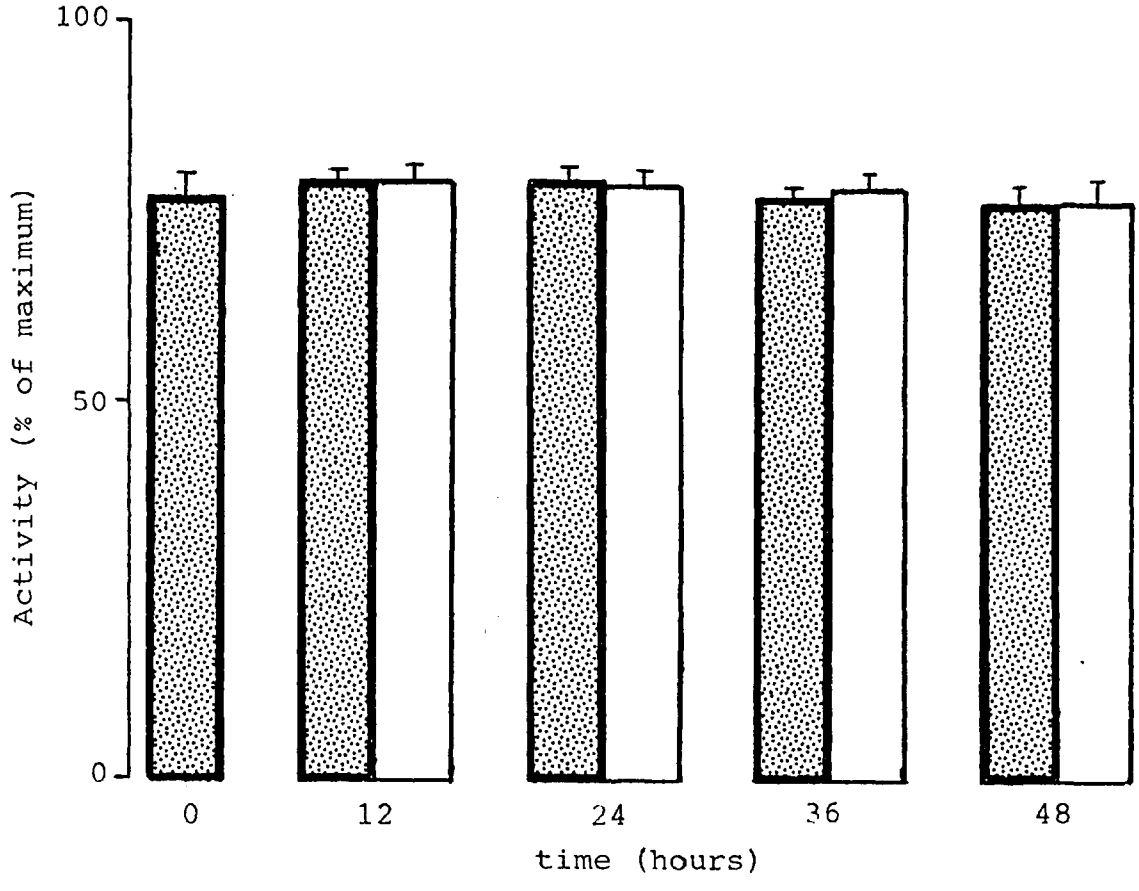


Fig. 3.14 Latent N-acetyl- β-glucosaminidase in pituitaries from lactating and post-lactating rats. (n= 7 animals each group).

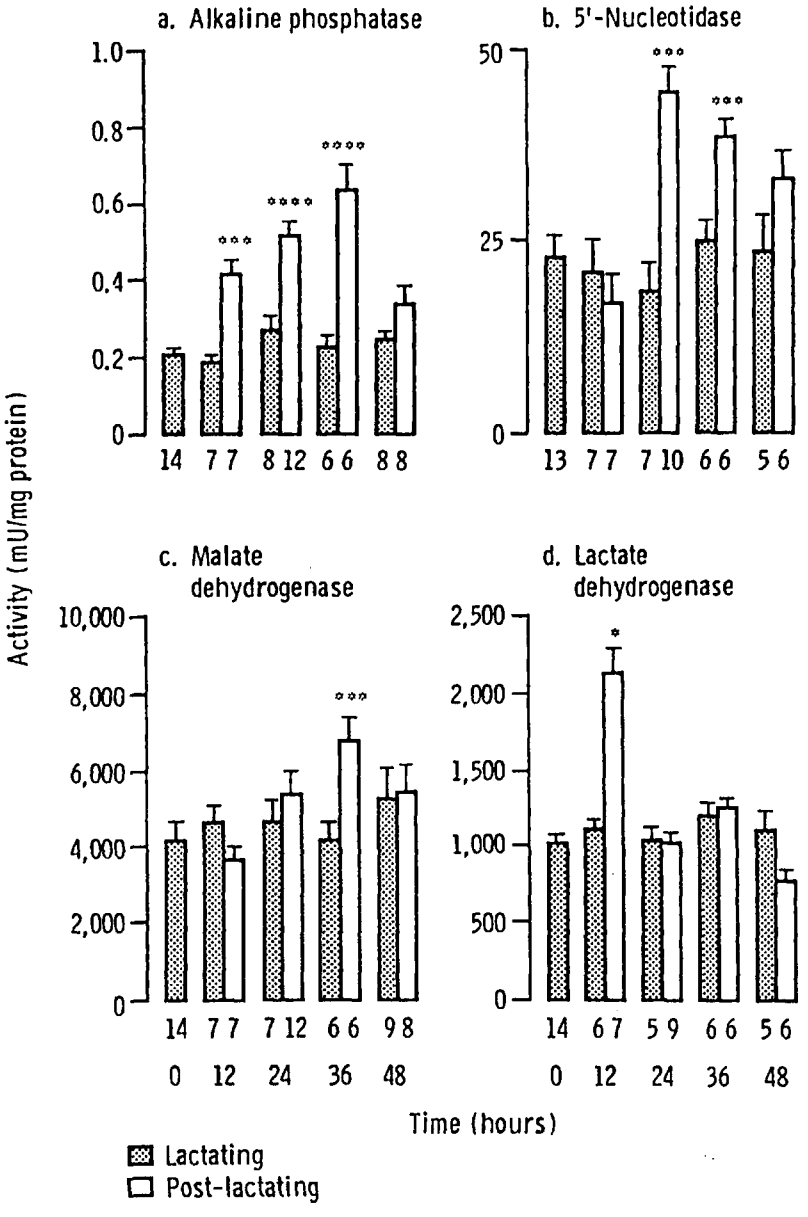


Fig. 3.15 Enzymic activities (milliunits per mg protein) of alkaline phosphatase (plasma membrane), 5'nucleotidase (plasma membrane), malate dehydrogenase (mitochondria), and lactate dehydrogenase (cytosol) in the pituitaries of post-lactating rats at various times after removal of the pups compared to lactating rats in which suckling was uninhibited. Details as in Fig. 3.11. *, $p < 0.05$; ***, $p < 0.01$; ****, $p < 0.001$.

(Figure 3.16) shows a predominance of rough-surfaced endoplasmic reticulum and few mature secretory granules. A lactotroph from a 24 h post-lactating rat is illustrated in Figure 3.17 which shows free ribosomes, a less prominent endoplasmic reticulum, polymorphous (aggregating) secretory granules and an increased number of lytic bodies (lysosomes). Figure 3.18 shows the lytic bodies from a lactotroph cell of a 24 h post-lactating rat at a higher power.

(iii) Subcellular fractionation

To determine whether the changes observed in total organelle marker enzyme activities in the pituitary homogenates were reflected in the pattern of distribution of these organelles, analytical subcellular fractionation of a pituitary from a post-lactating rat, which had her litters removed 24 h before being sacrificed, was carried out. The results (Figures 3.19 and 3.20) show the distribution of some subcellular organelles and prolactin compared with those obtained for lactating rats (Section 3.1.2).

Figure 3.19 shows the distribution of three lysosomal marker enzymes and of prolactin granules. The distribution profile of N-acetyl-β-glucosaminidase and prolactin on the gradient was unchanged when compared with that of pituitaries from lactating rats. However, β-glucuronidase showed a different pattern represented by increased activity in the soluble fraction of the gradient as well as two peaks of activity at densities

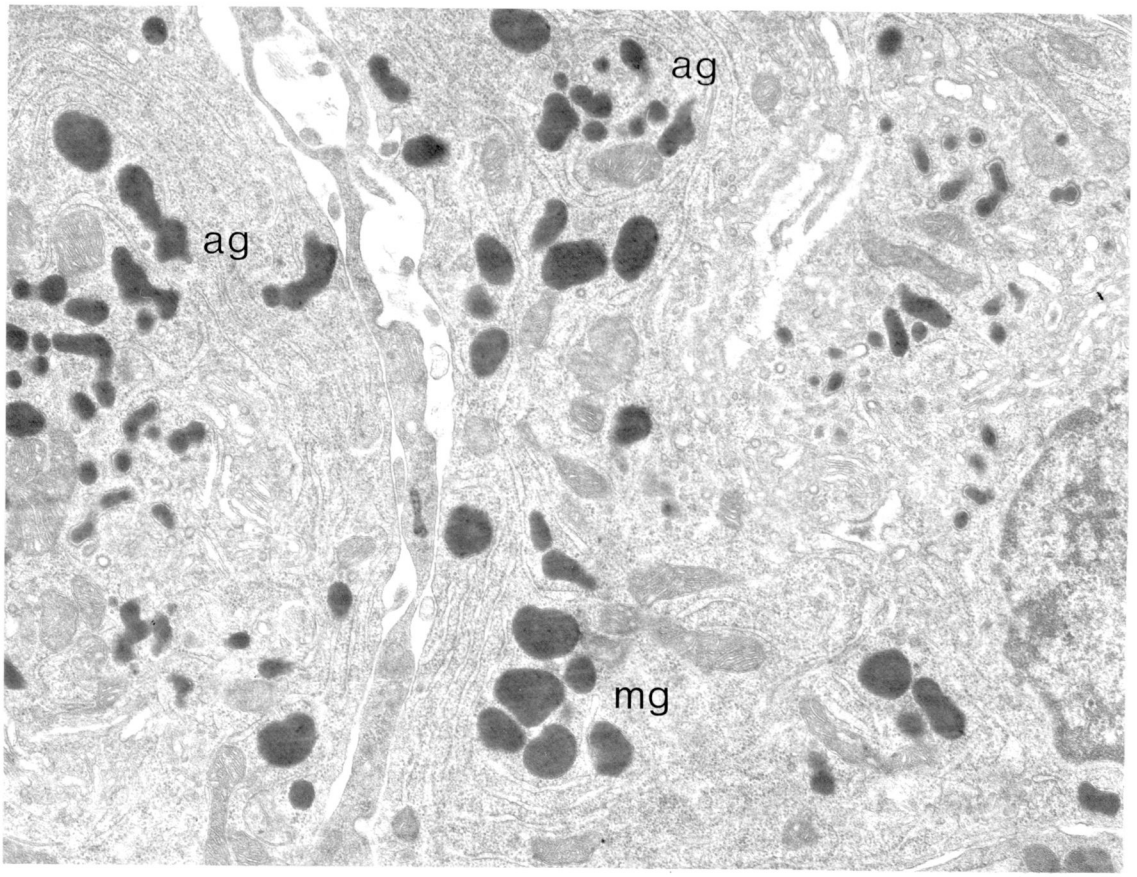


Fig. 3.16 Electron micrograph of a part of a lactotroph cell from a 3 day lactating rat showing a well developed rough-surfaced endoplasmic reticulum, aggregating secretory granules (ag) and mature secretory granules (mg). Glutaraldehyde/osmium tetroxide fixation. Stained with lead citrate and uranyl acetate. (x 22,500).

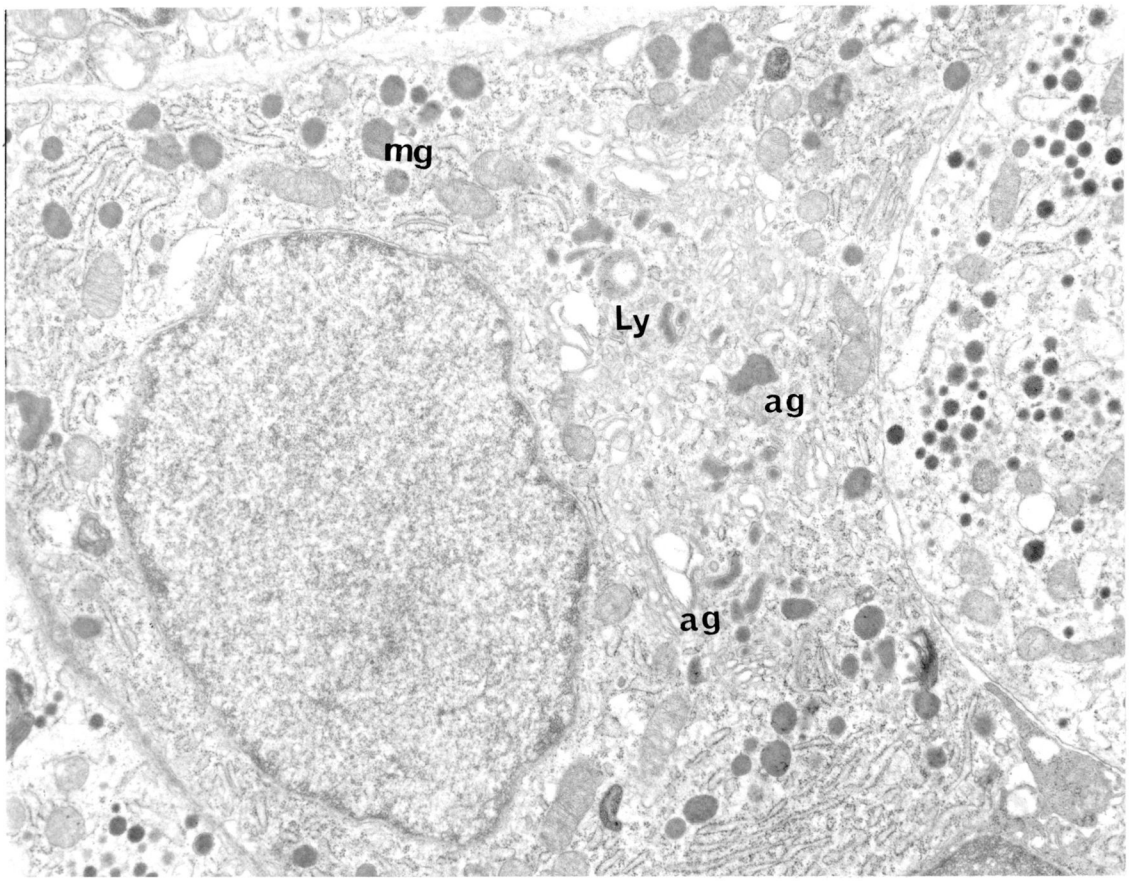


Fig. 3.17 Electron micrograph of a lactotroph cell from a 24 h post-lactating rat showing an undeveloped rough-endoplasmic reticulum, aggregating secretory granules (ag), mature secretory granules (mg) and a secondary lysosome (ly). Glutaraldehyde/osmium tetroxide fixation. Stained with lead citrate and uranyl acetate. (x 15,000).

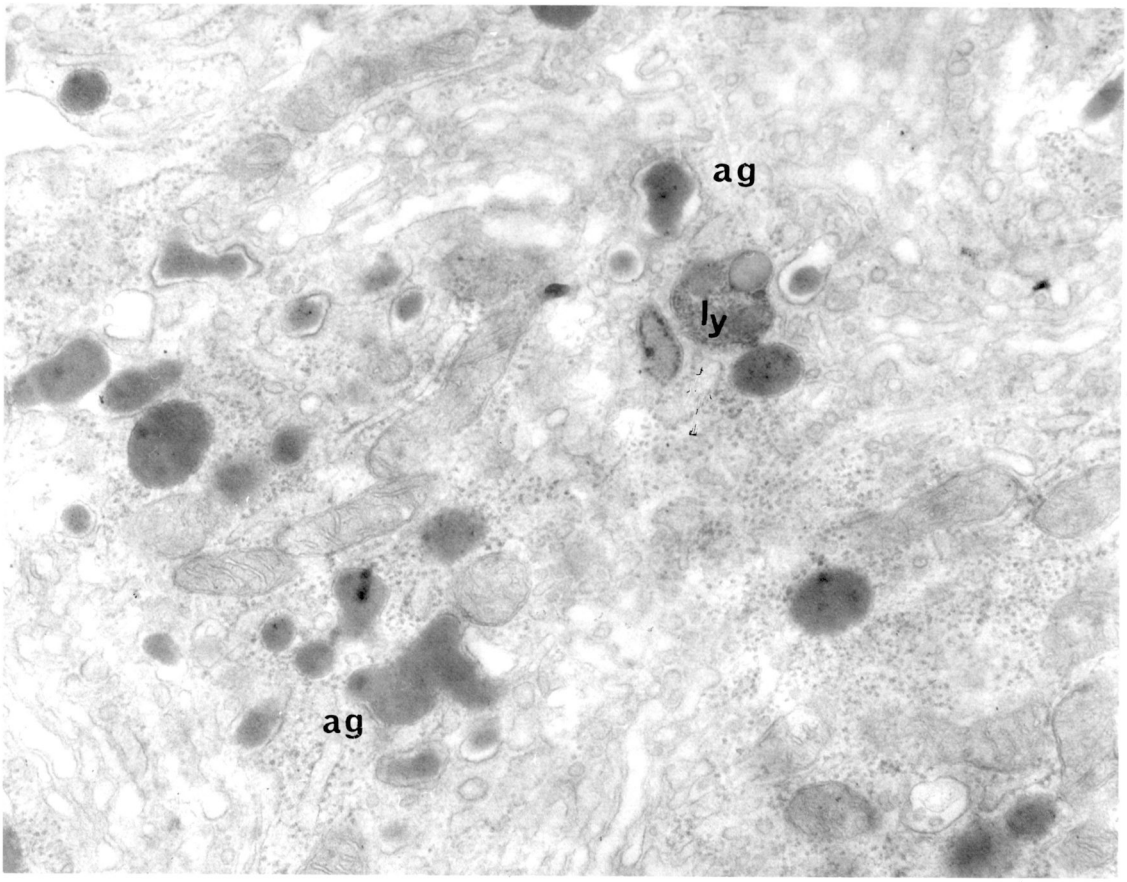


Fig. 3.18 Electron micrograph of a part of a lactotroph cell from a 24 h post-lactating rat showing aggregating secretory granules (ag) and a secondary lysosome (ly). Glutaraldehyde/osmium tetroxide fixation. Stained with lead citrate and uranyl acetate. (x 45,000).

1.17 and 1.22 g/ml respectively. In addition, the acid prolactin protease, a lysosomal marker enzyme, showed negligible activity in the soluble fraction and a small shift in the modal density of its peak activity which was now less dense, at 1.18 g/ml, more closely followed that of N-acetyl-β-glucosaminidase. The density distribution of prolactin was unchanged when compared with that obtained from lactating rats.

Figure 3.20 shows the distribution profile of 5' nucleotidase (plasma membrane), malate dehydrogenase (mitochondria), neutral α-glucosidase (endoplasmic reticulum) and lactate dehydrogenase (cytosol). There was no significant change in the enzyme distributions when compared with lactating rats.

It has been shown previously (Section 3.1.2) that the use of digitonin selectively disrupts the lysosomes in the density gradient. Fractionation of a pituitary homogenate from a 24 h post-lactating rat in sucrose medium containing 0.2 mmol/l digitonin (Figure 3.21) gave results similar to those obtained with pituitary homogenates from lactating rats. Activities of N-acetyl-β-glucosaminidase, β-glucuronidase and acid prolactin protease were localised in the soluble fraction whereas prolactin was unaffected by digitonin treatment, confirming their localisation to lysosomes in these animals.

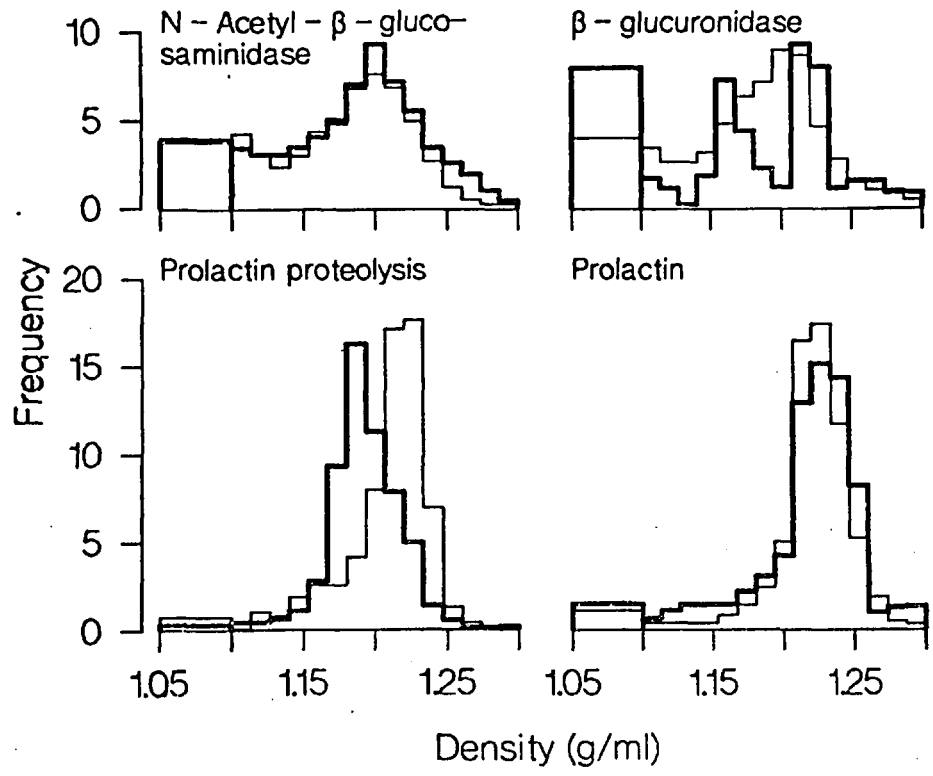


Fig. 3.19 Isopycnic centrifugation of 6000 x g-min supernatant homogenate of anterior pituitary from a 24 h post-lactating rat (thick line). Average controls (lactating rats) from Fig. 3.1 are shown by the thin line. Details are as in Fig. 3.1. The percentage of activities recovered are: N-acetyl-β-glucosaminidase, 104; β-glucuronidase, 77; prolactin proteolysis, 91; prolactin, 94.

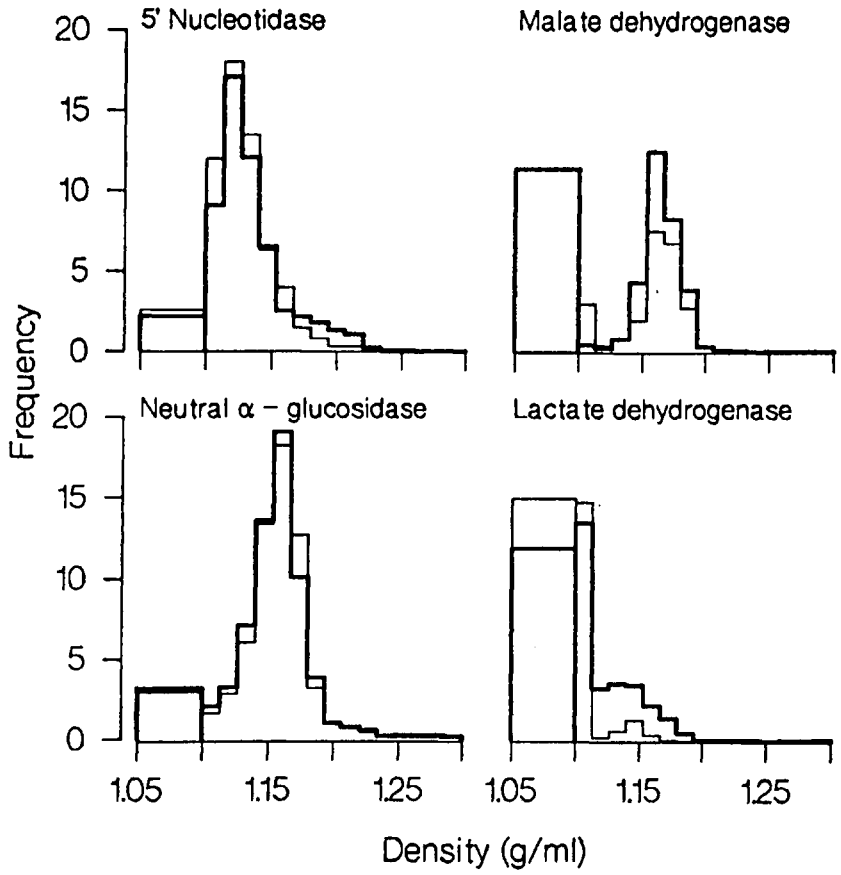


Fig. 3.20 Isopycnic centrifugation of 60000 x g-min supernatant of an anterior pituitary from a 24 h post-lactating rat (thick line). Average controls (lactating rats) from Fig. 3.1 are shown by the thin line. Details as in Fig. 3.1. The percentage of recovered activities are: 5'nucleotidase, 86; malate dehydrogenase, 96; neutral α-glucosidase, 83; lactate dehydrogenase, 94.

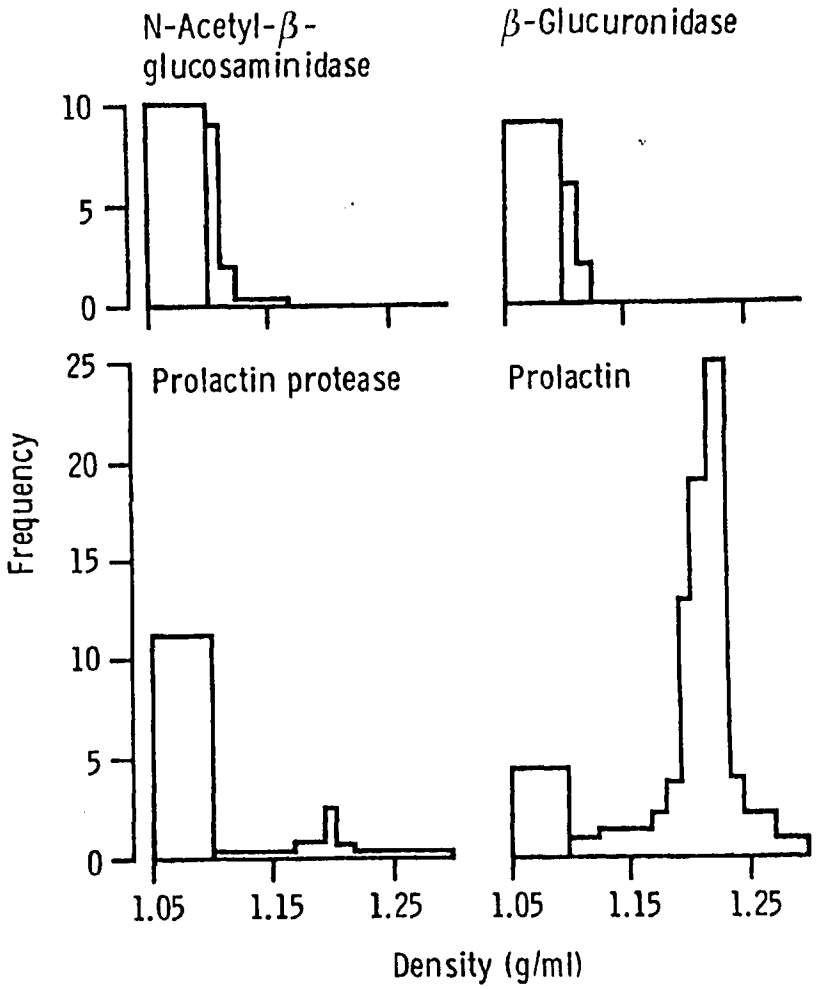


Fig. 3.21 Isopycnic centrifugation of 6000 x g-min supernatant from a 24 h post-lactating rat anterior pituitary homogenized in iso-osmotic sucrose containing 0.2 mmol/l digitonin. Details are as in Fig. 3.1. The percentage of activities recovered (digitonin-treated) are: N-acetyl-β-glucosaminidase 103; β-glucuronidase, 90; prolactin protease, 94; prolactin, 104.

3.4 PITUITARY ENZYME ACTIVITIES AND SUBCELLULAR FRACTIONATION STUDIES IN LACTATING AND POST-LACTATING RATS TREATED WITH BROMOCRIPTINE

3.4.1 Introduction

Prolactin secretion has been shown to be under inhibitory control by the hypothalamus (MacLeod and Lehmeyer, 1974; Shaar and Clemens, 1974; Gibbs and Neill, 1978). The results in Section 3.2 showed that bromocriptine and dopamine exerted no direct action on the enzymic prolactin degrading activity in broken cell preparations. To investigate the inhibitory dopaminergic process further, the effects of bromocriptine were studied in intact animals according to the experimental plan described previously in Section 2.1. Lactating rats were sacrificed 24 h after the injection of single doses of bromocriptine ranging from 0.5 to 500 µg. This time was chosen because ultrastructural studies have shown that pituitary tissue fixed 18 h after the drug showed no granule extrusion and more intracellular prolactin granules were observed (Ectors, Dangui and Pasteels, 1972).

3.4.2 Results

(i) Whole homogenates

The highest dose of bromocriptine caused a significant fall in plasma prolactin from 350 ng/ml to 40 ng/ml, lower concentrations of the drug having little, if any, effect (Figure 3.22).

In contrast, pituitary prolactin in the same animals, was significantly elevated with all doses of bromocriptine to at least 300% of the starting values although a clear dose response relationship could not be demonstrated. Total protein content was unchanged, whereas all doses of bromocriptine caused a 50% reduction in total cell DNA as shown in Figure 3.22, but again there was no dose response relationship although the degree of significance was increased.

The highest concentration of bromocriptine used, 500 µg, caused an 8-fold increase in the activity of the lysosomal acid prolactin protease with lower doses also inducing a marked rise in the activity of the enzyme. However, in marked contrast, activities of N-acetyl-β-glucosaminidase and β-glucuronidase, also lysosomal marker enzymes, remained unchanged following administration of the drug (Figure 3.23). Acid phosphatase was significantly increased at 24 h in rats treated with 0.5, 5 and 500 µg but with 50 µg bromocriptine the increase was not statistically significant.

The activities of the plasma membrane marker enzyme 5'-nucleotidase and of alkaline phosphatase (Figure 3.24) were elevated at least two-fold by 24 h in treated animals. Neutral α-glucosidase (endoplasmic reticulum) was unaffected by bromocriptine but catalase (peroxisomes) was significantly higher in all groups, showing an increase in activity of approximately 100% over control levels. Malate dehydrogenase did not show any difference between the control and experimental groups (Figure 3.24).

To determine whether the mechanism(s) of action of bromocriptine was similar to that obtained with the physiological removal of suckling stimulus, both lactating and post-lactating rats were given a single dose of 500 µg bromocriptine. The results are summarised in Figures 3.25 to 3.27. Twelve and 24 h following removal of the litters there was a marked fall in plasma prolactin (post-lactating rats) to normal values for non-lactating animals. These changes were paralleled by an increase in pituitary prolactin which was maximal at 12 h. As in the previous experiment, lactating rats treated with 500 µg bromocriptine also showed a highly significant fall in plasma prolactin at 12 and 24 h, accompanied by a rise in pituitary prolactin, that was higher than that attained in the post-lactating rats. Post-lactating rats treated with bromocriptine showed similar falls in plasma prolactin but with much higher pituitary prolactin levels than the post-lactating control rats not treated with bromocriptine (Figure 3.25). Although none of the treatment changed the total protein content of the pituitaries, the administration of bromocriptine induced a significant fall in total DNA in both lactating and post-lactating rats (Figure 3.25).

The lysosomal enzymes acid prolactin protease, N-acetyl-β-glucosaminidase and β-glucuronidase were all shown to be increased 24 h after the removal of the suckling stimulus alone. However, treatment with bromocriptine in the lactating and post-lactating animals had a differential effect on these enzymes,

with acid prolactin protease showing an 18-fold increase in both groups at 12 and 24 h. These increases were significantly greater than those produced by litter removal alone. In contrast, whereas litter removal significantly increased the activities of N-acetyl-β-glucosaminidase and β-glucuronidase at 24 h, bromocriptine administration blocked these increments (Figure 3.26). Acid phosphatase showed a significant increase 24 h following litter removal that was also observed at 12 and 24 h in lactating and post-lactating rats treated with bromocriptine (Figure 3.26).

The activities of marker enzymes for plasma membrane, mitochondria and endoplasmic reticulum are illustrated in Figure 3.27. Alkaline phosphatase showed a marked increase in activity both at 12 and 24 h after removal of the litters and 5' nucleotidase was increased only at 24 h. Administration of bromocriptine induced more significant increases at both 12 and 24 h for 5' nucleotidase but not for alkaline phosphatase in lactating and post-lactating groups. Malate dehydrogenase was increased 24 h after litter removal, an effect that was blocked by bromocriptine. Neutral α-glucosidase was unchanged between any treatment groups.

(ii) Subcellular fractionation

An anterior pituitary from a lactating rat that had received 500 μg bromocriptine subcutaneously 24 h before sacrifice was subjected to analytical subcellular fractionation as described previously (Section 2.4). The distribution of N-acetyl-β-

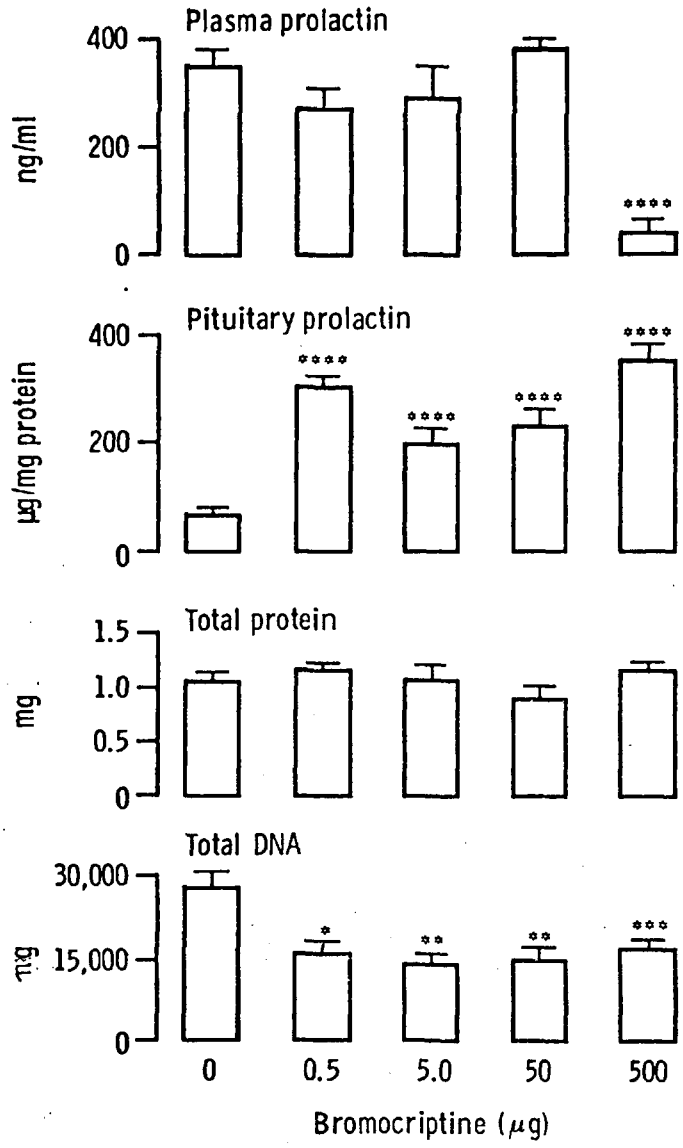


Fig. 3.22 Effect of varying doses of bromocriptine on plasma PRL (ng/ml), and pituitary PRL (µg/mg protein), total protein (mg) and total DNA (µg) in lactating rats sacrificed 24 h after drug administration. The number of pituitaries analysed was, 8 for control and 500 µg bromocriptine, 4 for the other groups. Results are expressed as the mean±SE; *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$; ****, $p < 0.001$; vs. control.

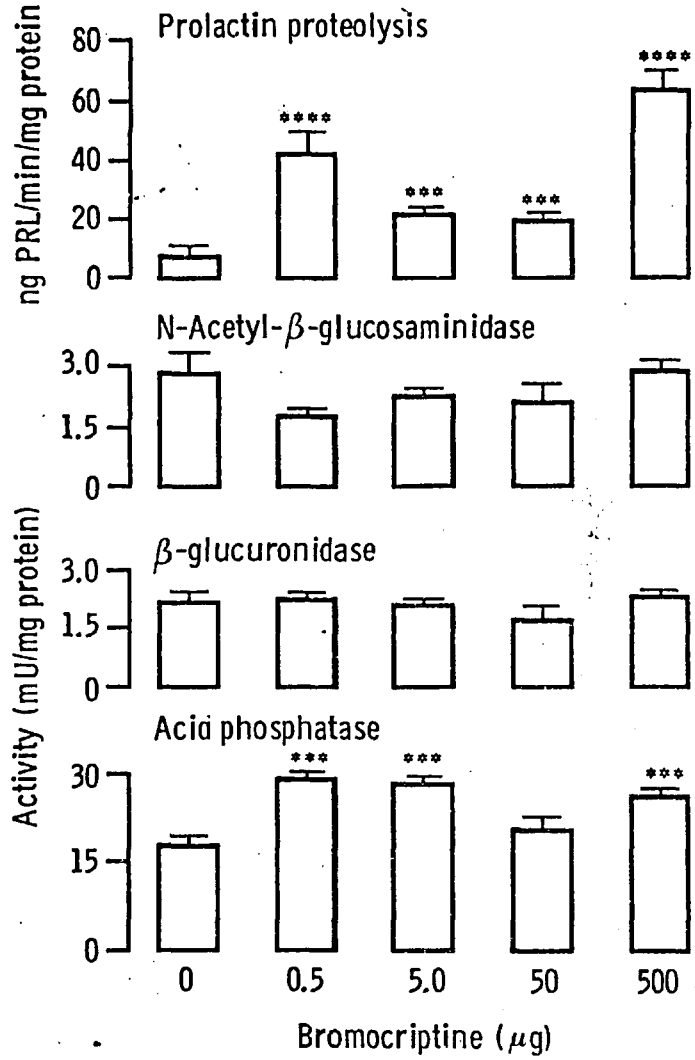


Fig. 3.23 Effect of varying doses of bromocriptine on the enzymic activity of the lysosomal marker enzymes acid PRL protease (ng PRL/ degraded/mg protein/min), N-acetyl-β-glucosaminidase (mU/mg protein), β-glucuronidase (mU/mg protein) and the lysosomal/endoplasmic reticulum marker acid phosphatase (mU/mg protein) in the pituitaries of lactating rats sacrificed 24 h after drug administration. The number of pituitaries analysed was, 8 for control and 500 μg bromocriptine, 4 for the other groups. Results are expressed as mean ± SE; ***, $p < 0.01$; ****, $p < 0.001$; vs. control.

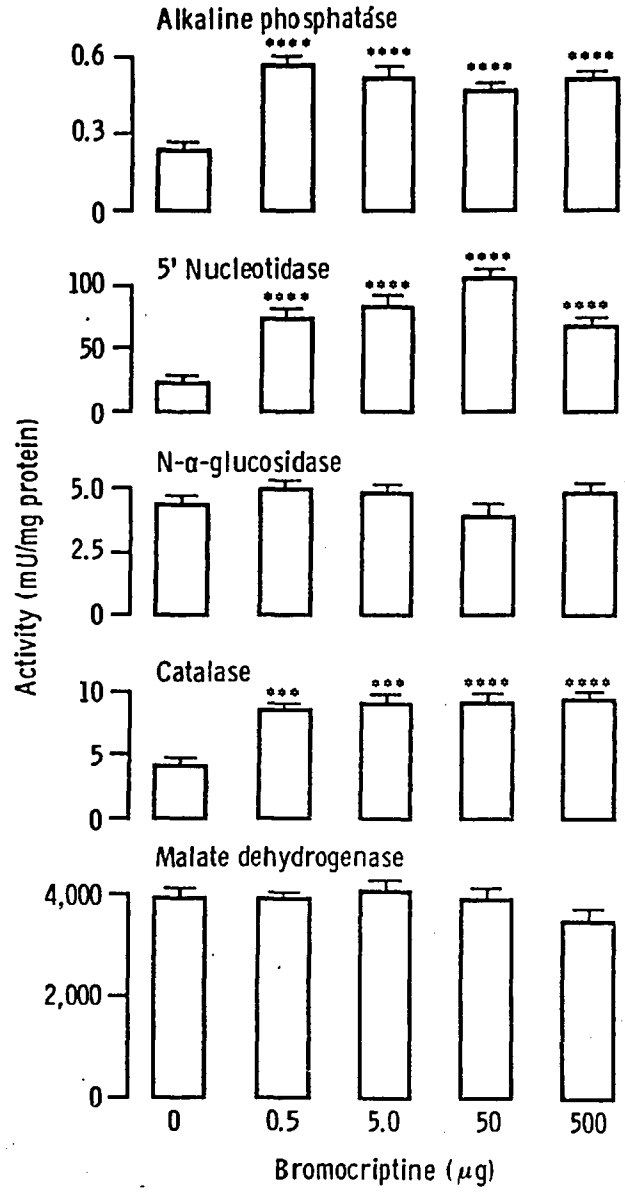


Fig. 3.24 Effect of varying doses of bromocriptine on the enzymic activity (mU/mg protein) of alkaline phosphatase, 5'nucleotidase (plasma membrane), neutral-α-glucosidase (endoplasmic reticulum), catalase (peroxisome) and malate dehydrogenase (mitochondria) in the pituitaries of lactating rats sacrificed 24 h after drug administration. The number of pituitaries analysed was, 8 for control and 500 μg bromocriptine, 4 for the other groups. Results are expressed as mean ± SE; ***, $p < 0.01$, ****, $p < 0.0001$, vs. control.

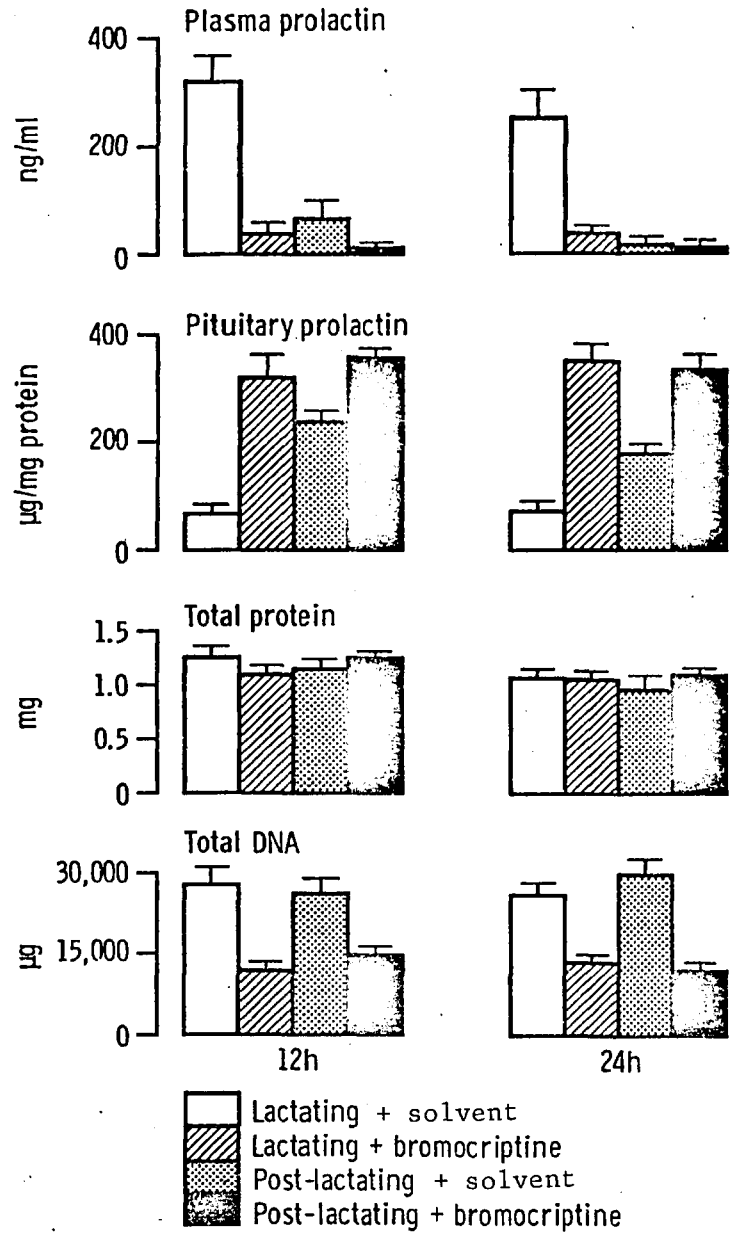


Fig. 3.25 Plasma and pituitary PRL, total pituitary protein and DNA in lactating and post-lactating rats 12 and 24 h after administration of 500 µg bromocriptine or solvent (150 mmol/l NaCl containing 5.5 mmol/l ethanol) compared to controls. Each group comprised 4 - 12 rats. Results are expressed as the mean ± SE.

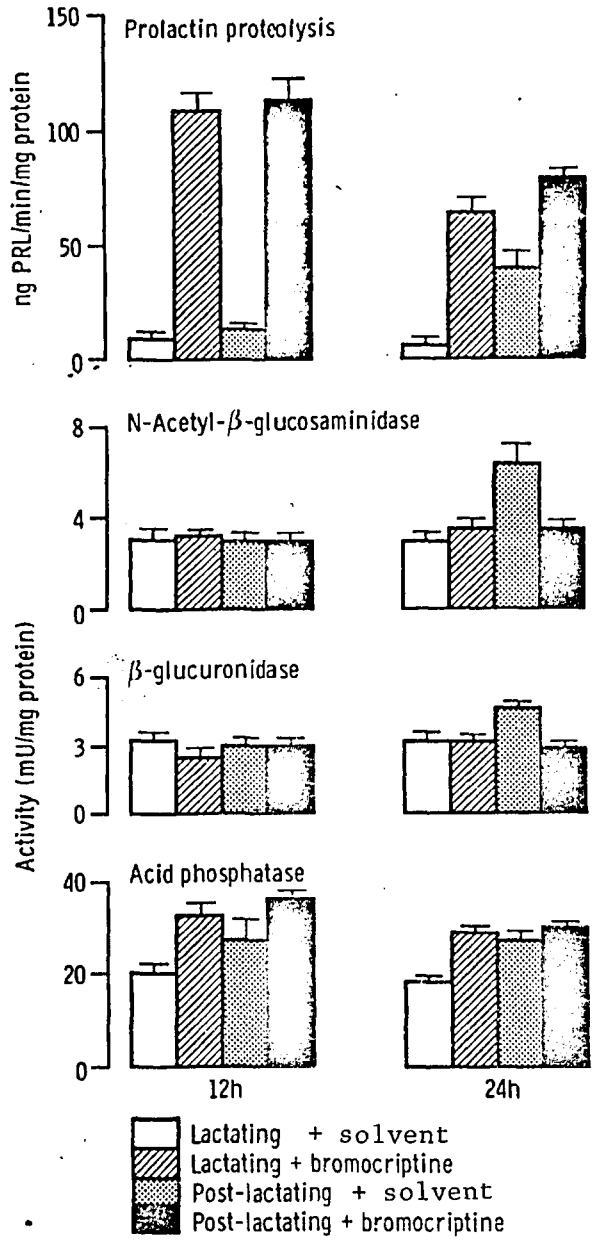


Fig. 3.26 Enzymic activities of lysosomal PRL protease (ng PRL degraded/mg protein/min), N-acetyl-β-glucosaminidase (mU/mg protein), β-glucuronidase (mU/mg protein) and the lysosomal/endoplasmic reticulum marker acid phosphatase (mU/mg protein) in the pituitaries of lactating and post-lactating rats 12 and 24 h after administration of 500 µg bromocriptine compared to controls given solvent (150 mmol/l NaCl containing 5.5 mmol/l ethanol). Each group comprised 4 - 12 rats. Results are expressed as the mean ± SE.

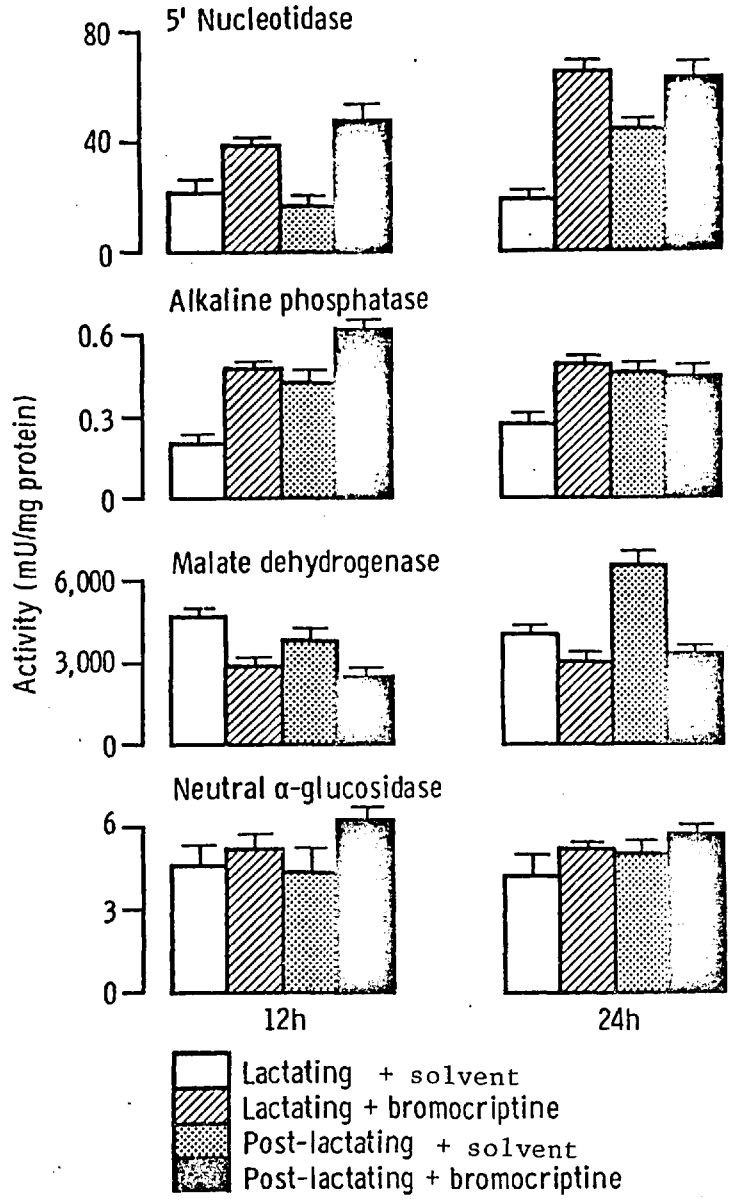


Fig. 3.27 Enzymic activities (mU/mg protein) of 5'nucleotidase, alkaline phosphatase (plasma membrane), malate dehydrogenase (mitochondria) and neutral-α-glucosidase (endoplasmic reticulum) in the pituitaries of lactating and post-lactating rats 12 and 24 h after administration of 500 µg bromocriptine compared to controls given solvent (150 mmol/l NaCl containing 5.5 mmol/l ethanol). Each group comprised 4 - 12 rats. Results are expressed as the mean ± SE.

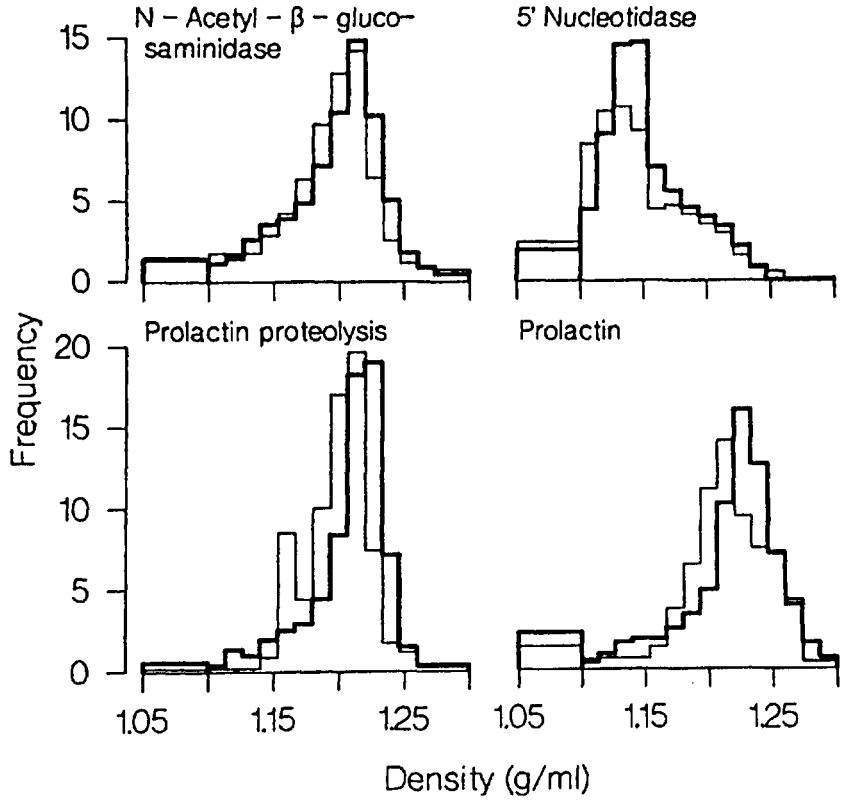


Fig. 3.28 Isopycnic centrifugation of 6000 x g-min supernatant homogenate of an anterior pituitary from a lactating rat treated with 500 µg bromocriptine (thin line). Average control from Fig. 3.1 is shown by the thick line. Details are as in Fig. 3.1. The percentage of activities recovered are: N-acetyl-β-glucosaminidase, 93; 5'nucleotidase, 70; prolactin proteolysis, 79; prolactin, 93.

glucosaminidase, prolactin proteolysis (lysosomes), 5' nucleotidase (plasma membrane) and prolactin granules are compared with that of lactating animals (Figure 3.28). The profile of all four did not show any significant difference to that obtained with pituitaries from lactating rats not receiving bromocriptine.

3.5 CHARACTERISATION OF HUMAN PITUITARY ORGANELLES IN NORMAL AND ADENOMATOUS TISSUE

3.5.1 Introduction

The study of human pituitary glands has relied largely on the application of various morphological techniques (Pasteels, Gausset, Dangui, Ectors, Nicoll and Varavudhi, 1972; Horvath and Kovacs, 1976; Pelletier, Robert and Hardy, 1978; Moriarty, 1973; Lewis and Van Noorden, 1974; Kovacs, Ryan, Horvath, Ezrin and Penz, 1978). Tissue dissociation techniques have been utilised to study the basal and modulated hormone secretion by normal anterior pituitary tissue (Bridson and Kohler, 1970; Adams, Brajkovich and Mashiter, 1981). In addition, secretion of pituitary tumours have also been under investigation by explant or isolated cell culture techniques (Kohler, Bridson, Rayford and Kohler, 1969; Peillon, Gourmelen, Dannadieu, Brandi, Sevaux and Pam Huu Trung, 1975; Mashiter, Van Noorden, De Marco, Adams and Joplin, 1979; Adams, Brajkovich and Mashiter, 1979; Mashiter, Adams, Gillies, Van Noorden and Ratter, 1980).

Having established methods for subcellular fractionation and organelle marker enzymes, and shown marked changes in a number of these, particularly those associated with lysosomes, a study was made of human pituitary tissue to seek information on the underlying pathology of hormone hypersecretion. To determine and characterise the subcellular localisation of the organelles of human pituitaries as well as obtain information about the activities of enzyme markers in normal and abnormal pituitary tissue, a combination of sucrose density centrifugation and biochemical determinations were utilised.

3.5.2 Results: Normal Pituitary Tissue

The results shown in this section describe the characterisation of the subcellular organelles and hormone-containing granules of three normal pituitaries by sucrose density centrifugation.

The density distribution of lysosomal, endoplasmic reticulum, mitochondria and cytosol marker enzymes is shown in Figure 3.29. The frequency distribution of N-acetyl-β-glucosaminidase showed a single peak at modal density 1.20 g/ml. β-Glucuronidase, another lysosomal enzyme, had a similar peak at 1.20 g/ml with a subsidiary peak at 1.13 g/ml. Acid phosphatase, a dual marker for lysosomes and endoplasmic reticulum, showed a broad distribution of activity with a modal density of 1.16 g/ml. The distribution of neutral α-glucosidase also showed a peak at modal density 1.16 g/ml with some activity in the soluble fraction.

Malate dehydrogenase showed a substantial amount of activity in the soluble fraction of the gradient and a peak activity at modal density 1.15 g/ml whereas lactate dehydrogenase, a cytosol marker enzyme, did not migrate into the gradient, being almost entirely located to the soluble fraction.

The distribution of plasma membrane and peroxisomal enzyme markers as well as four pituitary hormones is illustrated in Figure 3.30. 5'-Nucleotidase showed a sharp peak of activity at density 1.12 g/ml and catalase had most of the activity located to the soluble fraction. There was a considerable overlap between the density distributions of all four hormones assayed but they showed distinct distribution patterns. Prolactin showed a peak activity at modal density 1.23 g/ml with very little activity in the soluble fraction. Growth hormone showed activity localised to the soluble fraction as well as a relatively sharp peak at modal densities 1.24 to 1.26 g/ml. Luteinising hormone (LH) showed the activity localised to densities 1.22 to 1.26 g/ml whereas follicle-stimulating hormone (FSH) was also located at those densities but presented with more activity in the soluble fraction of the gradient.

3.5.3 Results: Prolactin-Secreting Adenomas

Analytical subcellular fractionation of five separate prolactin-secreting adenomas yielded the data illustrated in Figures 3.31 to 3.33 and the results are compared with the distribution of the organelles previously described for the

normal tissue. Similar distributions were obtained for most of the subcellular organelle marker enzymes with the exception of β-glucuronidase (Figure 3.31), a lysosomal marker, which showed a broad activity between densities 1.15 and 1.21 g/ml, in contrast to normal tissue which showed two distinct peaks. In addition, the median density of 5'-nucleotidase was slightly increased in the prolactin-secreting tumours and the median density of neutral α-glucosidase showed a shift to a ligher density distribution (Figure 3.32). Human prolactin protease, an enzyme marker described in detail in Section 3.7, showed a sharp peak of activity at densities 1.20 to 1.22 g/ml but control studies were not performed. Growth hormone was found in three of the five adenomas subjected to density fractionation, with a modal density of 1.24 g/ml and most probably reflected contamination with normal tissue.

3.5.4 Results: Growth Hormone-Secreting Adenomas

Three growth hormone-secreting adenomas from acromegalic patients were subjected to subcellular fractionation. One of these pituitary adenomas was unusual as the patient had a relatively low serum growth hormone but with a large tumour and gross facies of an acromegalic. The pituitary homogenate from this patient was subjected to density fractionation and showed a different profile for the growth hormone-containing granules, as well as very low secretion of the hormone in the cell culture system. In addition, morphological studies showed

several autophagic vacuoles surrounded by hormone granules. This case will be discussed further in Section 3.8.

The distribution profile of lysosomal, endoplasmic reticulum, mitochondria and cytosol marker enzymes in the density gradient fractions is illustrated in Figure 3.34 and is compared with that of normal pituitaries. N-Acetyl-β-glucosaminidase showed a peak activity at modal density 1.20 g/ml with somewhat less activity in the soluble fraction. β-Glucuronidase, another lysosomal marker, showed a bimodal distribution, with peak activities present at densities 1.13 and 1.20 g/ml, very similar to normal pituitary tissue. Acid phosphatase had more activity in the soluble fraction when compared with normal tissue but showed a broad distribution of activity in the sucrose density fractions, whereas neutral α-glucosidase showed a sharper peak of activity at modal density 1.15 g/ml than control. Malate dehydrogenase was localised at modal density 1.17 g/ml with activity present in the soluble fraction, as in normal tissue, and lactate dehydrogenase was entirely located to the soluble fraction.

Figure 3.35 shows the distribution profile of plasma membrane and peroxisome marker enzymes as well as hormone-containing granules. 5' Nucleotidase showed a denser distribution pattern to that found in normal pituitary tissue with a modal density of 1.15 g/ml. Catalase was largely recovered in the soluble fraction for both patient groups. Growth hormone was found to have a density distribution very similar to that found in normal tissue, at modal density 1.24 g/ml.

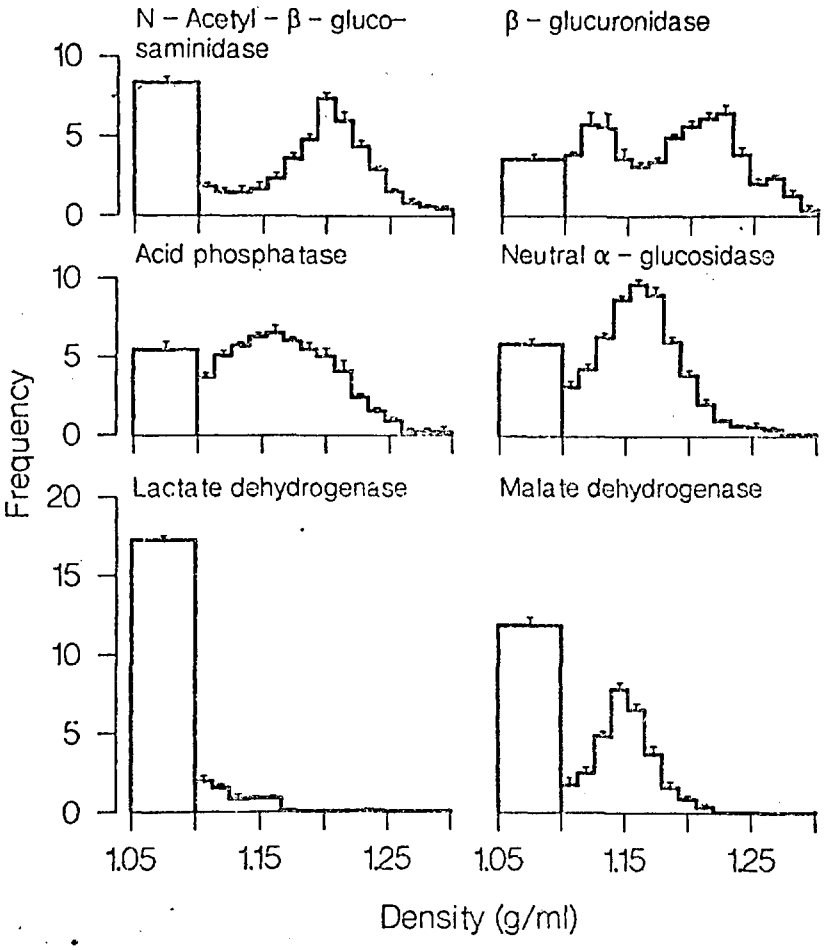


Fig. 3.29 Isopycnic centrifugation of 6000 x g-min supernatant from normal human anterior pituitaries. Details as in Fig. 3.1. The mean percentage of recovered activities are: N-acetyl-β-glucosaminidase, 122 (3); β-glucuronidase, 96 (3); acid phosphatase, 128 (3); neutral α-glucosidase, 89 (3); lactate dehydrogenase, 79 (3); malate dehydrogenase, 116 (3).

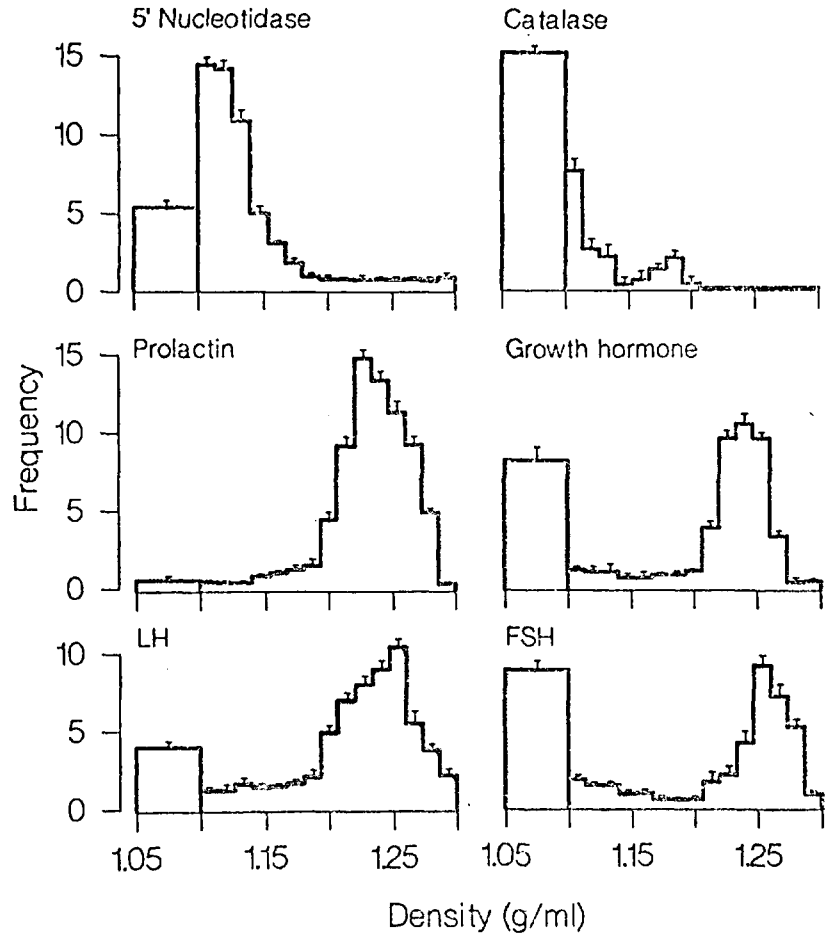


Fig. 3.30 Isopycnic centrifugation of 6000 x g-min supernatant from normal human anterior pituitaries. Details as in Fig. 3.1. The mean percentage of recovered activities are: 5'nucleotidase, 80(3); catalase, 89(3); PRL. 109(3); GH, 69(3); LH, 77(3); FSH, 85(3).

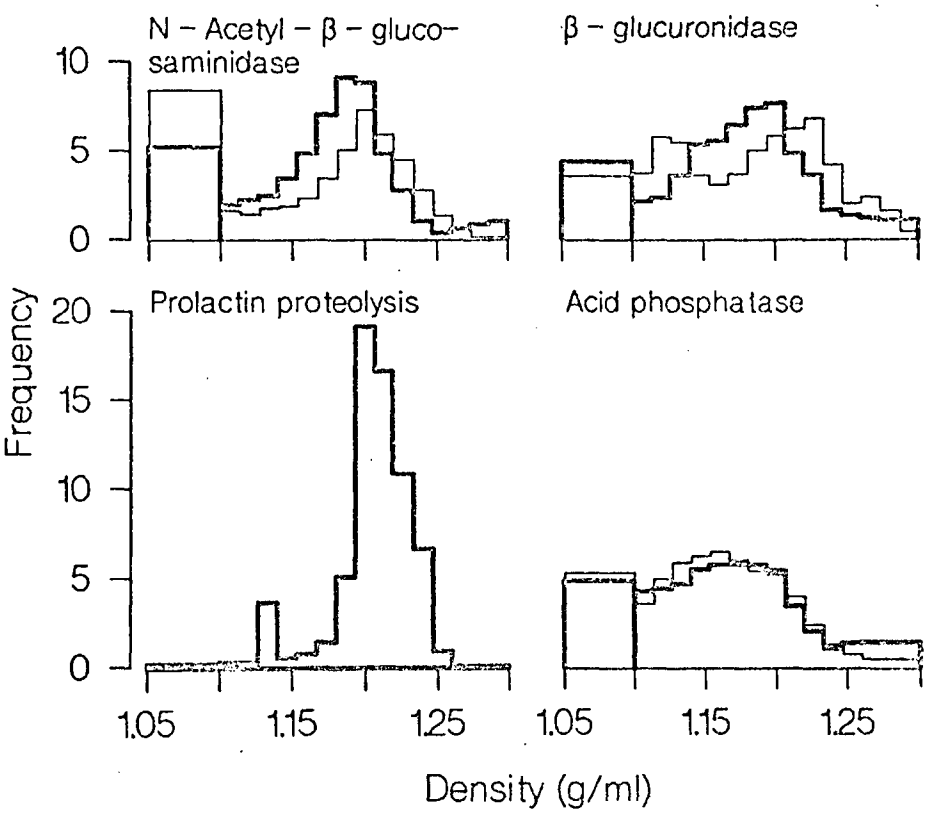


Fig. 3.31 Isopycnic centrifugation of 6000 x g-min supernatant of human anterior pituitaries from patients with prolactin-secreting adenomas (thick line) as compared with normal anterior pituitaries (thin line). Details as in Fig. 3.1. The mean percentage of recovered activities are: N-acetyl-β- glucosaminidase, 91(5); β -glucuronidase, 86(5); prolactin proteolysis, 115(2); acid phosphatase, 79(4).

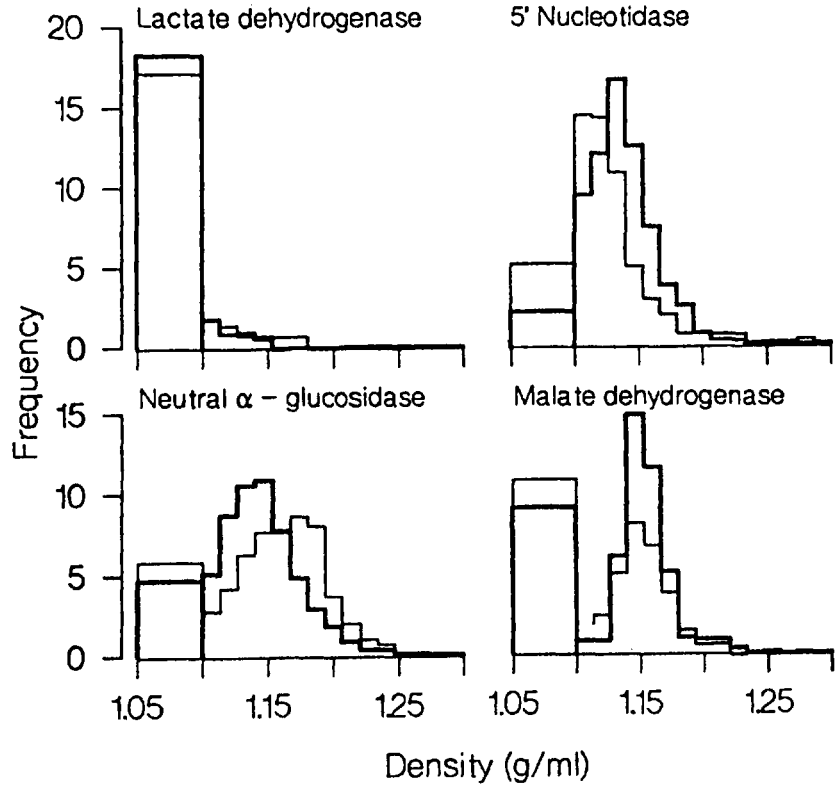


Fig. 3.32 Isopycnic centrifugation of 6000 x g-min supernatant of human anterior pituitaries from patients with prolactin-secreting adenomas (thick line) as compared with normal anterior pituitaries (thin line). Details as in Fig. 3.1. The mean percentage of recovered activities are: lactate dehydrogenase, 99(4); 5'nucleotidase, 107(5); neutral α-glucosidase, 88(5); malate dehydrogenase, 101(4).

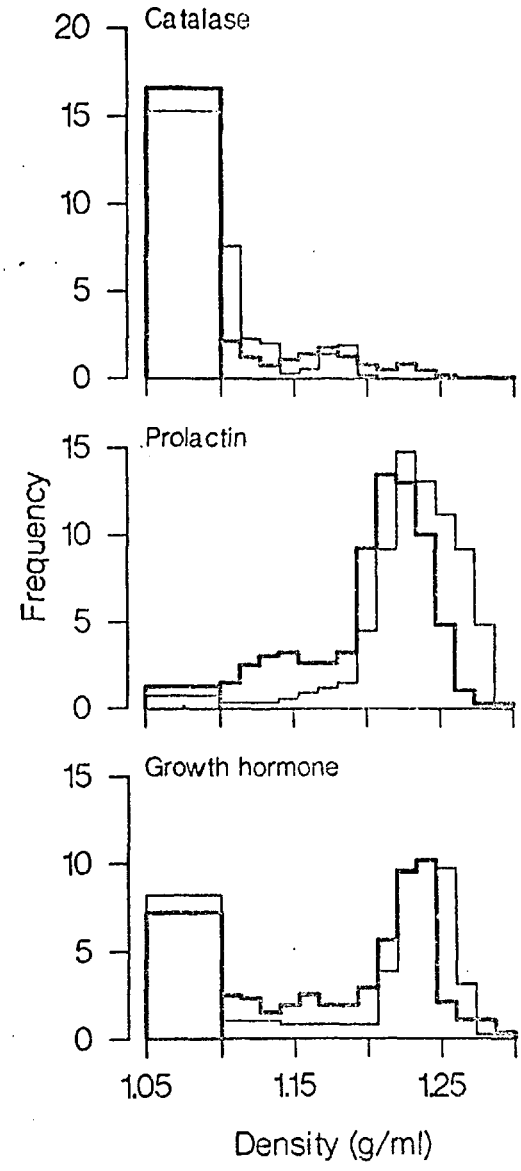


Fig. 3.33 Isopycnic centrifugation of 6000 x g-min supernatant of human anterior pituitaries from patients with prolactin-secreting adenomas (thick line) as compared with normal anterior pituitaries (thin line). Details as in Fig. 3.1. The mean percentage of recovered activities are: catalase, 73(3); prolactin, 92(5); growth hormone, 82(2).

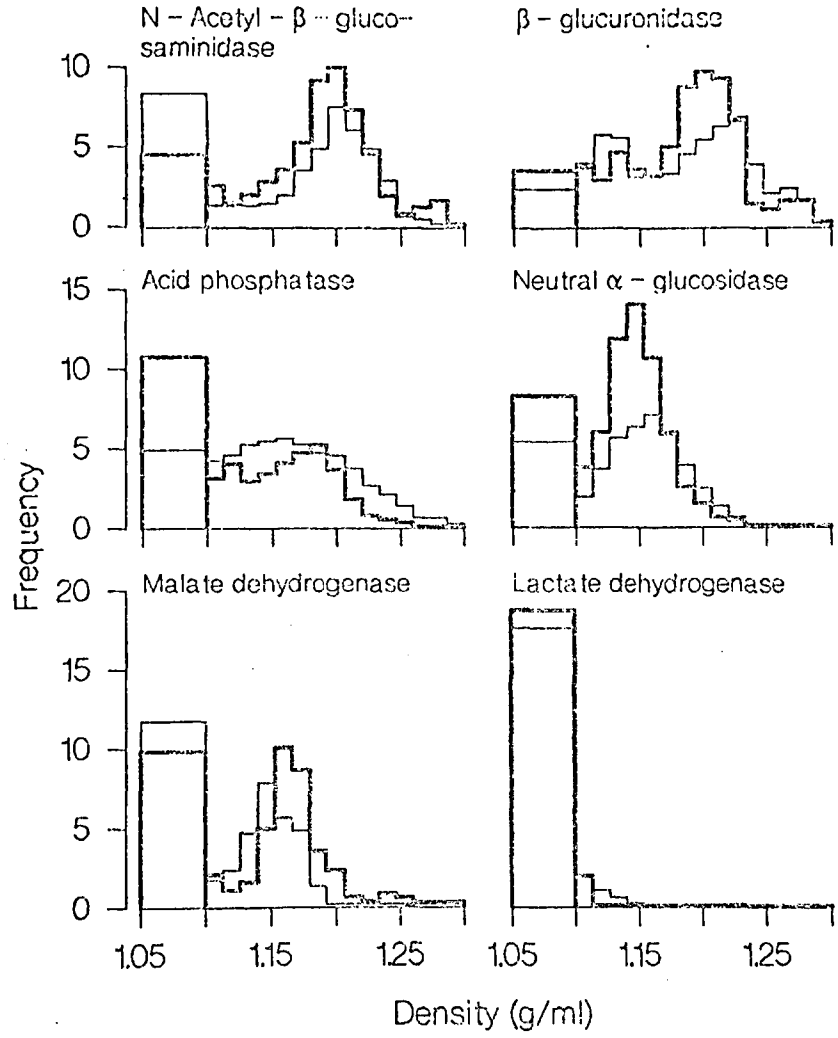


Fig. 3.34 Isopycnic centrifugation of 6000 x g-min supernatant of human anterior pituitaries from patients with growth hormone-secreting adenomas (thick line) as compared with normal anterior pituitaries (thin line). Details as in Fig. 3.1. The mean percentage of recovered activities are: N-acetyl- β-glucosaminidase, 76(3); β-glucuronidase, 77(2); acid phosphatase, 116(3); neutral α-glucosidase, 113(3); malate dehydrogenase, 78(3); lactate dehydrogenase, 69(3).

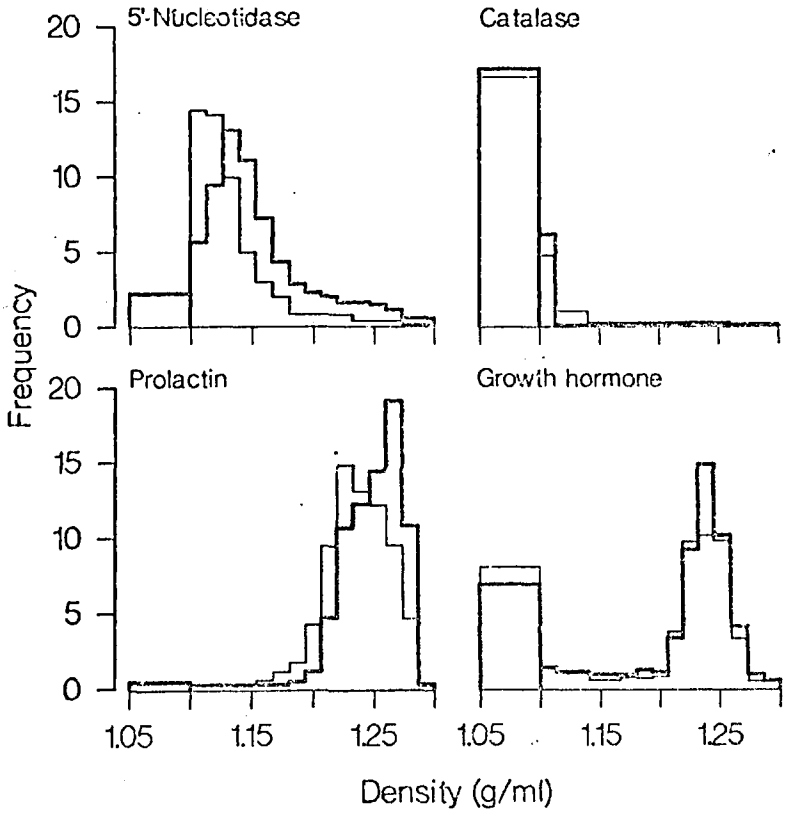


Fig. 3.35 Isopycnic centrifugation of 6000 x g-min supernatant of human anterior pituitaries from patients with growth hormone-secreting adenomas (thick line) as compared with normal anterior pituitaries (thin line). Details as in Fig. 3.1. The mean percentage of recovered activities are: 5'nucleotidase, 79(3); catalase, 65(2); prolactin, 79(2); growth hormone, 67(2).

The presence of prolactin-containing granules probably reflected contamination with normal tissue, although showing a small shift to a more dense distribution with peak activity now found at modal density 1.27 g/ml.

3.6 ENZYME ACTIVITIES AND HORMONE CONTENT IN HUMAN PITUITARY TISSUE HOMOGENATES

The results described in this section record the activities of several enzymes and the hormonal content of seven normal pituitaries, ten prolactin-secreting adenomas, fourteen growth hormone-secreting adenomas and eight functionless pituitary tumours. The individual data for the enzyme activities, hormone content and pre-operative serum levels in the patients are shown in Appendices 1 to 8.

Analysis of their mean hormonal content (Appendices 1 to 8) showed that, although the tumourous hormone predominates, a number of the specimens contained other hormones, indicating some degree of contamination with normal pituitary tissue. This was confirmed by immunocytochemistry and cell culture studies performed by other members of this laboratory. Varying amounts of each hormone assayed were found in all seven normal pituitary homogenates (Appendix 1). Pre-operative serum levels of these patients were unobtainable.

Appendix 2 shows the hormone content for the ten prolactin-secreting adenomas. Prolactin values ranged from 1,990 ng/mg protein to 21,500 ng/mg protein. In addition, some growth hormone was detected in most of these adenomas, with values ranging from 103 to 17,400 ng/mg protein. No growth hormone was found in tumours 11 and 15. The only abnormality in the serum of all patients with prolactin-secreting adenomas was high prolactin levels, as reported by the doctor-in-charge (range 117 to 6,600 ng/ml).

Hormone content for the fourteen growth hormone-secreting adenomas is shown in Appendix 3. Growth hormone content varied widely, ranging from 850 to 265,000 mIU/mg protein. The majority of these adenomas contained only growth hormone, with little prolactin, LH or FSH present. Pre-operative serum levels of the patients showed some to have a raised serum prolactin (range 6 to 75 ng/ml) further to the expected rise in serum growth hormone, which ranged from 7 to 183 mIU/ml. The raised serum prolactin was probably due to tumour compression of the pituitary stalk.

Patients with functionless tumours presented with pre-operative serum levels within the normal limits. In some cases, no values were given, but the doctor's letter stated that the levels were normal. Patient 37 was the only exception, as the serum FSH levels were high, consistent with her being post-menopausal; however, her serum LH was inappropriately low. The tumour of patient 36 only contained GH yet the patient was

not acromegalic and had a normal serum LH. Otherwise, the pituitary hormonal content of these functionless tumours varied widely and often contained LH and FSH, however, the frequent presence of GH could probably represent contamination with normal pituitary.

Enzymic activities for lysosomal, endoplasmic reticulum, mitochondrial, peroxisomal and cytosolic organelles in normal pituitary tissue are shown in Figure 3.36 and these are compared with the enzyme activities of prolactin-secreting adenomas, growth hormone-secreting adenomas and functionless tumours.

Activity of the lysosomal marker enzyme N-acetyl-β-glucosaminidase was significantly reduced in the prolactin-secreting adenomas. Although the activity of this enzyme in the functionless tumours was not different to that of normal tissue, it was significantly higher when compared with either prolactin or growth hormone-secreting adenomas. Another lysosomal marker enzyme, β-glucuronidase, also had significantly reduced activity in prolactin and growth hormone-secreting adenomas. The activity in functionless tumours was also decreased but did not reach statistical significance. Cathepsin C, a further lysosomal marker, and neutral α-glucosidase, an endoplasmic reticulum marker, did not show any difference between the four groups. The activity of acid phosphatase, a dual marker for lysosomes and endoplasmic reticulum, was significantly reduced in the growth hormone-secreting adenomas when compared with normal pituitary tissue. Neither the activities of catalase, malate dehydrogenase nor lactate dehydrogenase showed any statistical significant difference between the four groups.

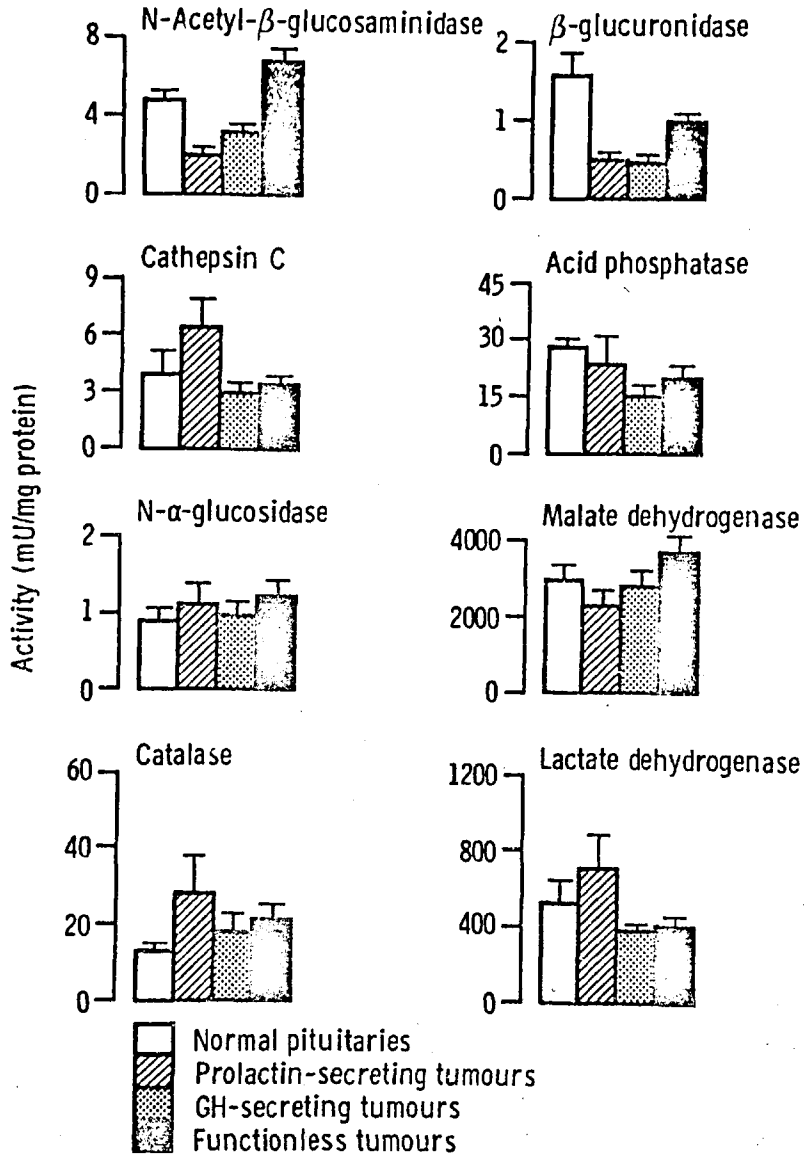


Fig. 3.36 Pituitary enzyme activities in 'normal' tissue, PRL-secreting adenomas, GH-secreting adenomas and functionless tumours.

3.7 HUMAN PROLACTIN PROTEASE

Having described in Section 3.2 the existence of a rat acid prolactin protease capable of degrading prolactin, an assay for human prolactin protease was also performed with both normal and tumourous human pituitary tissue obtained during neurosurgical exploration. The procedure was identical to that for rat prolactin protease assay. A single peak of human prolactin degrading activity occurred at pH 4.5 in the prolactin-secreting adenomas, normal pituitary tissue and functionless tumours (Figure 3.37). At this optimal pH of 4.5 and after a lag period of approximately 8 min, the degradation of prolactin from prolactin-secreting adenoma homogenates proceeded linearly with time up to 3 h (Figure 3.38). The initial lag period is probably due to the time required for a number of consecutive proteolytic attacks to be made on the protein moiety of prolactin before it was broken down to peptides small enough to be soluble in trichloroacetic acid. The proteolytic degradation of ^{125}I-labelled human prolactin was not linear in relation to the concentration of pituitary cell homogenate for less than 20 µg tissue protein (Figure 3.39). In addition, it is noteworthy to show that the activity of human prolactin protease is about 200-fold less than that of rat prolactin protease and this difference could be due to the very small amount of enzyme present in the human pituitary homogenate. This, taken in the context of lower activities of the other lysosomal marker enzymes, N-acetyl-β-glucosaminidase and β-glucuronidase, may be of significance in explaining the hormone hypersecretion of human pituitary tumours.

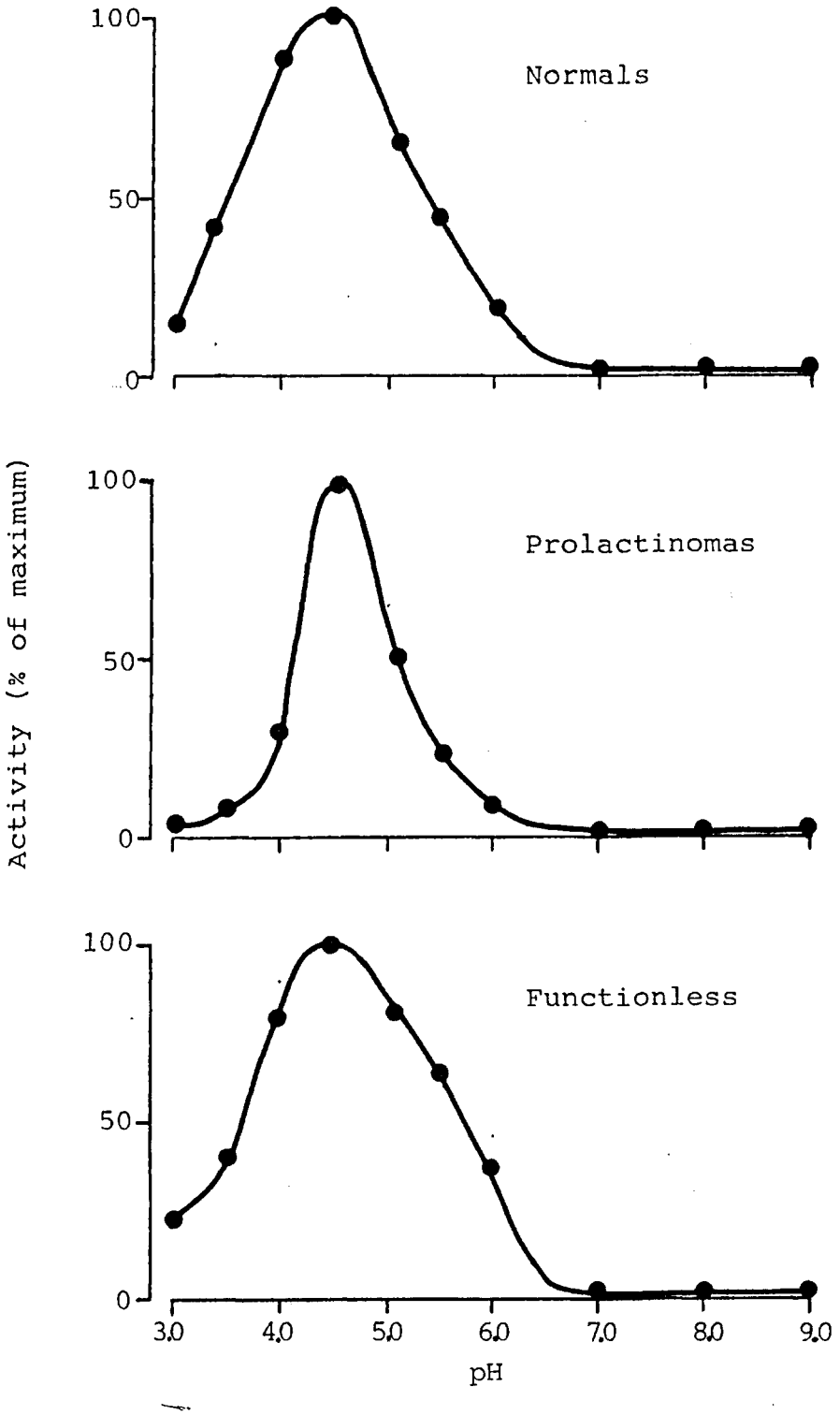


Fig. 3.37 pH dependence of human PRL degradation. ^{125}I-PRL was incubated at various pH values for 90 min with either sucrose medium (blank) or homogenates from normal pituitaries, PRL-secreting adenomas and functionless tumours. A sodium acetate-acetic acid buffer (final concentration 0.1mol/l) was used between pH 3 and 6 and a Tris-HCl buffer between pH 6 and 9.

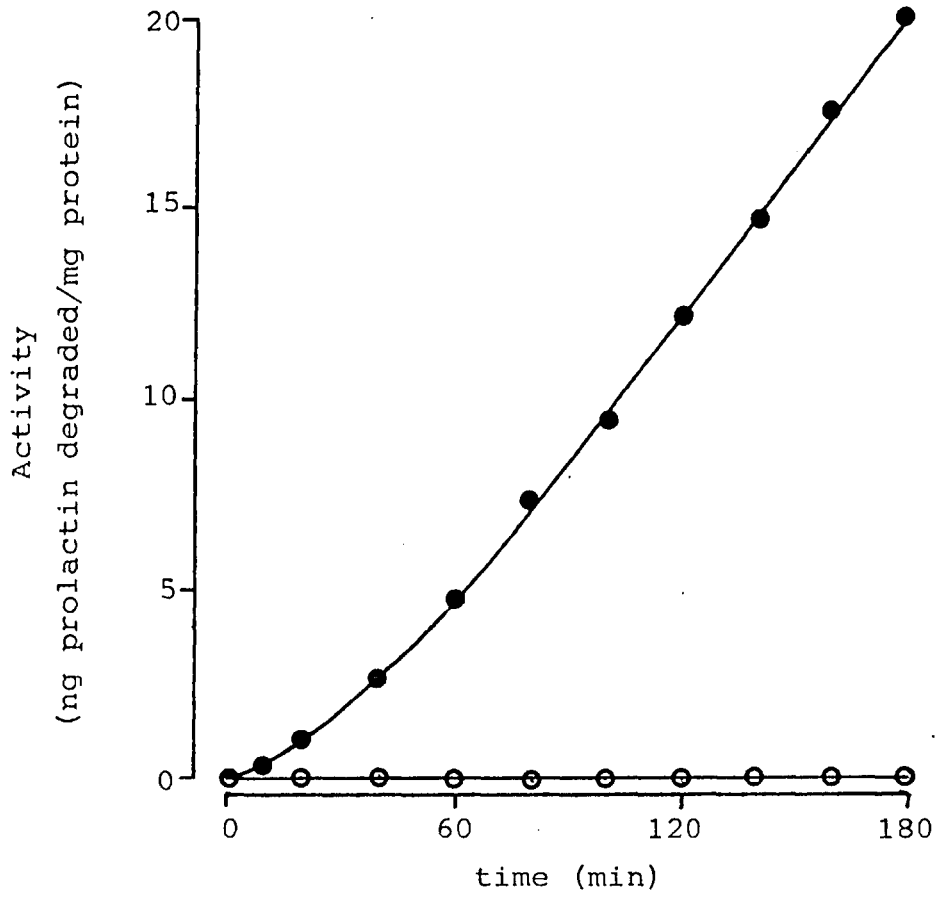


Fig. 3.38 Variation with time of the rate of degradation of human PRL

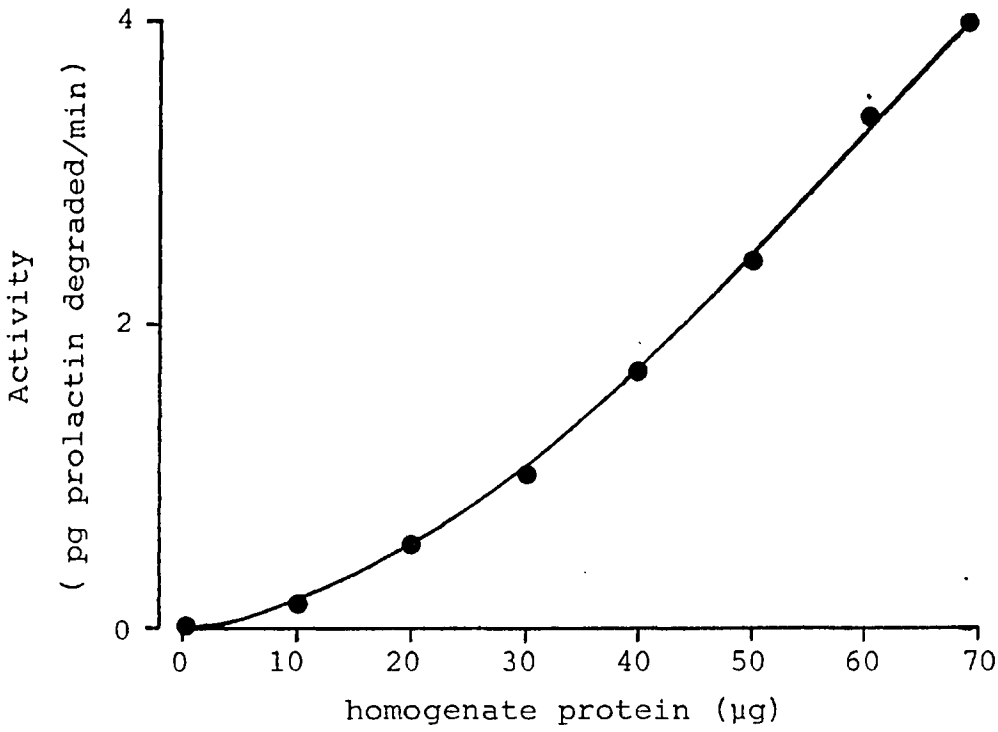


Fig. 3.39 Variation of activity with amount of human pituitary homogenate protein.

3.8 CASE REPORT: INAPPROPRIATELY LOW SERUM GH IN AN ACROMEGALIC- LYSOSOMAL INVOLVEMENT IN INTRACELLULAR HORMONE DEGRADATION

The patient, a 29-year-old female, was referred to Hammersmith Hospital with a history of 2 years of oligomenorrhoea followed by amenorrhoea for one year. She also complained of lethargy, frequent headaches, joint stiffness, excessive sweating, and enlarging extremities. Her shoe size had increased from 5½ to 6½ and she was no longer able to wear her wedding ring. She had gained 19 kg in the previous 2 years and had spontaneous galactorrhoea for 4 years. There was retrosternal pain on exertion that was relieved by propranolol.

On examination, she had obvious acromegalic features: a large jaw, hands and feet. Her skin was thickened and sweaty and there was facial acne. She was clinically euthyroid. Coned views of the pituitary fossa and an air encephalogram with tomography showed a balloned fossa with an intact lamina dura that contained a solid tissue mass with a dome-shaped bulge above the sella. Fossa area was 378 mm^2.

The patient was treated by interstitial irradiation with an implant of a single seed of ^{90}Yttrium (50,000 rads at the tumour periphery), at which time a needle aspiration biopsy was obtained for pathology. The tumour texture was considered normal. One year later there was a marked improvement in her condition. There was a significant reduction in her sweating and lethargy, regression of the acromegalic facial appearance, and reduction in foot size. Galactorrhoea had ceased and her periods resumed; however, corticosteroid hormone replacement was required.

Prior to, and 1 year after treatment, pituitary function was tested (Harsoulis, Marshall, Kuku, Burke, London and Fraser, 1973). Serum GH levels were assessed during a 50 g GTT and the mean of the values at 60, 90 and 120 min will be reported. Serum GH,LH,FSH,TSH and PRL were measured by specific radioimmunoassay as previously described (Hartog, Gaafar, Meisser and Fraser, 1964; Marshall, Anderson, Burke, Galvao-Teles and Russel Fraser, 1972; Marshall, Anderson, Fraser and Harsoulis, 1973; Kuku, Harsoulis, Young and Fraser, 1974; Gwee and Mashiter,1978) and cortisol by a competitive protein binding assay (Beardwell, Burke and Cope, 1968).

3.8.1 Results

(i) Serum hormones

Pre-operatively, the basal serum GH was raised at 22.5 mIU/l and did not suppress with glucose (Table 2.5). However, since a relationship between tumour size and serum GH in an acromegalic patients has been suggested previously (Wright, McLachlan, Doyle and Fraser, 1969), this level was inappropriately low. Prolactin was also raised basally and could be further stimulated by thyrotrophin-releasing hormone (TRH). Basal gonadotrophins were reduced but responded adequately to gonadotrophin-releasing hormone (LHRH). The patient was biochemically euthyroid with a serum T_3 of 2.3 nmol/l and serum T_4 of 113 nmol/l, and serum TSH responded normally to TRH. Basal serum cortisol levels were normal, although the response to hypoglycaemia may have been blunted by propanolol therapy.

One year post-operatively, the serum GH level was normal and suppressed to undetectable levels with oral glucose. Basal PRL was also normal, as was the TSH response to TRH. Gonadotrophin responses

were somewhat reduced and cortisol levels below normal.

(ii) Immunocytochemistry

The adenoma tissue stained positively for GH but not for any of the other anterior pituitary hormones (Fig. 3.40). Most of the cells showed sparse positive granules suggesting a low intracellular GH content. No staining was present when the antibody was pre-incubated with excess GH (Fig. 3.41).

(iii) Tissue culture

Only GH was secreted by the adenoma tissue in dispersed cell culture, confirming this to be composed solely of somatotrophs. The amount of GH secreted was extremely low when compared (Fig. 3.42) with that from the same number of cells of similarly treated adenomas removed from 5 other unselected active acromegalics whose serum GH ranged from 149 - 750 mIU/l and less than that from normal pituitary cells, only half of which are likely to be somatotrophs.

(iv) Subcellular fractionation and tissue hormone content

The activity of the lysosomal marker enzyme N-acetyl-β-glucosaminidase was markedly increased in the homogenate from the patient's adenoma (4.9 mU/mg protein) when compared to that of 8 adenomas obtained from other active acromegalics (2.5 ± 0.3 mU/mg protein). Acid phosphatase activity in the patient's tumour was not different from the other eight. The level of radioimmunoassayable GH (4,400 mIU/mg protein) in the patient's adenomas was low, being less than 20% of that found in 5 normal pituitaries (22,500 ± 10,500 mIU/mg protein) and less than 4% of that found in eight

similarly studied functioning acromegalic adenomas (131,000 ± 30,900 mIU/mg protein).

Subcellular fractionation (Fig. 3.43) showed that the distribution of lysosomes as indicated by the marker enzyme N-acetyl--glucosaminidase on the density gradient for the patient was the same as the average of the other two tumours (modal density 1.17 g/ml). However, there was a marked difference in the distribution of the GH granules such that in the 2 control adenomas these were localised at a modal density of 1.26 g/ml, clearly separated from the lysosomes. In contrast, the patient's GH granules were located at a modal density of 1.17 g/ml, which was identical with that of the lysosomes.

(v) Electron microscopy

The cells of the patient's solid adenoma were usually regular and rounded, with cytoplasm of variable electron density (Fig. 3.44). Nuclei were rounded, with prominent nucleoli, and occasional giant cells with two or three nuclei were seen. Secretory granules were sparse and small (89 - 100 nm diameter) when compared to those found in normal somatotrophs (300 - 400 nm diameter).An unusual feature was the presence of numerous intracellular profiles of low electron density, enclosed in a double membrane and occasionally containing secretory granules and recognizable parts of mitochondria (Fig. 3.45). The profiles were nearly always completely surrounded by secretory granules apparently fused with the outer membrane. These structures were tentatively identified as autophagic vacuoles (secondary lysosomes).

Table 2.5

Serum hormones

	Pre-implant		Post-implant (1 year)	
Hormone and test	Basal	Response	Basal	Response
GH during 50 g GTT	22.5 (<5mIU/1)*	19.5 (<5mIU/1)	<0.5	<0.5
Cortisol during ITT (0.1 U insulin kg/ body wt.)	287 (100-500 nmol/1)	281 (>500 nmol/1)	<90	N.T. +
LH response to 100 µg GnRH I.V.	1.0 (3-8 U/1)	17.0 (4-34 U/1)	3.5	6.6
FSH response to 100 µg GnRH I.V.	2.0 (2-8 U/1)	8.0 (2-11 U/1)	4.6	5.9
TSH response to 200 µg TRH I.V.	1.2 (< 4 mU/1)	8.9 (4-20 mU/1)	1.0	8.5
Prolactin response to 200 µg TRH I.V. (pre-implant) 10 mg metoclopramide I.V. (post-implant)	40 (5-25 µg/1)	90 (20-105µg/1)	11.7	2.9

* Normal values in paretheses
\+ Not tested

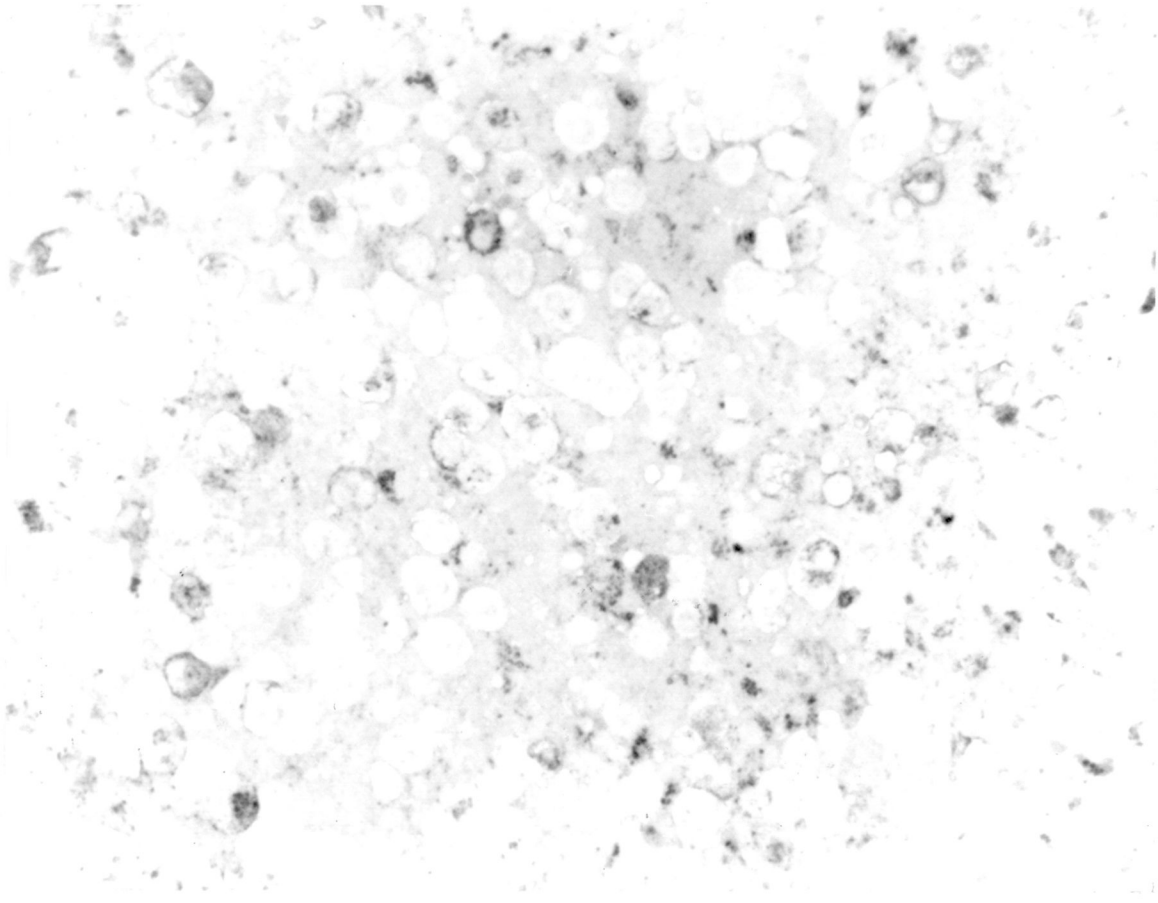


Fig. 3.40 Immunostain (PAP) for growth hormone showing positive immunoreactivity 1/16,000. Nuclei lightly counter stained with haematoxylin; 0.7 µm section of formalin-fixed, resin-embedded (x400).

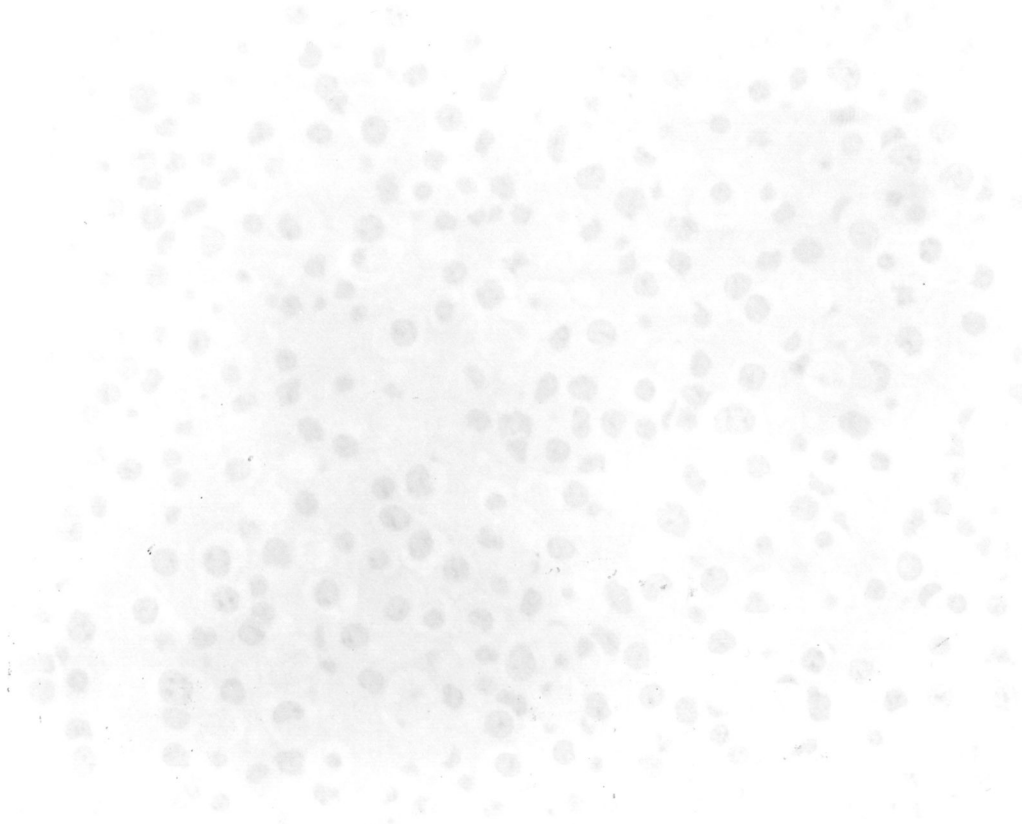


Fig. 3.41 Immunostain (PAP) for growth hormone showing negative staining when the antibody was pre-absorbed with excess GH. Nuclei lightly counter stained with haematoxylin; 0.7 µm section of formalin-fixed, resin-embedded tissue. (x400).

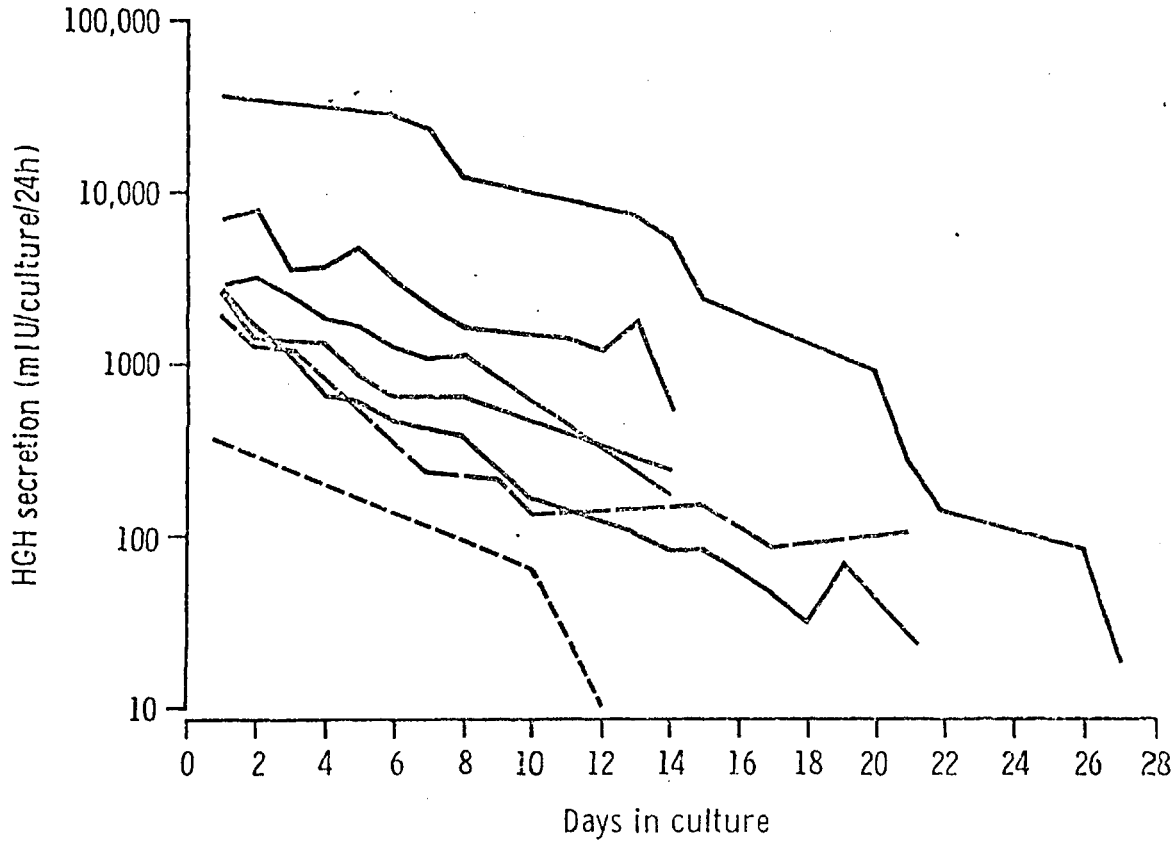


Fig. 3.42 GH secretion by cultures of 2 x 10^5 dispersed cells of adenomas from 5 acromegalics (———), a normal pituitary (- - - -) and the adenoma from the acromegalic under investigation (— ——). Cell viability was > 95% (trypan blue exclusion). The results are the means of 2-10 replicates. Culture medium was changed every 1-3 days and the total hormone secreted per 24 h plotted against time in culture.

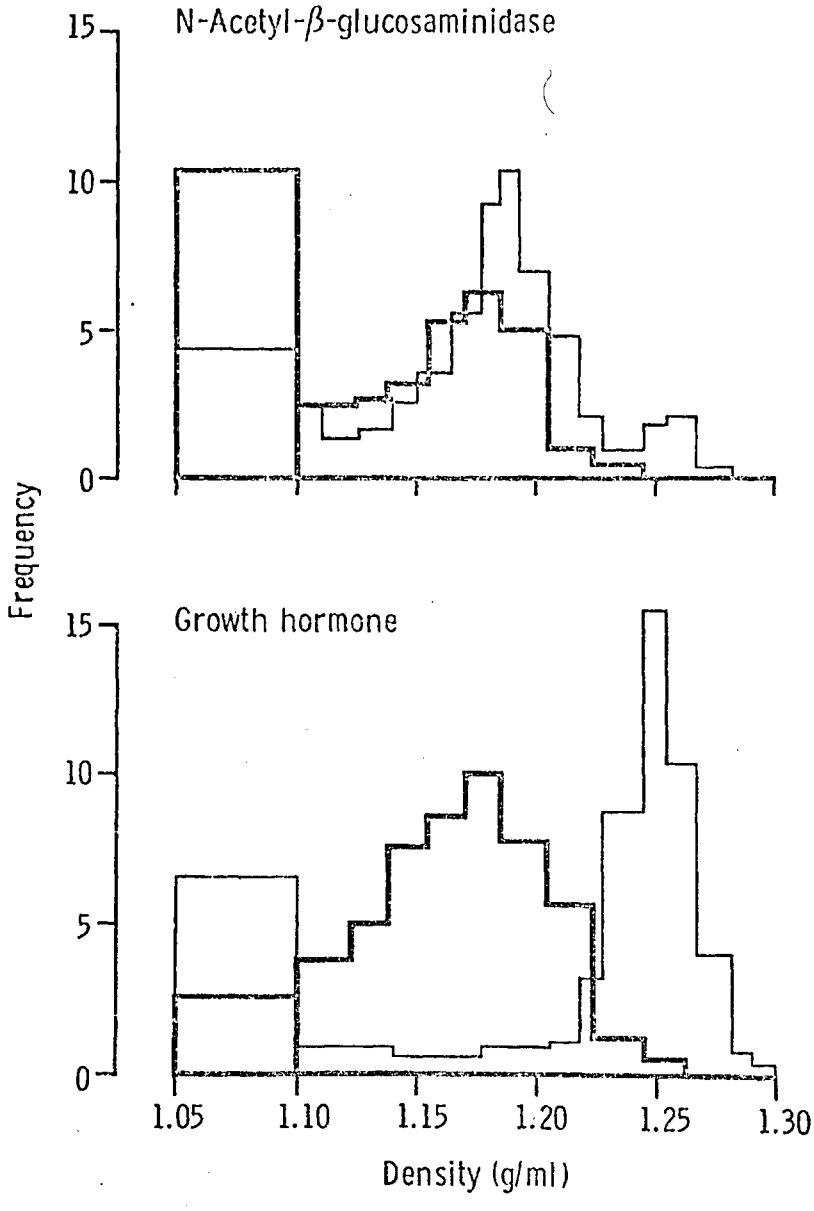


Fig. 3.43 Isopycnic centrifugation of 6000 x g-min supernatant of pituitary biopsy homogenate from the patient (——) and from another 2 patients with the typical clinico-pathological features of acromegaly (———). Details are as in Fig. 3.1.

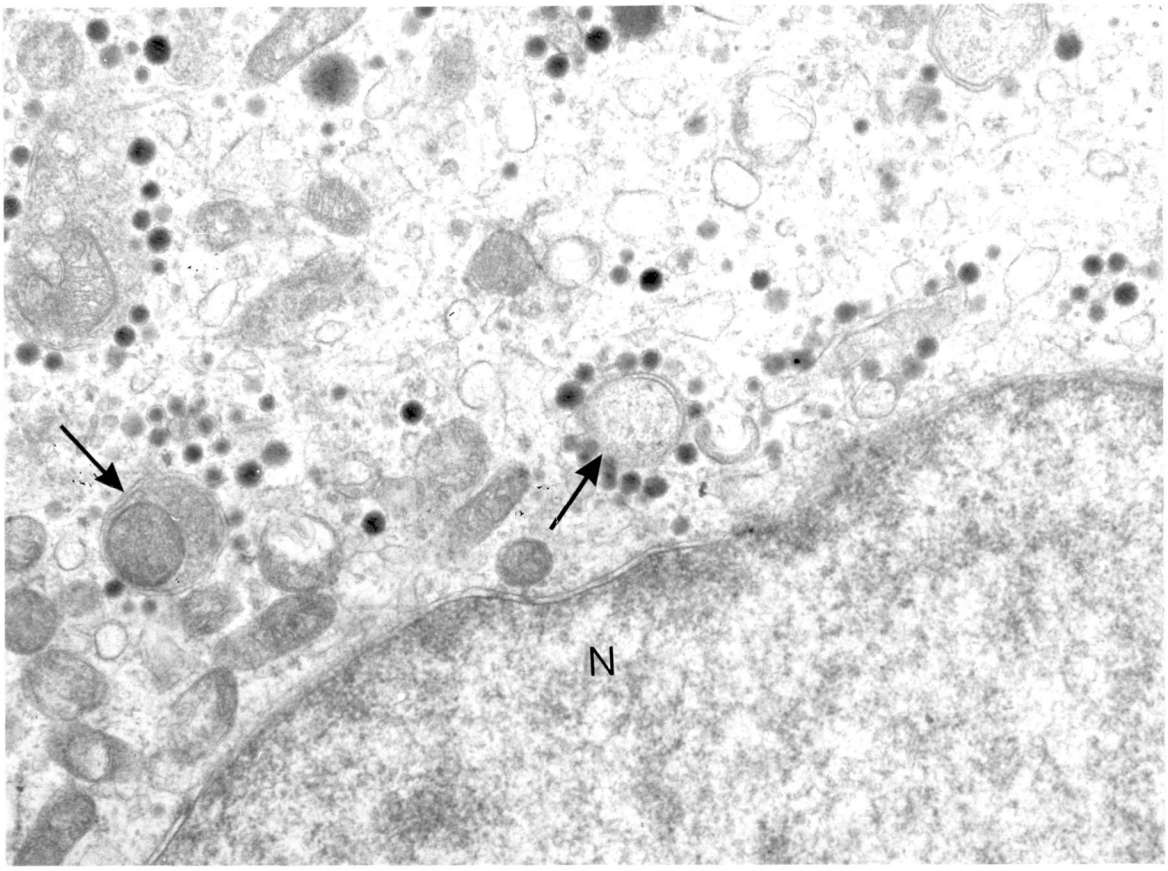


Fig. 3.44 Electron micrograph of a part of a tumour cell showing structures tentatively identified as autophagic vacuoles (arrows). They are surrounded by secretory granules and some contain cytoplasmic organelles. Glutaraldehyde/osmium tetroxide fixation. Stained with lead citrate and uranyl acetate. (x 18,750). N, nucleus.

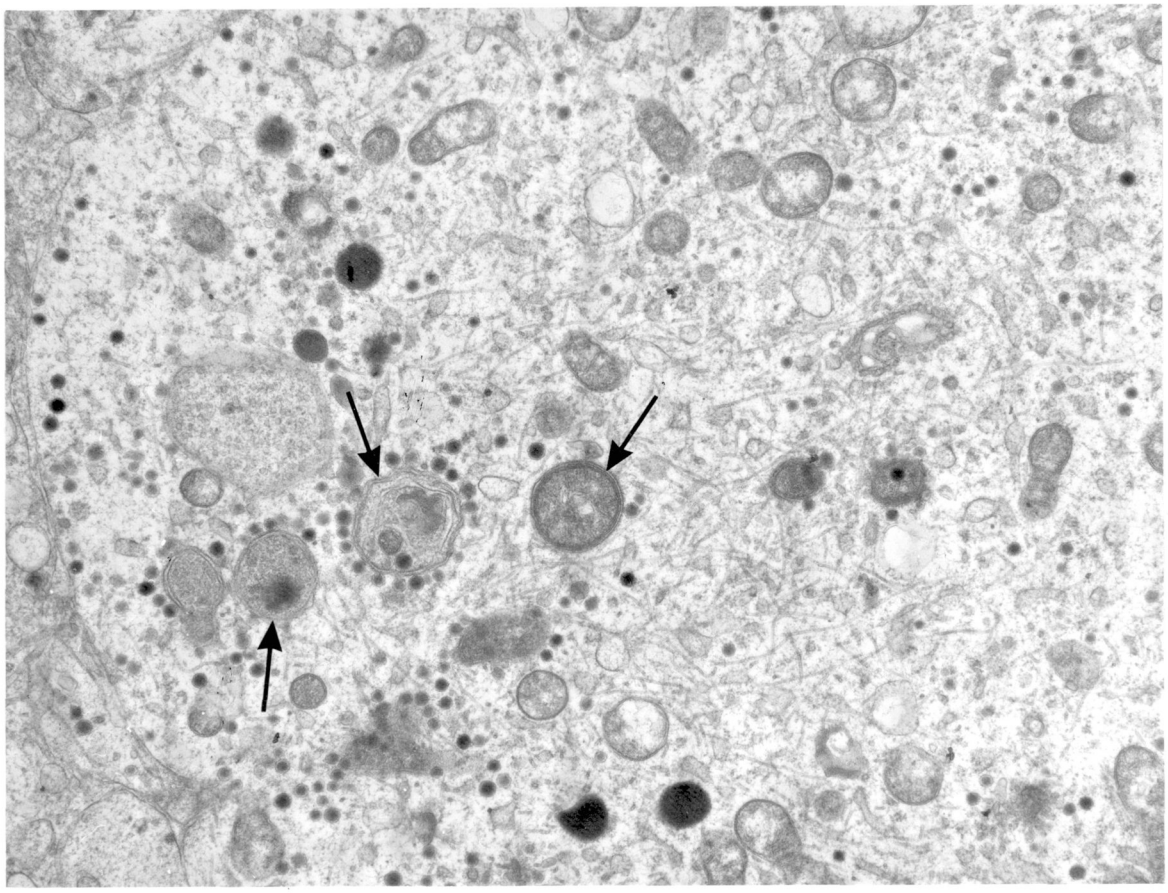


Fig. 3.45 Electron micrograph of a part of a tumour cell showing a group of structures tentatively identified as autophagic vacuoles (arrows), one clearly containing a secretory granule. Glutaraldehyde/osmium tetroxide fixation. Stained with lead citrate and uranyl acetate. (x 18,750).

CHAPTER FOUR

DISCUSSION

4.1 Introduction

4.2 Rat studies

4.3 Human studies

4.4 Summary

CHAPTER 4

DISCUSSION

4.1 INTRODUCTION

The work reported in this thesis examines the role of subcellular organelles in pituitary hormone secretion, particularly the intracellular mechanisms controlling the synthesis, secretion and release of PRL with a special emphasis on the role of lysosomes in the degradation of PRL. Methods have been developed to characterise the hormone granules as well as the various intracellular organelles of the rat and human pituitary and their properties have been studied under physiological, experimental and pathological conditions.

The premise for this study was the morphological studies of Smith and Farquhar (1966) who suggested that lysosomes may dispose of excess PRL by lysosomal action, a process they called crinophagy. These authors demonstrated that, in pituitary lactotrophs, as well as in other pituitary cells (Smith and Farquhar, 1966; Farquhar, 1969), lysosomes could play a role in regulating the hormone secretory process by disposing of the contents of undischarged granules. Morphological studies showed that cells are stimulated to discharge their secretory product (e.g. in the lactating animal with suckling young), the granules move towards, fuse with the cell membrane and

are discharged by exocytosis. If secretion is suppressed (e.g. removal of the suckling young), 24 to 48 h later the granules move towards and fuse with lysosomes.

To investigate the biochemical basis of this process, it was necessary to develop techniques, including enzymic analysis and subcellular fractionation, and apply them to a suitable animal model. The lactating rat model of Smith and Farquhar (1966) was used because 75% of the pituitary cells are lactotrophs (Everett and Baker, 1945; Goluboff and Ezrin, 1969) and allows rapid modulation of hormone levels.

The experiments have employed a single step centrifugation procedure which satisfactorily allowed resolution of the major organelles from pituitary specimens when as little as 0.2 mg protein was present. The technique of analytical subcellular fractionation, in combination with enzymic microanalysis, has previously been applied to milligram quantities of tissue obtained by closed biopsy procedures (Peters, 1977, 1981). This approach has defined the properties of various organelles in normal rat liver (Selden, Wootton, Moss and Peters, 1978) as well as normal and diseased human liver (Peters and Seymour, 1978; Seymour and Peters, 1978; Peters, Jenkins and Dubowitz, 1980), human leukocytes (Smith and Peters, 1981), intestine (Peters, 1976) and heart muscle (Bloomfield and Peters, 1974). In addition, regulatory peptide granules of the gastrointestinal tract have been characterised by this technique (Dawson, Bryant, Bloom and Peters, 1980; Dawson, Bryant, Cox, Christofides, Bloom and Peters, 1980). In addition to analytical subcellular

fractionation, the technique of counter-current partition was also employed in an attempt to further separate and characterise subcellular organelles and hormone granules.

Intracellular organelles contain specific marker enzymes by which they can be identified (de Duve, 1971), therefore, cell function can be studied by examining the marker enzyme activities of the subcellular organelles. Differences between normal and experimental or pathological situations may be related to either the enzyme content of the organelle or to the properties of the organelle and, therefore, may reflect differences in cell function.

The validation of this approach depended upon the reliability of enzyme assays. There have only been a few studies of pituitary enzyme activity (McShan, Rozich and Meyer, 1953; LaBella and Brown, 1958, 1959; Perdue and McShan, 1962) and these applied different procedures. It was necessary therefore to establish optimal conditions for all enzymes measured in our experiments.

Acid hydrolases assayed with the substrate 4-methylumbelliferyl showed good reliability, as Hultberg and Okerman (1972) had previously established, even when multiple forms of the enzyme exist as in the case of N-acetyl-β-glucosaminidase, β-glucuronidase and acid phosphatase (Robinson, 1974). The blank values and the stability of the substrates stored in a dessicator were consistently satisfactory, as previously reported (Guilbault, Sadar, Glazer and Haynes, 1968). It was important to use freshly prepared buffers, as minimal bacterial contamination could give elevated blank readings.

The substrates were prepared as 10 mmol/l solutions in 2-methoxyethanol, mainly for convenience and also because some of the substrates were not very soluble in water. The solutions, stable at 4°C, were diluted with the appropriate buffer immediately before use, to give a final substrate concentration of 0.21 mmol/l.

Considering the lysosomal enzymes of the anterior pituitary, expressed as μmol substrate transformed per min per mg protein, acid phosphatase was most active, followed by N-acetyl-β-glucosaminidase, β-glucuronidase and cathepsin C. This variation may reflect different populations of lysosomes and is further complicated by the dual intracellular localisation of some enzymes. Thus, as in human liver (Peters and Seymour, 1978), acid phosphatase also has a major extra-lysosomal component and such subcellular variations will be discussed later.

Alkaline phosphatase was assayed at high sensitivity with 4-methylumbelliferyl phosphate as a substrate. This substrate has been compared with several other phosphate derivatives and was found to be most satisfactory for the alkaline phosphatase assay (Guilbault, Sadar, Glazer and Haynes, 1968) although, in our assay, the pH optimum of 9.25 was lower than that usually recorded for alkaline phosphatase, especially human liver (Seymour and Peters, 1977) but this was probably due to low substrate concentration employed in the present work (Skillen and Harrison, 1973). The addition of magnesium chloride has been shown to enhance the activity of the enzyme in several

tissues including intestine, liver (Clark and Porteous, 1965; Seymour and Peters, 1977) and leukocytes (Raja, Smith and Peters, 1981). In the anterior pituitary a 1.4 mmol/l concentration gave a maximum enhancement.

5' Nucleotidase is probably a family of enzymes differing in many respects but sharing the ability to hydrolyse nucleoside 5'-phosphates (Goldberg, 1976). The radiometric assay of Avruch and Wallach (1971) was adopted and a number of modifications similar to those of Douglas, Kerley and Isselbacher (1972) were employed to enhance activity. A further specificity of the assay towards adenosine monophosphate was obtained by adding 12 mmol/l 2-glycerophosphate to the medium as a substrate divertor for alkaline phosphatase (Belfield and Goldberg, 1968).

The data obtained from the rat and human studies will be discussed as separate entities, as the rat studies formed the basis for the studies on human pituitary tumours.

4.2 RAT STUDIES

The establishment of these biochemical techniques as procedures applicable to the pituitary tissue allowed new tools for investigating Smith and Farquhar's (1966) model of crinophagy.

The results of the present study provide new supporting biochemical evidence for the involvement of intracellular organelles, especially lysosomes, in the intracellular control of PRL secretion. Our data show that, as the PRL accumulates

in the pituitary between 12 and 36 h after the removal of the litters, plasma PRL falls dramatically. By 48 h, pituitary PRL returns to control lactating levels, but plasma PRL remains low. These results are in agreement with those of Amenomori, Chen and Meites (1970) who showed that within 3 h after the removal of the litters, on the fourth day of lactating, there was a fall in serum PRL from 70.0 to 8.0 ng/ml. By 12 h, the pituitary PRL content was doubled.

In this situation, PRL accumulates in the pituitary. After a short time has elapsed, pituitary PRL returns to control values without being released into the general circulation. Therefore, an intracellular mechanism by which secretory granules are degraded must be in existence. The temporal relationship of the phenomena agrees well with our biochemical studies. Our findings of increased activity of certain lysosomal enzymes support the postulated role of these organelles in the removal of excess accumulated hormone granules. The differences in time response of the various lysosomal enzymes suggest them to have some degree of heterogeneity in the lactotroph cell. Davies (1975) has shown heterogeneity of lysosomes in the liver and Peters (1976), employing analytical subcellular fractionation to human jejunal homogenates, demonstrated the existence of three populations of lysosomes, although their functional significance remains to be established.

It was of interest to note that pituitary cathepsin C (EC 3.4.14.1) which is also called dipeptidylpeptidase I, did not change at any time after the mother rats had the suckling

stimulus removed, suggesting that this enzyme is not induced in the breakdown of proteins in the anterior pituitary.

The lysosomes of most animal cells possess a diverse array of hydrolytic enzymes; around 60 enzymes are known to be present in the lysosomes, these include several proteinases, glycosidases, nucleases, phospholipases, phosphatases and sulphatases (Dean, 1975). Most lysosomal enzymes are glycoproteins and the heterogeneity of the carbohydrate moiety contributes to the multiple forms most lysosomal enzymes exhibit; this carbohydrate moiety may control intra-lysosomal distribution by affecting the degree to which an enzyme interacts hydrophobically and electrostatically with the inside of lysosomal membranes (Dean, 1975).

Enzymes which hydrolyse peptide bonds form a major group among the enzymes of lysosomes and related organelles and are named proteases. Proteases comprise two distinct sub-groups; the exopeptidases, which cleave bonds only near the end of the polypeptide chain; and endopeptidases, which cleave bonds away from the ends of the polypeptide chain, although they can also cleave near the ends (Barrett, 1980).

Cathepsins are a heterogeneous group of peptide hydrolases, with a pH optimum in the acid range, which are located to lysosomes. Cathepsin D, a carboxyl endopeptidase (EC 3.4.23.6) is the most easily detectable lysosomal proteinase, cleaving polypeptides primarily in clusters of hydrophobic residues (Barrett, 1972). Adams and Smith (1951) showed that two different proteinases were present in the pituitary gland and that, with denatured haemoglobin as substrate, they catalysed

maximal hydrolysis of hog anterior pituitary at pH 3.8 and 8,3; these proteinases being called proteinase I and II, respectively. Proteinase I was subsequently isolated by Ellis (1960) who demonstrated its capability of degrading both sheep PRL and beef GH.

4.2.1 Acid PRL Protease

The proteolytic degradation of ^{125}I-labelled PRL by homogenates of anterior pituitaries from lactating rats was studied with an assay that could detect the breakdown of nanogram amounts of endogenous PRL. The precipitation extraction procedure was carried out at 0°C because it was previously shown by Leake and Peters (1981) that precipitation of ^{125}I-labelled LDL from homogenates of smooth muscle cells by trichloroacetic acid was more effective at this temperature than at room temperature. A major problem with this type of assay is that dehalogenase acting both on the undegraded protein and the peptides, releases significant quantities of inorganic iodide which is found in the trichloroacetic acid supernatant. This contributes to a high blank value in the assay. Oxidation of free iodide to iodine allows its selective extraction by chloroform. It was important to limit the time of oxidation by H_2O_2 to 5 min, because if longer times were used the blank values increased considerably. When oxidation time was limited to 5 min, the radioactivity in the blanks was approximately 0.1%

of the total radioactivity originally present in the substrate. We demonstrated a sharp pH optimum of 4.3 with only trace amounts of a neutral protease present. This pH optimum was very similar to that found by Adams and Smith (1951) in their preparation. Apparent saturation of the protease(s) was achieved with amounts of endogenous PRL greater than 1 µg/assay tube and a linear relationship between the amount of PRL degraded and the concentration of pituitary homogenate was found. However, it was not possible to obtain sufficient unlabelled purified rat PRL to ensure that saturating levels of substrate were achieved in all experiments.

To determine if this PRL proteolytic activity was distributed in the particulate or sedimentable parts of the density gradient, gradient fractions were incubated with ^{125}I-labelled PRL. It was found that most of the activity had a density distribution closely resembling that of lysosomes with very little activity in the soluble part of the gradient. This distribution, particularly taken together with the effect of the selective membrane perturbant digitonin, and the acidic pH optimum of the activity, strongly suggests that this enzyme is of lysosomal origin.

To determine which cathepsins were responsible for rat PRL proteolysis, the effects of specific inhibitors were studied. The contributions which individual cathepsins make to the degradation of various proteins depend largely on the nature of the protein. Thus, haemoglobin is usually broken down mainly by cathepsin D, whereas albumin is usually broken down mainly by cathepsin B (Barrett, 1977).

Pepstatin A, a known cathepsin D and E inhibitor (Barrett and Dingle, 1972) completely blocked the degradation of PRL in the protease assay. Similarly, antipain, an inhibitor of cathepsins A and B (Ikezawa,Yamada,Aoyagi,Tekeuchi and Umezawa,1972), iodoacetic acid, an inhibitor of cathepsins A, B and C (Misaka and Tapel, 1971; Huisman, Bouma and Gruber, 1974); and leupeptin, an inhibitor of the endopeptidase cathepsin B (Huisman, Lanting, Doddema, Bouma and Gruber, 1974) also blocked PRL proteolysis, causing 90% inhibition.

These studies clearly show that both cathepsins B and D are necessary for PRL degradation, as they are also implicated in the proteolysis of low density lipoprotein by smooth muscle cell lysosomes (Leake and Peters, 1981). In other tissues, such as rat liver, cathepsin D has been shown not to be essential in the degradation of albumin and other proteins by lysosomes (Huisman, Lanting, Doddema, Bouma and Gruber, 1974).

Supporting evidence that degradation of PRL occurs within the pituitary gland was suggested by Shenai and Wallis (1979) who, in experiments on the rate of incorporation of [^{3}H]leucine into rat pituitary PRL found this to be less than anticipated, postulating that this was due to rapid intracellular lysosomal degradation of PRL.

Subsequent to our investigation being completed, Nansel, Gudelsky, Reymond and Porter (1981) found that the oestrogen-induced antagonism of the inhibitory action of dopamine on PRL release was accompanied by a reduction in the capacity of dopamine

to stimulate the activity of β-glucuronidase, a lysosomal enzyme, in the pituitary gland, suggesting that lysosomes could be not only involved in regulation of PRL secretion as a secondary mechanism but also as a primary mechanism.

4.2.2 Subcellular Fractionation

The heterogeneity of rat pituitary lysosomes could be demonstrated by density gradient fractionation. The equilibrium densities of N-acetyl-β-glucosaminidase, β-glucuronidase and cathepsin C were the same (1.19 g/ml), all having a common pattern of distribution. However, PRL protease had a slightly denser peak of activity (1.22 g/ml) and a profile which was different from the other three enzymes mentioned above. The demonstration that these were of lysosomal origin was achieved with the use of digitonin, a detergent which disrupts membranes containing cholesterol. The lysosomes were selectively disrupted and their enzymes released into the soluble fraction. Mitochondria, through the marker enzyme malate dehydrogenase, was not affected by digitonin treatment and the increase in density of the plasma membrane marker 5' nucleotidase was similar to that found in rat liver (Tilleray and Peters, 1976), reflecting its high concentration of digitonin-complexable cholesterol (Amar-Costesec, Wibo, Thinès-Sempoux, Beaufay and Berthet, 1974; Mitropoulos, Venkatesan, Balasubramaniam and Peters, 1978). In addition, PRL granules must also contain significant amounts of cholesterol, as they also shifted to a denser distribution after digitonin treatment.

LaBella and Brown (1958) attempted to separate, by differential centrifugation, the subcellular fractions of beef and pig anterior pituitaries and found that acid phosphatase and proteinase I had a similar localisation, to the mitochondrial and supernatant fractions, but could not resolve further the location of these enzymes in the mitochondrial fraction. In a further experiment, these same authors (LaBella and Brown, 1959), employing higher centrifugal forces, assumed that acid proteinase I was localised only to particulate elements.

When anterior pituitary tissue from a 24 h post-lactating rat was subjected to density gradient centrifugation, the heterodensity of lysosomes was even more apparent. Whereas the distribution of N-acetyl-β-glucosaminidase did not change, that of β-glucuronidase showed an increase in the sedimentable fraction as well as two populations of peak activities, one at density 1.16 g/ml and the other at 1.19 g/ml. In addition, PRL proteolysis showed a highly significant shift to density 1.18 g/ml, becoming less dense.

4.2.3 The Enzymic Changes

The specificity of lysosomal changes in controlling PRL levels was confirmed by the lack of change in total pituitary protein and DNA content. The use of neutral α-glucosidase as a marker for endoplasmic reticulum has been clearly demonstrated for rat liver (Tilleray and Peters, 1976), smooth muscle cells (Peters and de Duve, 1974), cardiac muscle (Bloomfield and Peters, 1974) and human liver (Gamklou and Schérsten, 1972; Seymour and

Peters, 1978). The absence of any alterations in the activity of this enzyme and its subcellular distribution, together with the unchanged total pituitary protein content suggested that major changes in protein synthesis do not occur in the immediate post-lactation period, although more specific studies of protein synthetic mechanisms would be necessary before this conclusion is fully substantiated.

The increase in 5' nucleotidase, the plasma membrane marker enzyme (Solyom and Trams, 1972), after pup removal was particularly striking. The explanation of this finding is uncertain, but two possibilities occur. It has been suggested that adenosine may modulate hormone secretion in adrenal and pancreatic cells (Kowal and Fiedler, 1969; Ismail, El Denshary and Montague, 1977). An increase in tissue 5' nucleotidase could lead to an increase in tissue adenosine and this might trigger the process that prevents PRL granule discharge. Alternatively, the cessation of PRL secretion would be expected to affect the dynamics of intracellular membranes, particularly plasma membrane retrieval (Farquhar, 1978). The membrane of the secretory granules is smooth-surfaced until it contacts and fuses with the plasma membrane. The empty caveola left by discharge of the granule then appears to acquire a clathrin coat (invagination or coated vesicle). Its neck presumably constricts and the membrane of the secretory granule is retrieved directly, without even being completely incorporated in the plasma membrane (Fawcett, 1981). The equilibrium density of 1.13 g/ml of 5' nucleotidase was

significantly lower than for rat liver (1.15 g/ml) when similar centrifugation procedures were used (Peters and Shio, 1976; Tilleray and Peters, 1976) and this could reflect different subcomponents of the plasma membrane or adventitious association of the enzymes to subcellular fragments of the cell wall (Wallach and Sun Li, 1973).

The activity of alkaline phosphatase showed a progressive increase 12 h after the removal of the suckling young and an abrupt return to control levels by 48 h; it was not clear why this enzyme was increased, although this enzyme has been demonstrated to be associated with various intracellular membranes of the secretory process (Smith and Farquhar, 1969). The subcellular localisation of this enzyme showed it to have a complex density distribution to plasma membrane, cytosol and hormone granules (Caughey and De Marco, unpublished results) although the total alkaline phosphatase activity in rat pituitary was significantly lower than in the rat liver.

The activity of lactate dehydrogenase was increased soon after the removal of suckling young and this may reflect a reduced release of cytosolic enzymes which normally occurs during secretion, particularly in phagocytic cells. In addition, total lactate dehydrogenase activity in lactating rat pituitary homogenates was considerably higher than that found in rat liver whole homogenates (Selden, Wootton, Moss and Peters, 1978). The distribution of lactate dehydrogenase in the gradient was found to be almost exclusively localised to the soluble fractions with very little binding of the enzyme to membranes.

Malate dehydrogenase also showed a transient increase in activity 36 h after the removal of the litters, but the cause of this increase remains obscure. A greater proportion of the activity was localised to the soluble fraction, a finding which is consistent with a lower potential for mitochondrial TCA activity, but further mitochondrial enzyme assays and detailed metabolic studies would be necessary to substantiate this suggestion.

The existence of catalase, a marker for peroxisomes, has been conclusively demonstrated in rat liver (Peters and Shio, 1976) and other tissues (de Duve and Baudhuin, 1966). The significant lower density of the particulate enzyme in the rat pituitary homogenates might be related to the absence of uricase from the homogenates. In rat liver, the cores of the peroxisomes contain most of the uricase and contribute to their high density (de Duve and Baudhuin, 1966).

In summary, employing refined biochemical techniques, it was possible to characterise the principal organelles from rat pituitary and good resolution of plasma membrane, mitochondria, lysosomes and PRL granules was obtained, their separate entities being confirmed by the centrifugation of tissue extracts which had been prepared in the presence of low concentrations of digitonin. In addition, pituitary enzyme activities in whole homogenates of lactating and post-lactating rats were determined, showing striking changes in lysosomal enzyme activities during lactotroph involution induced by cessation of lactation.

4.2.4 Effects of Bromocriptine

It is well established that dopamine release from tuberoinfundibular neurons into hypophyseal portal vessels inhibits the secretion of PRL by the anterior pituitary gland (Shaar and Clemens, 1974; MacLeod and Lehmeyer, 1974; Ben-Jonathan, Oliver, Weiner, Mical and Porter, 1977). The dopamine agonist 2-bromo-α-ergocryptine (bromocriptine) has been shown to suppress excessive hormone secretion in patients with hyperprolactinaemia and pituitary tumour (Sobrinho, Nunes, Santos and Mauricio, 1977; Nillius, Bergh, Lundberg, Stahle and Wide, 1978; Wass, Moult, Thorner, Dacie, Charlesworth, Jones and Besser, 1979; Corenblum and Hanley, 1981) and Mashiter, Adams, Beard and Holley (1977) demonstrated this to occur by direct action on the tumourous lactotrophs.

There is morphological evidence that rats treated with ergocornine and bromocriptine show a decrease in hormone exocytosis and an increase in autophagic vacuoles (Ectors, Dangui and Pasteels, 1972; Hausler, Rohr, Marbach and Fluckiger, 1978). In addition, there was also evidence that dopamine could become internalised and associated with PRL granules as well as increasing activities of the lysosomal marker enzymes β-glucuronidase and acid phosphatase (Nansel, Gudelsky and Porter, 1979; Nansel, Gudelsky, Reymond, Neaves and Porter, 1981).

Having demonstrated the existence of a lysosomal acid PRL protease in rat pituitary lactotrophs (Section 3.2) and also found striking changes of this and other lysosomal enzymes during lactotroph involution following cessation of lactation, a study was

performed to investigate the role of subcellular organelles, especially lysosomes, in pituitaries from lactating and post-lactating rats treated with bromocriptine. The purpose was to examine whether the mechanisms of action of this substance were similar to the mechanisms derived from the physiological cessation of lactation.

The results obtained both confirmed and extended the previous observations that removal of the suckling stimulus from lactating rats resulted 12 to 24 h later in a lowering of plasma PRL, an accumulation of intracellular PRL with marked changes in certain organelle marker enzymes. Administration of bromocriptine resulted in even more marked changes in plasma and pituitary PRL in both lactating and post-lactating animals consistent with its known suppressive effect on PRL secretion. However, there was a striking differential effect on the activities of lysosomal marker enzymes. Acid PRL protease, an enzyme characterised in Section 3.2, was markedly increased by bromocriptine treatment in both lactating and post-lactating rats whereas the lysosomal marker enzymes N-acetyl-β-glucosaminidase and β-glucuronidase were unchanged in lactating rats given bromocriptine. Furthermore, the significant increases in N-acetyl-β-glucosaminidase and β-glucuronidase activities produced by litter removal were abolished by co-administration of bromocriptine. Nansel, Gudelsky, Reymond, Neaves and Porter (1981) have provided both *in vitro* and *in vivo* evidence that dopamine can act directly on pituitary to increase lysosomal enzyme activities as measured

by β-glucuronidase and acid phosphatase and this was closely correlated with suppression of PRL release. These results are consistent with the data obtained in this thesis; however, as bromocriptine is believed to act through dopaminergic mechanisms, an increase in β-glucuronidase activity might also have been anticipated in our study. Lack of activation of this enzyme in lactating, and blockade of its increase in post-lactating rats treated with bromocriptine may suggest that this drug has other than dopaminergic effects. Similar differences between the action of dopamine and bromocriptine has been reported in respect of LH secretion and other endocrine glands (Martin, Rogol, Kaiser and Thorner, 1981). These authors showed that bromocriptine was ineffective in suppressing LH secretion in normal ovulating women but dopamine decreased LH secretion by 30% thus suggesting the presence of multiple dopamine receptors. Alternatively, the differences may be related to the different times of investigation (8 h versus 12 and 24 h), or the use of pituitaries having a preponderance of lactotrophs in this study whereas Nansel, Gudelsky, Reymond, Neaves and Porter (1981) could not be certain that dopamine affected β-glucuronidase in the lactotroph or the other pituitary cells.

Bromocriptine's selective and striking activation of the lysosomal acid PRL protease is entirely consistent with the hypothesis that lysosomes may degrade excess accumulated PRL granules in the lactotroph cells and suggests these proteases may be the major activities responsible for this effect.

The findings support the suggestion of Dannies and Rudnick (1979) that lysosomes may be responsible for PRL degradation. They found that rat pituitary cells, cultured in the presence of bromocriptine, showed enhanced degradation of newly synthesized (^{3}H)-PRL compared to control cultures. The apparent rate of synthesis after 4 days of chronic treatment with bromocriptine, measured by the incorporation of (^{3}H) leucine for 1 or 2 h was 1.4 to 2.8 fold less than controls. In addition, Maurer (1980) showed that, in pituitary cultures from female rats containing bromocriptine in the chase incubation medium, there was a 50% decrease in labelled PRL after 24 h and the addition of cycloheximide blocked the ability of bromocriptine to induce PRL degradation when the two substances were added simultaneously. Furthermore, chloroquine, a weak base which is taken up selectively into lysosomes and inhibits several of its constituent enzymes (de Duve, de Barsy, Poole, Trouet, Tulkens and Van Hoof, 1974) was able to partially inhibit the ability of bromocriptine to stimulate PRL degradation in pituitary cell cultures (Maurer, 1980). When bromocriptine was directly added to pituitary homogenates in the present study and acid PRL protease assayed, there was no direct effect on the enzymic degradation of PRL. These results suggest that the cell must be intact for bromocriptine effects to be exerted on the enzymic degradation of PRL. It is likely that bromocriptine reduced cellular discharge of PRL granules leading to intracellular accumulation of the hormone which in turn increases lysosomal PRL protease activity. Indeed, the analytical

subcellular fractionation studies showed that the localization of acid PRL protease in tissue extracts from lactating rats treated with bromocriptine was similar to the one observed for a post-lactating animal whereas N-acetyl-β-glucosaminidase and 5'nucleotidase were unaffected by bromocriptine treatment. It is of particular interest that the peak activity of prolactin granules in bromocriptine-treated animals was at the same density as the lysosomal enzymes possibly reflecting fusion of these two organelles as a part of the degradative process.

Bromocriptine significantly increased the activities of plasma membrane marker 5'nucleotidase and alkaline phosphatase (partial) in both lactating and post-lactating rats to a similar or greater degree to those invoked by suckling withdrawal alone and these changes, as previously speculated, might be due to intracellular changes in adenosine or alterations in the dynamics of plasma membrane retrieval. The activity of malate dehydrogenase was increased by suckling withdrawal alone whereas this effect was blocked by bromocriptine but the reason for this effect remain obscure.

The striking decrease in DNA content of pituitaries from lactating and post-lactating rats treated with bromocriptine is of particular interest in view of the increasing evidence that ergot alkaloids can reduce the size of PRL-secreting pituitary tumours both in animal models (MacLeod and Lehmeyer, 1973) and man (Corenblum, Webster, Mortimer and Ezrin, 1975; Nillius, Bergh, Lundberg, Stahle and Wide, 1978; Wass, Moult Thorner, Dacie, Charlesworth, Jones and Besser, 1978; Thorner

Martin, Rogol, Morris, Perryman, Conway, Howards and MacLeod, 1980; Sobrinho, Nunes, Calhaz-Jorge, Mauricio and Santos, 1981; Corenblum and Hanley, 1981). In vitro studies on pituitaries from normal and pregnant rats as well as those with oestrogen-induced pituitary tumours (Lloyd, Meares and Jacobi, 1975; Lloyd, Jacobi and Meares, 1978; Kalberman, Machiavelli, De Nicola, Weissenberg and Burdman, 1980; Kalberman, Szijan and Burdman, 1979) have also shown that bromocriptine can decrease their DNA content and have indicated a relationship between pituitary PRL content and DNA replication. Hence, an increase in pituitary PRL content as produced in the last weeks of pregnancy (Kalberman, Szijan and Burdman, 1979) or by bromocriptine (Lloyd, Meares and Jacobi, 1975) can both lead to an inhibition of mitosis. Conversely, a temporary depeletion of PRL produced either by sulpiride stimulation or by continuous secretion from tumour cells is enough to trigger a series of events culminating in increased DNA synthesis and cell proliferation. Recently, it has been shown that synthesis of DNA, as measured by the in-vitro incorporation of (^{3}H)thymidine into pituitaries from virgin rats was decreased by bromocriptine treatment (MacLeod, 1982 - personal communication). Although the data presented in this thesis with bromocriptine-treated rats is consistent with all these studies, the increase in pituitary PRL induced by suckling withdrawal alone did not change cellular DNA. It cannot be excluded therefore, that bromocriptine has effects on DNA that are not directly related to intracellular accumulation of PRL. Indeed, there is evidence suggesting that

bromocriptine only acts on the newly synthesized hormone, having no effect on the secretion of the pre-existing pool of PRL (Weinstein, Schenker, Gloger, DeGroot, Hochberg and Folman, 1981). These authors, in studying the in-vitro incorporation of (^{3}H)leucine or (^{3}H)uridine to female rat pituitaries in the presence or absence of bromocriptine showed that the inhibitory effect of bromocriptine was much more profound in the poly(A(+))RNA synthesis (85%) than in protein synthesis, suggesting that the primary site of action for this substance is on transcription. The conclusion inferred by the results obtained in this thesis, together with the available data, demonstrates that bromocriptine causes profound changes in organelle marker enzymes, particularly the lysosomes, and DNA, and these changes could provide biochemical basis for its mode of action.

4.2.5 Summary

The studies on rat pituitaries have shown that the development of assays for enzyme marker of subcellular organelles and techniques for density gradient fractionation is of value for characterizing and analysing these organelles during physiological and experimental variations. The methods have shown marked changes occurring in certain organelles in association with alterations in hormone secretion and accumulation in the lactotroph cells. Activities of lysosomal enzymes are closely linked with intracellular hormone granule control at least for the lactotroph cell. The results confirm the presence of the

crinophagic process and demonstrate the existence and characterization of an acid protease of lysosomal origin capable of degrading PRL. Bromocriptine has been confirmed as a major pharmacological suppressant of PRL secretion also inducing marked changes in the PRL protease most probably due to the intracellular accumulation of PRL granules. Bromocriptine causes a number of other changes in cellular enzymes particularly of the plasma membrane and significantly reduces cellular DNA. This latter finding may be of relevance to the reported tumour shrinking effect of this drug on human PRL-secreting pituitary adenomas.

4.3 Human pituitary tissue

Despite intensive investigation of subjects with pituitary tumours, there is as yet little information as to the cause of the patient's pituitary pathology. In-vivo experimentation of the effects of various pharmacological agents has shown a number of disturbances in hypothalamic-pituitary relationships. For example, administration of antidopaminergic drugs has demonstrated increased dopaminergic tone in patients with PRL-secreting adenomas leading in turn to hypersensitivity of thyrotroph cells once this suppression is removed (Quigley, Judd, Gilliland and Yen, 1979; Scanlon, Rodriguez-Arnao, McGregor, Weightman, Lewis, Cook, Gomez-Pan and Hall, 1981). However, it is most likely that these changes occur as a consequence of the presence of the tumour rather than being causal. Tumour cells have shown little change in their responses

compared to normal pituitary cells in vivo, although these are exceptions such as the GH rise following TRH in patients with acromegaly that may point to the presence of altered receptors. Only in the past few years have investigations of tumour tissue been undertaken in-vitro, however, these have been very largely confined to tissue culture techniques and measurement of hormone secretion in response to various agents.

Although these studies have provided considerable information about the factors regulating hormone secretion, they have yet to reveal the primary disturbances in tumour cells. If there is an underlying pathology in these cells that gives rise to hormone hypersecretion, it may be revealed by studies at a subcellular level. Matsakura, Kakita, Hirata, Yoshimi, Fukase, Iwasaki, Kato and Imura (1977) demonstrated the presence of multiple hormone receptors in GH or ACTH producing pituitary adenomas and ectopic ACTH producing tumours. In addition, Bression, Brandi, Martres, Nousbaum, Cesselin, Racadot and Peillon (1980) found similar number of binding sites in normal pituitaries and PRL-secreting adenomas, although the latter has 2 - 3 times more PRL cells thus suggesting a defect in the dopaminergic control in PRL-secreting adenomas.

The single step analytical subcellular fractionation and sensitive marker enzyme assays techniques developed as part of this work for the study of the rat pituitary model were found to be equally applicable to human tissue. It was a logical step therefore, to examine the subcellular organelles of tumourous human pituitary tissue, particularly in relation to

lysosomal enzyme activities, since the finding of the previous section showed these to be inextricably linked to hormone secretory processes. A comprehensive literature search has not revealed any previous work on either the characterization of subcellular organelles or comparison of their activities in normal or tumourous human pituitary tissue removed from patients with the various endocrinopathies.

To be able to determine the changes in tumourous tissue, a control group of 'normal' pituitaries was obtained from patients undergoing hypophysectomy for palliation of breast or prostate carcinoma. It is recognised that such comparisons may be not ideal on two grounds. Firstly, the normal pituitary is heterogeneous in terms of its cell type and comparison are being made with tumourous tissue which is still heterogeneous but probably to a lesser degree (a number of tumour specimens were shown to contain more than one hormone). Secondly, it can not be proven that the pituitaries of these patients are normal in all respects since many of these carcinomas appear to be responsive to endocrine therapy. However, there are strict limitations on the use of human pituitary material which excluded other sources such as post mortem tissue, indeed this was probably unsuitable because of the necessary time delay before collection.

The tumourous tissue was obtained as part of an ongoing collaborative research programme between the laboratory and various neurosurgeons. Whereas transport of these tissue over 2 or 3 days in culture medium has been found acceptable for

cell culture, care was taken to work, where possible, only with local material so that processing could start as soon as possible.

The studies showed that resolution of the major sub-cellular organelles and hormone granules was achieved in both types of tissue. There was little difference however in the organelle distribution patterns between normal pituitary tissue and PRL, GH or functionless tumours apart from the finding of two peaks of activity for β-glucuronidase (lysosome) in the normal tissue but only one in PRL-secreting tumours.

Measurement of total enzyme activities in whole homogenate revealed some marked differences, particularly in respect of lysosomal marker enzyme activities. In the tumour tissue taken from patients with known hormone hypersecretion, activities of N-acetyl-β-glucosaminidase and β-glucuronidase were significantly lower than in normal tissue. In contrast, activity of these enzymes in quiescent, functionless tumours were normal or greater than normal.

It is known that actively secreting tumours store relatively little hormone and these results therefore are consistent with the findings in the rat that pituitary lysosomal enzyme activities are related to the cellular hormone content. That is the increased accumulation of hormone following suckling withdrawal or bromocriptine leads to increases in hormone degrading activity. That such proteases are present in human pituitary tumour tissue was shown by the study of the PRL protease in PRL-secreting tumour tissue. The characteristics

of this enzyme were similar to those of normal pituitary tissue and functionless tumours.

It cannot be ruled out that the reduced hormone degrading activity of pituitary tumours results in hormone hypersecretion. The study of an acromegalic patient with large tumours and a surprisingly low serum GH may be important in this respect although demonstrating the reverse phenomena. Indeed, complete absence of endocrine hyperactivity in a patient with a corticotrophic cell adenoma has been attributed to increased lysosome accumulation and crinophagy (Kovacs, Horvath, Bayley, Hassaram and Ezrin, 1978).

4.3.1 Case report: discussion

This patient presented with clinically obvious acromegaly with a raised serum GH and PRL. Although 20 - 40% of acromegalics have been found to have a raised serum prolactin (Jacobs and Daughaday, 1973; Aubert, Grumbach and Kaplan, 1975; Jacobs and Franks, 1975) and evidence has been provided for mixed pituitary adenomas (Zimmerman, Defendini and Frantz, 1974; Corenblum, Sirek, Horvath and Kovacs, 1976; Halmi and Duello, 1976; Guyda, Cole and Hardy, 1973; Mashiter, Van Noorden, De Marco, Adams, and Joplin, 1979) this patient's tumour, if our small sample was typical, was composed solely of somatotrophic cells. No prolactin was secreted in culture, and none was found in the tissue by immunocytochemistry or by direct analysis. Since the tumour was fairly large, it is probable that it impinged on the hypothalamic-hypophysial vessels in

such a way that there was reduction in the amount of prolactin-inhibitory factor (PIF) reaching the normal prolactin secreting cells, leading to a raised prolactin, which could have been the cause of her presenting complaints of amenorrhoea and galactorrhoea.

The serum growth hormone was strikingly low in this patient in view of the degree of acromegaly, duration of disease, and the size of the tumour. Moderate correlations have been established previously between tumour size and circulating GH, (Wright, MacLachlan, Doyle and Fraser, 1969) although in a number of patients, including this one, the results clearly do not fit this pattern. There was little to suggest that the tumour had undergone necrosis, since the AEG showed a small upwards bulge of the tumour above the diaphragma of the sella, and organelle marker enzyme levels (apart from those marking lysosomes) were not different from those in adenomas from other acromegalics. Furthermore, the cell culture experiments were carried out with cells having a viability of greater than 95%; nevertheless, these cultures released remarkably little GH when compared to adenomas from 5 other acromegalics or even a normal pituitary, suggesting that the low serum GH levels were a direct result of a subnormal release of GH. In addition, the immunocytochemistry and direct measurement of tissue GH showed that the reduced GH secretion most probably resulted from a low intracellular hormone content. Association of a number of experimental data and comparison with adenomas from other active acromegalics lead us to believe that our patient's adenoma was undergoing regression

of its secretory activity by the process of crinophagy (lysosome-hormone granule fusion). This would be followed by the sequestration of the secretory granules within lysosomes, so that intracellular degradation of growth hormone would be enhanced.

When compared with other adenomas, the tumour showed the remarkable feature of numerous autophagic vacuoles (secondary lysosomes) surrounded by and containing hormone granules. Activity of the lysosomal marker enzyme N-acetyl-β-glucosaminidase was significantly increased, and subcellular fractionation showed a different distribution pattern such that GH granules were found in the same fraction of the lysosomes rather than being separate. Since it was previously shown that pituitary lysosomes have proteases capable of degrading hormones in both rat and man (sections3.2 and3.7), these results provide strong evidence for lysosomal degradation resulting in the low hormone content. We cannot exclude the possibility that the hormone in the tissue or released by the somatotroph cells had an altered molecular structure, with increased biological or low immunoassayable activity (Lewis, Dunn, Bonewald, Seavey and VanderLaan, 1978).

This case has a number of features of similarity with that of a silent corticotrophic cell adenoma described by Kovacs, Horvath, Bayley, Hassaram and Ezrin, 1978). The major difference is that our patient must have had a more active phase to her disease at one time, whereas no endocrine abnormality was observed by Kovacs et al. in their case.

We conclude that the simultaneous occurrence in our patient of a relatively low serum GH together with a large tumour and obvious acromegaly can be rationalized by the striking finding of crinophagy - disposal of hormone secretory granules within the somatotroph cells themselves. The occurrence of 'burnt-out' acromegaly has been known to endocrinologists for some time and has usually been attributed to spontaneous infarction (Rigolosi, Schwartz and Glick, 1968; Ewer and Kotheimer, 1970). The present study may go some way to suggesting an additional biochemical mechanism.

4.3.2 Summary

The studies have shown that the biochemical techniques developed for the study of normal rat pituitary subcellular organelles are of value for the investigation of human pituitary pathology. A number of different types of human pituitary tumours and normal tissue have been studied and the activities and distribution of the subcellular organelles and hormone granules examined. Despite the tissue and cell heterogeneity, certain tentative conclusions have been drawn. These relate particularly to the significantly decreased activity of lysosomal enzymes in tissue that is hypersecreting hormones compared with normal, or quiescent functionless tissue. The results are consistent with the hypothesis formulated in the rat studies that lysosomal enzyme activity is related to intracellular hormone content. A case report of a patient showing low hormone secretion from a large tumour and increased lysosomal enzyme activity provided further supportive evidence for this hypothesis.

Chapter Five

CONCLUSION

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CONCLUSION

5. CONCLUSION

The studies reported in this thesis provide techniques for the study of hormone granules and subcellular organelles in normal rat as well as normal and tumourous human pituitaries.

Biochemical evidence is provided for a role of lysosomes in the degradation of hormone granules within the pituitary cells (crinophagy) as a regulating process intrinsically linked to hormone secretion. Lysosomal proteases responsible for this degradation are described and characterised. These studies provide a basis for further study of this regulatory process in human pituitary pathology.

APPENDIX 1

Pituitary hormone levels in controls (normal glands)

Patient	Pituitary			
	PRL ng/mg protein	GH mIU/mg protein	LH U/mg protein	FSH U/mg protein
1	11,084	23,300	152	6,338
2	1,706	14,119	38,041	24,938
3	3,347	12,134	756	180
4	3,064	10,405	16,300	636
5	844	10,417	10,917	3,592
6	1,304	64,783	10,917	10,917
7	331	4,865	11,135	1,405
$\bar{x}$	3,097	20,003	12,603	6,858
SD	3,691	20,516	12,675	8,826
SE	1,393	7,742	4,783	3,330
n	7	7	7	7

APPENDIX 2

Pituitary and plasma/serum hormone levels in patients with prolactin-secreting adenomas

Patient	Pituitary				Plasma/Serum			
	PRL ng/mg protein	GH mIU/mg protein	LH U/mg protein	FSH U/mg protein	PRL µg/l	GH mIU/l	LH U/l	FSH U/l
8	3,488	653	0	0	175	3.8	2.3	5.5
9	19,727	275	0	0	666	1.0	2.8	3.9
10	2,558	17,412	0	0	na	na	na	na
11	21,471	0	0	0	1,350	0.5	1.0	2.4
12	2,180	3,065	262	0	117	na	na	na
13	3,741	103	0	0	na	na	na	na
14	10,750	250	1,308	615	970	0.6	1.0	1.0
15	3,000	0	600	275	400	na	na	na
16	4,380	350	333	0	120	na	na	na
17	1,985	713	0	0	6,660	na	na	na
$\bar{x}$	7,328	2,282	250	89				
SD	7,441	5,392	425	204				
SE	2,355	1,706	134	65				
n	10	10	10	10				

na = not available

APPENDIX 3

Pituitary and plasma/serum hormone levels in patients with growth hormone-secreting adenomas

Patient	Pituitary				Plasma/Serum			
	PRL ng/mg protein	GH mIU/mg protein	LH U/mg protein	FSH U/mg protein	PRL µg/l	GH mIU/l	LH U/l	FSH U/l
18	0	850	0	0	75	65	2.3	3.6
19	0	264,900	255	0	60	163	3.0	4.8
20	49	11,980	0	0	12	88	1.0	2.1
21	114	143,985	40	32	41	170	1.7	8.0
22	0	173,876	143	0	6	82	4.3	15.0
23	146	46,949	0	80	41	183	2.3	2.3
24	0	6,450	450	0	n	30	n	n
25	0	120,000	0	0	53	143	2.2	3.7
26	2,640	200,000	0	0	n	30	n	n
27	0	2,000	1,000	371	17	7	2.3	2.3
28	384	24,038	0	0	n	12	n	n
29	0	2,045	0	57	n	100	n	n
30	0	964	1,084	0	n	30	n	n
31	0	4,400	0	0	40	23	1.0	2.0
$\bar{x}$	166	71,603	210	38.6				
SD	437	90,620	375	99.0				
SE	117	24,230	100.4	26.5				
n	14	14	14	14				

n = normal

APPENDIX 4

Pituitary and plasma/serum hormone levels in patients with functionless tumours

Patient	Pituitary				Plasma/Serum			
	PRL ng/mg protein	GH mIU/mg protein	LH U/mg protein	FSH U/mg protein	PRL µg/l	GH mIU/l	LH U/l	FSH U/l
32	252	6,791	548	0	n	0.5	2.2	2.8
33	0	11	0	0	12	2.9	4.4	2.0
34	0	9,620	10,000	1,100	n	n	n	n
35	3,488	1,281	121	85	n	n	n	n
36	0	48,148	0	0	n	n	n	n
37	0	1,000	1,400	400	35	1.6	1.5	18.6
38	0	1,320	260	0	n	n	n	n
39	1,100	1,000	250	125	n	n	n	n

n = normal

APPENDIX 5

Enzyme activities in human normal pituitary homogenates (mU/mg protein)

Patient	Protein (mg/ml)	DNA (µg/ml)	N-Acetyl-β-glucosaminidase	β-Glucuronidase	α-Glucosidase	Acid phosphatase	Catalase	Malate dehydrogenase	Lactate dehydrogenase	Cathepsin C
1	0.142	-	5.1	-	0.6	21.4	-	3,115	-	-
2	0.646	-	4.9	2.7	1.0	21.2	23.1	4,373	950	-
3	0.239	16,900	2.3	1.6	0.3	21.1	10.3	1,716	189	5.0
4	0.173	6,600	6.3	2.1	1.1	33.9	13.1	2,618	959	8.2
5	0.960	17,600	3.6	1.1	0.5	33.6	7.8	3,248	374	0.7
6	0.115	5,850	4.5	1.1	0.6	31.8	11.6	3,284	297	3.0
7	0.185	7,120	6.0	1.0	1.5	35.3	13.5	-	281	1.7
$\bar{x}$			4.7	1.6	0.8	28.3	13.2	3,059	508	3.7
SD			1.4	0.7	0.4	6.7	5.3	873	350	3.0
SE			0.5	0.3	0.2	2.5	2.2	356	142	
n			7	6	7	7	6	6	6	5

APPENDIX 6

Pituitary enzyme activities in prolactin-secreting adenomas (mU/mg protein)

Patient	Protein (mg/ml)	DNA (µg/ml)	N-Acetyl-β-glucosaminidase	β-Glucuronidase	α-Glucosidase	Acid phosphatase	Catalase	Malate dehydrogenase	Lactate dehydrogenase	Cathepsin C
8	3.110	-	3.0	0.2	3.0	25.9	77.2	-	367	-
9	0.110	-	4.7	2.8	1.3	101.5	62.4	5,243	656	-
10	0.533	-	3.6	0.3	0.6	4.7	-	714	185	14.5
11	0.210	-	2.7	0.5	0.7	8.5	-	-	-	2.8
12	0.061	2,400	3.1	0.8	0.9	15.6	8.7	1,177	280	7.7
13	0.058	5,600	2.9	1.3	1.6	16.8	6.7	4,568	246	11.1
14	0.026	1,200	1.3	0.3	0.1	12.0	16.3	2,630	1,027	2.6
15	0.040	2,800	0.8	0.3	0.4	25.1	12.1	2,564	496	2.2
16	0.024	1,640	0.5	0.6	1.5	16.1	19.1	2,493	267	1.5
17	0.068	-	1.3	0.2	0.6	12.5	-	3,645	578	2.2
$\bar{x}$			2.4	0.7	1.1	23.9	28.9	2,879	456	5.6
SD			1.3	0.8	0.8	28.1	28.6	1,555	268	4.9
SE			0.4	0.3	0.3	8.9	10.8	550	89	1.7
n			10	10	10	10	7	8	9	8

APPENDIX 7

Pituitary enzyme activities in homogenates of human growth hormone-secreting adenomas (mU/mg protein)

Patient	Protein (mg/ml)	DNA (µg/ml)	N-Acetyl-β-glucosaminidase	β-Glucuronidase	α-Glucosidase	Acid phosphatase	Catalase	Malate dehydrogenase	Lactate dehydrogenase	Cathepsin C
18	0.038	950	0.5	0.1	0.1	2.7	10.1	3,571	281	-
19	0.151	5,400	3.7	0.5	0.7	16.6	5.5	2.026	386	1.6
20	0.101	1,000	1.6	0.1	0.3	5.4	59.5	2,014	381	-
21	1.245	-	6.0	0.1	0.4	10.1	20.7	1,928	657	-
22	1.179	3,709	3.3	0.3	-	4.4	-	883	289	0.1
23	0.213	10,000	1.9	0.5	1.2	11.0	5.8	3,611	284	4.2
24	0.020	1,020	1.5	0.7	0.1	24.4	22.6	2,279	107	5.2
25	0.083	2,720	2.5	0.6	1.3	10.5	23.8	1,442	197	1.5
26	0.050	1,280	2.8	0.2	0.7	8.3	4.0	684	128	0.6
27	0.035	1,840	2.8	0.5	1.3	7.5	2.0	2,930	244	2.5
28	0.104	-	6.4	0.8	-	30.3	14.5	7,232	719	3.7
29	0.083	5,760	5.8	1.7	2.5	41.9	-	4,668	386	6.0
30	0.044	-	12.8	1.3	1.9	36.1	-	4,119	690	2.1
31	0.090	-	4.9	-	1.1	2.2	19.8	1,963	460	-
$\bar{x}$			4.0	0.6	1.0	15.1	17.1	2,811	372	2.8
SD			3.1	0.5	0.7	12.9	16.2	1,734	198	2.0
SE			0.8	0.1	0.2	3.5	4.9	646	53	0.6
n			14	13	12	14	11	14	14	10

APPENDIX 8

Pituitary enzyme activities in functionless tumours (mU/mg protein)

Patient	Protein (mg/ml)	DNA (µg/ml)	N-Acetyl-β-glucosaminidase	β-Glucuronidase	α-Glucosidase	Acid phosphatase	Catalase	Malate dehydrogenase	Lactate dehydrogenase	Cathepsin C
32	0.115	1,330	11.4	0.9	1.6	11.9	28.3	2,490	632	2.7
33	0.361	-	4.8	0.9	1.1	3.5	40.4	4,906	141	-
34	0.050	2,000	7.2	1.3	0.8	30.5	36.0	2,017	735	2.0
35	0.281	2,800	4.7	1.0	0.8	25.3	26.7	1,339	437	2.0
36	0.027	1,660	5.2	1.3	0.6	11.3	12.1	1,899	185	4.9
37	0.050	4,120	4.3	0.9	1.0	26.7	10.1	2,051	299	4.9
38	0.025	2,000	9.6	0.9	1.1	22.6	9.2	6,154	399	2.7
39	0.020	-	7.4	0.5	-	25.0	4.9	8,832	392	3.4
x			6.8	1.0	1.0	19.6	21.0	3,711	403	3.2
SD			2.6	0.3	0.3	9.5	13.5	2,663	204	1.2
SE			0.9	0.1	0.1	3.4	4.8	942	72	0.4
n			8	8	7	8	8	8	8	8

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ANALYTICAL SUBCELLULAR FRACTIONATION OF RAT PITUITARY HOMOGENATES, WITH SPECIAL REFERENCE TO PROLACTIN PROTEOLYSIS BY LYSOSOMES

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Prolactin proteolysis by rat pituitary homogenates was assayed by measuring the release of trichloroacetic acid-soluble peptides from ^{125}I-labelled rat prolactin. There was a distinct optimum at pH 4.3, with only trace amounts of activity at neutral and alkaline pH. Rat pituitary homogenates were subjected to analytical subcellular fractionation by sucrose density gradient centrifugation in a Beaufay automatic zonal rotor. The principal organelles were characterized by their respective marker enzymes, including: cytosol (lactate dehydrogenase); plasma membrane (5′-nucleotidase); lysosomes (*N*-acetyl-β-glucosaminidase, β-glucuronidase); mitochondria (particulate malate dehydrogenase); endoplasmic reticulum (neutral α-glucosidase); prolactin granules (radioimmunoassayable prolactin). Acid prolactin protease had a similar distribution to the lysosomal marker enzymes. A localisation of the activity to lysosomes was confirmed by subcellular fractionation experiments in which the lysosomes were selectively disrupted with low concentrations of the membrane perturbant, digitonin. Experiments with specific inhibitors of the lysosomal cathepsins indicate that both cathepsins B and D are implicated in pituitary prolactin proteolysis.

Introduction

In addition to the known processes regulating prolactin synthesis in the lactotroph cells of the anterior pituitary [1], it has also been suggested that there is a secondary mechanism, known as crinophagy, for the disposal of excess hormone granules. Smith and Farquhar [2] provided evidence for this process by demonstrating morphologically the fusion of prolactin granules with lysosomes; the granule contents presumably undergoing degradation within the lysosomes. Recent studies [3] demonstrated that the rate of incorporation of [^{3}H]leucine into rat pituitary prolactin was less than anticipated and attributed this to rapid intracellular lysosomal degradation of prolactin. There is however little direct biochemical evidence for this lysosomal degradation process.

The purpose of this study was to investigate, characterize and localise the prolactin-degrading activity in rat anterior pituitary cells and to determine the nature of the individual lysosomal cathepsins involved by the use of selective inhibitors.

Materials and Methods

Animals. The lactating rat was chosen for the source of anterior pituitary tissue because approximately 70% of the cells are lactotrophs [4,5]. Primiparous Sprague-Dawley rats were housed in individual cages with food (41B pellets) and water ad libitum. Following parturition, they were allowed to freely nurse 8–10 pups for 3 days. On the 4th day the mothers were killed by decapitation, the pituitary glands removed, and the posterior pituitary dissected off.

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Preparation of pituitary homogenates. Each anterior pituitary was disrupted by 12 strokes of a loose-fitting (type A) Dounce homogenizer containing 3 ml ice-cold 0.25 mmol/l sucrose, 1 mmol/l EDTA disodium salt, pH 7.2, and 20 mmol/l ethanol (sucrose medium) [6]. Samples of the homogenate were either subjected to analytical subcellular fractionation by sucrose density gradient or were stored at −30°C until assayed for enzymic activity, prolactin and protein content.

Subcellular fractionation. Rat anterior pituitary homogenate was centrifuged for 10 min at 800 × *g* at 4°C, the supernatant removed and kept at 4°C. The pellet was resuspended in a further 2 ml sucrose medium with three strokes of the Dounce homogenizer (type A pestle) and recentrifuged. The supernatants were combined and subjected to analytical subcellular fractionation by sucrose density gradient centrifugation with a Beaufay automatic zonal rotor as described by Peters [7]. The resulting 16 gradient fractions were analysed for marker enzyme activities, radioimmunoassayable prolactin content and prolactin protease. For each marker enzyme optimal assay conditions and linear kinetics were established.

Assay of prolactin protease. Suitably diluted homogenate or density gradient fraction, 0.1 ml, was incubated for 1 h at 37°C with 0.1 ml ^{125}I-labelled prolactin in 0.25 mol/l sodium acetate/actetic acid buffer, pH 4.3, containing 10 mmol/l dithiothreitol and 2 mmol/l EDTA disodium salt. The reaction was stopped by the addition of 2.2 ml ice-cold trichloroacetic acid (150 g/l) and immediately mixed thoroughly with 0.1 ml bovine serum albumin (100 mg/ml). The tubes were kept for 30 min in iced water and then centrifuged at 4°C for 30 min at 1500 × *g*. To 1.5 ml of the resulting supernatant, 0.02 ml of 2.4 mmol/l KI followed by 0.1 ml H_2O_2 solution (30% w/v) were added to oxidise any free iodide to iodine. After standing exactly 5 min at room temperature, the free iodine was removed from the supernatant by two washes with 3.25 ml $CHCl_3$, and 0.75 ml of the supernatant was then counted for radioactivity [8]. Appropriate blanks were performed in all experiments. A correction was made in the calculation of prolactin protease activity for the endogenous prolactin, determined by radioimmunoassay in the homogenate or appropriate fractions, according to the following formula:

ng prolactin degraded/min per mg tissue protein = total prolactin per assay tube × trichloroacetic acid-soluble radioactivity per assay tube/total radioactivity per assay tube × incubation time (min) × mg protein per assay tube.

Prolactin radioimmunoassay. Reagents for the prolactin measurement (NIAMDD rat prolactin I-2, anti-rat prolactin S-5 and rat prolactin RP-1) were generously donated by Dr. A.F. Parlow, NIAMDD, National Pituitary Hormone Distribution Program, NIH, Bethesda, U.S.A. Iodinated prolactin for the radioimmunoassay and prolactin protease assay was prepared with chloramine T according to the NIAMDD procedure (specific activity approx. 100 μCi/μg) and the NIAMDD double antibody procedure was used for the radioimmunoassay of prolactin.

Enzymic analysis. N-Acetyl-β-glucosaminidase (EC 3.2.1.30), β-glucuronidase (EC 3.2.1.31), neutral α-glucosidase (EC 3.2.1.20), lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (1.1.1.37), [6], 5′-nucleotidase (EC 3.1.3.5) [9] and protein [10] were assayed by appropriate methods.

Materials. The following reagents were supplied by Sigma London Ltd., Poole, Dorset: DL-dithiothreitol, bovine serum albumin (fraction V), leupeptin hemisulphate, antipain dihydrochloride, pepstatin A, iodoacetic acid, tartaric acid, L-ascorbic acid and digitonin. Plastic assay tubes (75 × 12 mm) were obtained from W. Sarstedt (U.K.) Ltd., Leicester, and Dounce homogenizers (Kontes Glass Company) from Unisciences Ltd., Cambridge. Dopamine hydrochloride was obtained from Arnar-Stone Laboratories, London, and bromocriptine mesylate was donated by Dr. W.P. Maclay, Sandoz Products Ltd., Feltham, Middlesex.

Results

Prolactin proteolysis. To determine the characteristics of prolactin-degrading activity, rat anterior pituitary homogenates were incubated with ^{125}I-labelled prolactin over a range of pH values. A single peak of prolactin-degrading activity occurred at pH 4.3 with low levels of activity at neutral or alkaline pH (Fig. 1). Control experiments showed that no significant degradation (under 0.001%) occurred with sucrose medium or with homogenate that had been

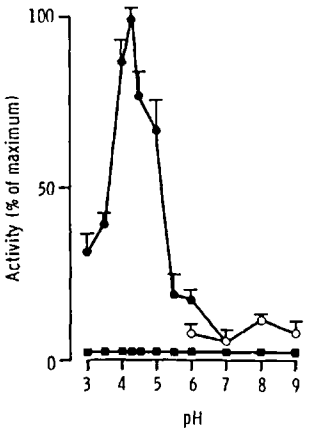

Fig. 1. pH Dependence of rat prolactin degradation. ^{125}I-labelled prolactin (100 μCi/μg) was incubated at various pH values for 90 min with either sucrose medium (▪) or pituitary homogenate (•, ○). A sodium acetate/acetic acid buffer (final concentration, 0.1 M) was used between pH 3 and 7 and a Tris-HCl buffer (0.1 M) between 6 and 9. Results are expressed as means ± S.E. for between three and five replicate determinations.

heated at 100°C for 5 min. All subsequent experiments were carried out at 37°C and pH 4.3. Prolactin degradation was found to be linear for at least 4 h (Fig. 2). Apparent saturation was achieved with amounts of prolactin greater than 1 μg/assay tube (Fig. 3). Fig. 4 shows a linear relationship between the amount of prolactin degraded and the concentration of pituitary homogenate.

Assays were routinely performed with 0.5–1 μg/

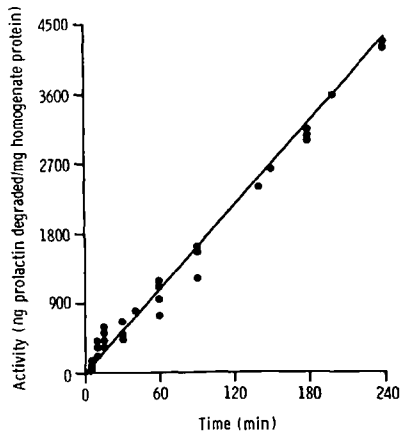

Fig. 2. Variation of degradation of rat prolactin with time.

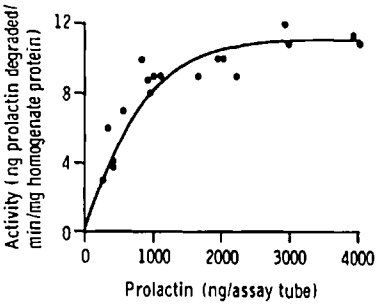

Fig. 3. Effect of varying prolactin concentration on the rate of degradation of rat prolactin.

assay tube. However, since less than 1% enzymic degradation was achieved, linear kinetics were obtained. It was not possible to obtain sufficient unlabelled purified rat prolactin to ensure that saturaring levels of substrate were achieved in all experiments.

Subcellular fractionation of rat pituitary homogenates. Analytical subcellular fractionation studies showed that certain organelles, as well as the prolactin granules, could be resolved (Fig. 5). The frequency distribution of *N*-acetyl-β-glucosaminidase and β-glucuronidase, both lysosomal markers, are almost identical. Prolactin proteolysis shows a similar modal density but has a different distribution pattern, which may reflect lysosomal heterogeneity. In addition, particulate malate dehydrogenase, a mitochondrial enzyme marker, was located as a distinct peak with a density of 1.23. Fractionation of a rat pituitary homogenate in sucrose medium containing 0.2 mmol/l digitonin, a selective lysosomal perturbant, resulted in a marked change in the distribution

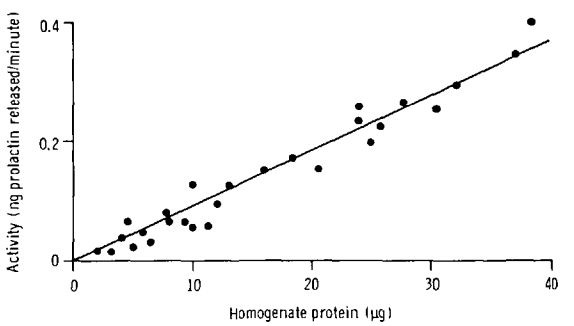

Fig. 4. Variation of activity with amount of pituitary homogenate protein.

pattern of *N*-acetyl-β-glucosaminidase and β-glucuronidase as well as of the prolactin-degrading activity so that activities were now mainly located in the soluble

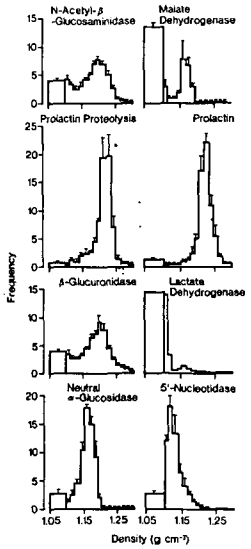

Fig. 5. Isopycnic centrifugation of 6000 X *g*/min supernatant from rat anterior pituitary homogenate. Frequency ± S.D. is defined as the fraction of total recovered activity in the gradient fraction divided by the density span covered. The activity over the abscissa interval 1.05–1.10 represents enzyme remaining in the sample layer and is presumed to reflect soluble activity. The mean percentage of activity recovered, with number of specimens analysed in parentheses, are: *N*-acetyl-β-glucosaminidase, 84 (5); malate dehydrogenase, 90 (3); prolactin proteolysis, 77 (4); prolactin, 75 (6); β-glucuronidase, 74 (5); lactate dehydrogenase, 114 (2); neutral α-glucosidase, 98 (4); 5'-nucleotidase, 89 (4).

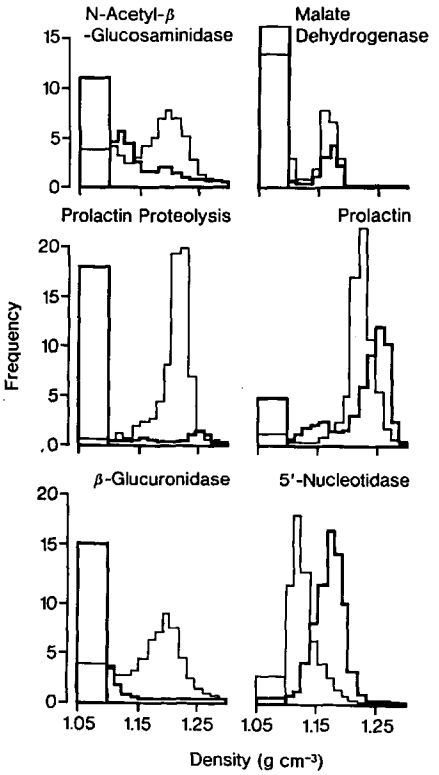

Fig. 6. Isopycnic centrifugation of 6000 X *g*/min supernatant from rat anterior pituitary homogenized in iso-osmotic sucrose containing 0.2 mmol/l digitonin. Averaged control data from Fig. 5 are shown by thin lines. Details are as in Fig. 5. The percentage recoveries (digitonin-treated) are: *N*-acetyl-β-glucosaminidase, 96; malate dehydrogenase, 72: prolactin proteolysis, 102; prolactin, 132; β-glucuronidase, 83; and 5'-nucleotidase, 90; Control, ———; digitonin-treated, ———.

fractions. The prolactin granules and particulate malate dehydrogenase activities were not disrupted by the digitonin treatment (Fig. 6).

Effect of specific cathepsin inhibitors on prolactin proteolysis. To determine which cathepsins were responsible for prolactin proteolysis, the effect of specific lysosomal cathepsin inhibitors was examined (Fig. 7). Pepstatin A (5 μM), a known cathepsin D (EC 3.4.23.5) and cathepsin E (EC 3.3.23.–) inhibitor [11] completely blocked the degradation of prolactin. Note that pepstatin was dissolved in methanol, final concentration in incubation mixture 85 mmol/l, and values for this inhibitor should be compared with the methanol control. Similarly, antipain (20 μM) an inhibitor of cathepsins A (EC 3.4.16.1) and B (EC 3.4.22.1) [12] and iodoacetic acid (10 mM), an inhibitor of cathepsins A, B and C (EC 3.4.14.1) [13,14] also blocked prolactin proteolysis. Leupeptin (35 mM), an inhibitor of the endopeptidase cathepsin B [15], was slightly less effective but still caused 90% inhibition.

In addition to the specific lysosomal inhibitors, the effects of two prolactin secretion inhibitors, dopamine and the dopamine agonist bromocriptine, were investigated in the prolactin protease assay. In three separate experiments, dopamine (1–100 ng/ml) and bromocriptine (0.1–100 μg/ml) had no effect on the enzymic degradation of prolactin.

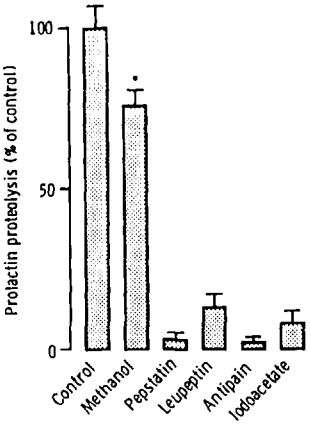

Fig. 7. Effect of catheptic inhibitors on the proteolytic degradation of ^{125}I-labelled prolactin. The mean ± S.E. of four observation is shown. *, control incubations for pepstatin, which was dissolved in methanol (final conc. 85 mmol/l).

Discussion

The present study characterizes the principal organelles from rat pituitary by analytical subcellular fractionation of tissue extracts with sucrose density gradient centrifugation. Good resolution of the plasma membrane mitochondria and lysosomes is obtained. The separation of lysosomes and prolactin granules on the sucrose gradients is small but they are clearly distinct. Their separate identities are confirmed by the centrifugation of tissue extracts which had been prepared in the presence of low concentrations of digitonin. The lysosomes are selectively disrupted and their enzymes are released into the soluble fraction. The granule density is increased somewhat and, thus, like plasma membrane, must contain significant amounts of cholesterol. A similar response to digitonin loading has been noted for certain leukocyte granules [16]. It is likely that a simple preparative procedure for the isolation of pure pituitary granules could be developed from this observation. Previous reports have also shown good preservation of pituitary cell granule integrity during sucrose density gradient centrifugation [17–20]. Their observed values for the equilibrium density of the granules are similar to those found in the present study.

The present results clearly show that lysosomal proteases are the major enzymes responsible for the intracellular degradation of prolactin in the pituitary. Previous studies by Adams and Smith [21] showed that aqueous extracts of hog anterior pituitary degrade haemoglobin and found two distinct pH optima of 3.8 and 8.3. The isolated acid protease was found to degrade both prolactin and growth hormone [22] but subcellular localisation studies were not performed. Only trace amounts of a neutral protease were detected in the present study and there was insufficient activity in the tissue extracts to characterise this activity further.

The studies with the selective cathepsin inhibitors clearly show that both capthepsins B and D are necessary for prolactin degradation. Inhibition of either activity almost completely abolished prolactin degradation. Similar studies [15] on the proteolysis of albumin by rat liver lysosomes have shown that cathepsin D was not important in the degradation of this protein or certain other proteins. However, Leake and Peters [8] in their studies of the degradation of the protein moiety of low density lipoprotein by smooth muscle cell lysosomes found that both cathepsins B and D were implicated in the proteolysis. Cathepsins A and C clearly have only a minor role in lysosomal proteolysis of intact prolactin.

Studies with dopamine and the dopamine agonist bromocriptine show that although these drugs appear to increase prolactin degradation by rat pituitary [23,24] they do not directly stimulate lysosomal cathepsins. These agents, therefore, are presumed to facilitate crinophagy, possibly by enhancing secretory granule-lysosome fusion. Although crinophagy was postulated as a process whereby excess hormone within secretory granules could be disposed of, there was little biochemical evidence to support this mechanism. The original observations of Smith and Farquhar [2] demonstrated acid phosphatase reaction product in fused lysosomes and granules and this view has been recently restated [25]. Further evidence for this process has been provided by cytochemical studies in a patient with a silent corticotroph cell adenoma [26] and by biochemical assay of several lysosomal enzymes, including acid prolactin protease in rats with experimentally induced mammotroph involution [27]. Clearly, further studies on the properties and kinetic of lysosomes in pituitary cells are indicated as their role in the control of hormone secretion is achieving increasing importance in both physiological and pathological situations [28].

Acknowledgments

We acknowledge the expert technical assistance of Mr. P. White, the NIAMDD, National Institute of Health, U.S.A. for the supply of reagents for the rat prolactin assay and financial support for L. De M. from Coordenaçao de Aoerfeiçoamento e Pesquisa de Pessoal de Nivel Superior, (CAPES), Brazil.

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Hormone Secretion by Human Somatotrophic, Lactotrophic, and Mixed Pituitary Adenomas in Culture*

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Endocrine Unit, Department of Medicine and the Department of Histochemistry (S.V.N.), Royal Postgraduate Medical School, London, United Kingdom

ABSTRACT. Hormone secretion by short and long term explant cultures from needle aspiration biopsies of human pituitary adenomas was measured. The results were compared with similarly studied specimens obtained by transsphenoidal hypophysectomy and related to tumor immunocytochemistry and the clinical presentation. There was a high degree of correlation between the pattern of hormone secretion from the pituitary biopsies, the hormones found in the tissue, and the clinical presentation of the patient. In the five aspiration biopsies studied, evidence was obtained that two adenomas secreted solely PRL, one secreted only GH, and two secreted a mixture (one with a preponderance of GH over PRL, and one with a preponderance of PRL over GH). In the latter, the GH and PRL were secreted by separate cells. The three specimens from transsphenoidal hypophysectomies secreted PRL, GH, LH, FSH, and TSH and contained both neoplastic and normal pituitary tissue.

In long term culture, GH, LH, FSH, and TSH secretion declined rapidly. PRL secretion from prolactinomas initially remained constant or, in a few cases, increased, but after 20 days again declined, although PRL remained detectable for up to 110 days.

It is concluded that short term culture of material obtained from the center of a pituitary by biopsy, but not necessarily specimens obtained by transsphenoidal hypophysectomy, can provide definitive information regarding secretory activity of the adenomas. (*J Clin Endocrinol Metab* **48:** 108, 1979)

CULTURES of surgically removed human pituitary adenomas have been shown to secrete not only GH but also smaller amounts of LH and TSH (1), indicating that such specimens often contain both neoplastic and normal pituitary tissue. Thus, definitive assessment of tumor activity *in vitro* under these circumstances is impaired by the cellular heterogeneity of the specimens. The purpose of the present study was to isolate human pituitary adenoma tissue by the use of biopsies obtained during implant of ^{90}Yttrium for therapeutic ablation. As the objective of the procedure is to place the radioactive seed into the center of the adenoma, needle aspiration biopsies obtained at this time should not be contaminated with normal tissue. Hormone secretion in short and long term culture and tissue immunocytochemistry in these biopsies were assessed and compared with specimens removed by transsphenoidal hypophysectomy.

Materials and Methods

Pituitary tumor tissue was obtained by needle aspiration from three women with hyperprolactinaemic-amenorrhoea (cases 1, 2, and 5) and two males with active acromegaly (cases 3 and 4) undergoing ^{90}Yttrium implant (2) for ablation of a radiologically evident pituitary adenoma. These specimens are referred to as biopsies in this report and are routinely taken for histological, immunocytochemical, and electron microscopic examination to establish the type and activity of the tumor. An additional three adenomas were obtained by transsphenoidal surgery from two males with active acromegaly (cases 6 and 7) and a woman with hyperprolactinaemic-amenorrhoea (case 8). These are referred to as surgical specimens. Basal serum PRL, GH, LH, FSH, and TSH were measured preoperatively in all patients. The GH values quoted are the minimum achieved during oral glucose suppression, and the PRL values are the means of at least four samples obtained under basal conditions on separate occasions.

Biopsy material was examined immediately after aspiration in a sterile Petri dish; a portion was transferred to medium for the culture experiments. The rest of the tissue was used for electron microscopy and immunocytochemistry. Surgical specimens were placed by the surgeon directly into culture medium; a portion subsequently was removed for immunocytochemistry.

Tissue for immunocytochemistry was freeze-dried and fixed in formaldehyde or *p*-benzoquinone vapor (3) before being embedded in wax. Five-micrometer secretions were used for immunostaining. Where possible, this was also carried out on 1-μm sections of resin-embedded tissue after fixation in neutral buffered formalin (2 h) or for a short time (5–15 min) in 2.5% vacuum-purified glutaraldehyde without postfixation in os-

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mium tetroxide. An indirect immunofluorescence technique was used (4). Rabbit antisera to human GH and ACTH (Wellcome Reagents, UK), and to human PRL, LH, FSH, TSH (NIAMDD) were used as the first layers at dilutions ranging from 1:200–1:1,000 and incubated at 4 C for 24 h. Fluorescein-conjugated goat antirabbit globulin (Hyland or Miles Laboratories) was then applied at dilutions of 1:10–1:40 for 1 h at room temperature. All antisera were diluted in 0.01 M phosphate-buffered normal saline. Controls for immunostaining included replacement of the first reactant with normal rabbit serum and, in previous experiments, establishment of the specificity of the antisera by loading with an excess of the pituitary hormones. The immunocytochemical analyses were reported independently of the results from the culture experiments.

For the culture experiments, aseptic conditions were employed. The adenoma tissue was minced into cubes of approximately 1 mm^3 and distributed in culture tubes (LABCO S100 WC) containing 2 ml culture medium (Eagles MEM and Hepes buffered with 10% fetal calf serum) and incubated at 37 C. The amount of tissue obtained and cultured from each patient was not standardized. The experiments were started on the third day after setting up the cultures and after a complete medium change and wash of the tissue in order to reduce any possibility of the results being influenced by hormone leakage from damaged cells. The short term culture experiments were then carried out over two consecutive periods of 3 days each (periods I and II), with a complete medium change at the end of each period. Culture tubes were centrifuged at 500 × *g* for 5 min to assist the removal of medium and retention of tissue. For the long term cultures, medium was similarly changed every 3 or 4 days throughout the experimental period and stored at −20 C for hormone assay.

RIA of PRL was carried out by a double antibody technique, as previously described (5), using human PRL VLS 1 as standard, VLS 3 for iodination, and anti-VLS 3 as antiserum. Intra- and interassay variations were 8.2% (n = 5) and 11.3% (n = 6), respectively, and the detection limit was 150 pg PRL/tube. GH was measured by RIA (6) using WHO First International Reference Preparation (MRC 66/217) as standard. Intra- and interassay variations were 5.4% (n = 6) and 13.7% (n = 21), respectively, and the assay could detect 0.05 μIU (0.025 ng) GH/tube. LH, FSH, and TSH were measured by previously described methods (7–9) using MRC 69/104 as standard for LH and FSH. MRC 69/104 has an assigned potency of 10 IU FSH and 25 IU LH/ampoule in terms of the Second International Reference Preparation of human menopausal gonadotropins and contains (by RIA) the equivalent of 485 μg NPA LER 907, from which it is derived (10). MRC 68/38 was used as standard for TSH. Samples were assayed at multiple dilutions in duplicate. It was found that the hormones in the culture medium diluted in a parallel manner to the standard in each assay. There was no cross-reaction between assays at the sample concentrations measured, nor were hormones detected in the culture medium at the dilutions employed.

Results

Pretreatment clinical details and the serum hormone levels for each patient are shown in Table 1. All patients, except one, demonstrated hypersecretion of either GH or PRL, in accordance with their clinical diagnosis. The exception was case 4, who presented with acromegaly yet without galactorrhoea and had a serum GH of 360 μIU/ml as well as an elevated serum PRL of 260 ng/ml. Since this patient had a large tumor, it was not apparent preoperatively whether the hyperprolactinemia resulted from secretion of PRL by the tumor itself or from compression of the pituitary stalk and reduction in the amount of PRL release-inhibiting factor (PIF) reaching the anterior pituitary. An air encephalogram showed no supressellar extension of the tumor. Serum TSH was normal in all patients and none were clinically or biochemically hypothyroid. Gonadotropin levels were normal in three patients (two prolactinomas and one acromegalic), partially reduced in three subjects (two acromegalics and one prolactinoma), and subnormal in two

TABLE 1. Clinical diagnosis and serum hormone levels of subjects with pituitary adenomas

Subject no.	Sex	Age (yr)	Diagnosis	GH (5 μIU/ml)[a]	PRL (5–25 ng/ml)	LH (3–8 mIU/ml)	FSH (2–8 mIU/ml)	TSH (<4 μU/ml)
Pituitary implants								
1	F	33	Amenorrhoea, galactorrhoea and headache	<1.0	300	5.0	2.5	<1.0
2	F	25	Amenorrhoea and galactorrhoea	6.2	450	<1.0	<1.0	1.1
3	M	32	Acromegaly and headache	28.5	4.7	2.2	3.5	1.6
4	M	45	Acromegaly and diabetes	360	260	1.7	1.4	<1.0
5	F	22	Amenorrhoea and galactorrhoea	<1.0	470	1.2	4.7	2.1
Transsphenoidal hypophysectomy								
6	M	52	Acromegaly and diabetes	36.5	5.2	4.6	6.5	<1.0
7	M	50	Acromegaly	140	10	1.5	2.8	<1.0
8	F	34	Amenorrhoea, galactorrhoea and headache	<1.0	680	6.5	6.2	2.6

[a] Normal range. For GH this is the minimum value during a 50-g oral glucose tolerance test with the exception of that for subject 2, which is a random value.

patients (one prolactinoma and one acromegalic).

Table 2 documents the hormone secretory patterns in short term tissue culture of the biopsies and cultures of the surgically removed specimens over the two consecutive 3-day periods. For each of the biopsies, with the exception of case 5, the only hormones found in the culture medium were those that had been hypersecreted *in vivo*, as indicated by elevated serum levels. Thus, biopsies from cases 1 and 2, who presented with amenorrhoea-galactorrhoea, secreted large (microgram) quantities of PRL in both culture periods, whereas no GH, LH, FSH, or TSH was detected. Immunocytochemical staining was similarly positive only with antisera to PRL and was negative to all other pituitary hormones including ACTH. The biopsy from the acromegalic patient (case 3) secreted only GH, in agreement with the sole elevation of serum GH and immunostaining with antisera to GH. However, the biopsy from the other acromegalic (case 4) secreted large (milligram) amounts of GH as well as microgram amounts of PRL, consistent with the preponderance of GH cells and the lesser number of PRL cells demonstrated by immunocytochemistry. Together these results confirmed that the increased serum levels of GH and PRL in this patient arose as a direct consequence of hypersecretion by a mixed pituitary adenoma.

The cultured material from the biopsy of the other prolactinoma patient (case 5) secreted large amounts of PRL as well as small but significant quantities of GH. Only the serum PRL was raised in this patient preoperatively. Immunocytochemistry of 1-μm sections (Fig. 1) demonstrated PRL immunofluorescence in the majority of cells. Certain cells, an example of which has been indicated, did not stain for PRL but did stain for GH in the serial section (Fig. 2), providing confirmatory evidence of a mixed tumor with a preponderance of PRL cells.

In contrast to the selective hormone secretory patterns

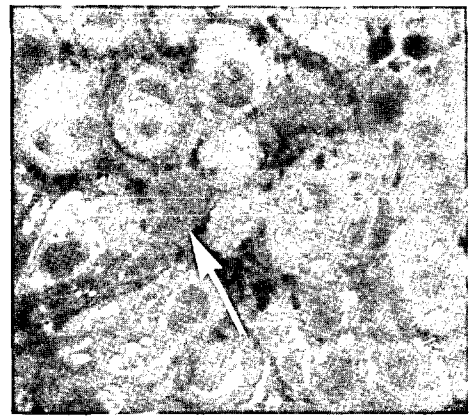

FIG. 1. Immunostaining of a 1-μm section of the biopsy of the adenoma of case 5, a woman with hyperprolactinemic-amenorrhoea but normal serum GH (×625). PRL immunofluorescence is shown over the Golgi area of the adenoma cells. One cell (indicated by the arrow) is unstained. Fixation was carried out in 2.5% vacuum-purified glutaraldehyde (pH 7.0) for 5 min, followed by embedding in araldite. Exposure to PRL antisera was for 24 h at 4 C and was followed by exposure to conjugated goat antirabbit globulin for 1 h at room temperature.

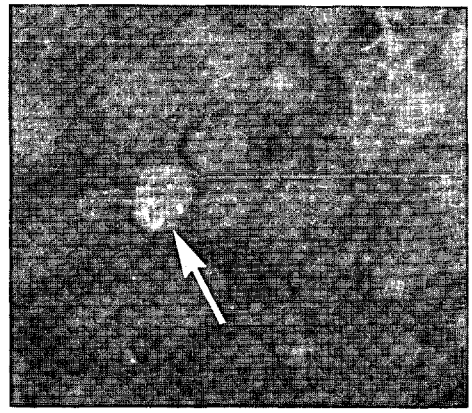

FIG. 2. The serial section to Fig. 1, immunostained for GH (×625). The only positively stained cell (indicated by the arrow) is that which did not stain for PRL.

TABLE 2. Hormone secretion by human pituitary adenoma in short term culture

Subject no.	PRL (μg)		GH (mIU)		LH (IU)		FSH (IU)		TSH (mU)		Immunocytochemistry[b]
	I[a]	II	I	II	I	II	I	II	I	II	
Needle aspiration biopsy											
1	92	123	ND[c]	ND	ND	ND	ND	ND	ND	ND	+++ PRL
2	10	4.25	ND	ND	ND	ND	ND	ND	ND	ND	+++ PRL
3	ND	ND	44.1	25.2	ND	ND	ND	ND	ND	ND	+++ GH
4	19.2	21.0	2.87×10^3	2.27×10^3	ND	ND	ND	ND	ND	ND	++ PRL, +++ GH
5	294	90.3	0.25	0.33	ND	ND	ND	ND	ND	ND	+++ PRL, + GH
Transsphenoidal hypophysectomy											
6	2.45	0.85	77	51.5	3.7	1.5	3.1	1.2	4.1	1.4	+++ GH
7	4.5	0.66	315	35.4	12.5	3.3	12.9	0.47	33.2	1.88	+++ GH
8	50.5	35.0	91	69	1.43	0.66	2.17	1.56	15.0	6.50	+++ PRL

[a] Cultures were incubated for two consecutive periods (I and II) of 3 days; values represent total radioimmunoassayable hormone in the culture medium during each of these periods.

[b] Refers to staining in the complete specimen for the biopsies (being negative for all other anterior pituitary hormones), but only refers to the adenoma itself for the transsphenoidal hypophysectomy specimens, since the latter all contained normal tissue (which stained for all of the anterior pituitary hormones). +, Relative number of positively staining cells.

[c] Not detectable.

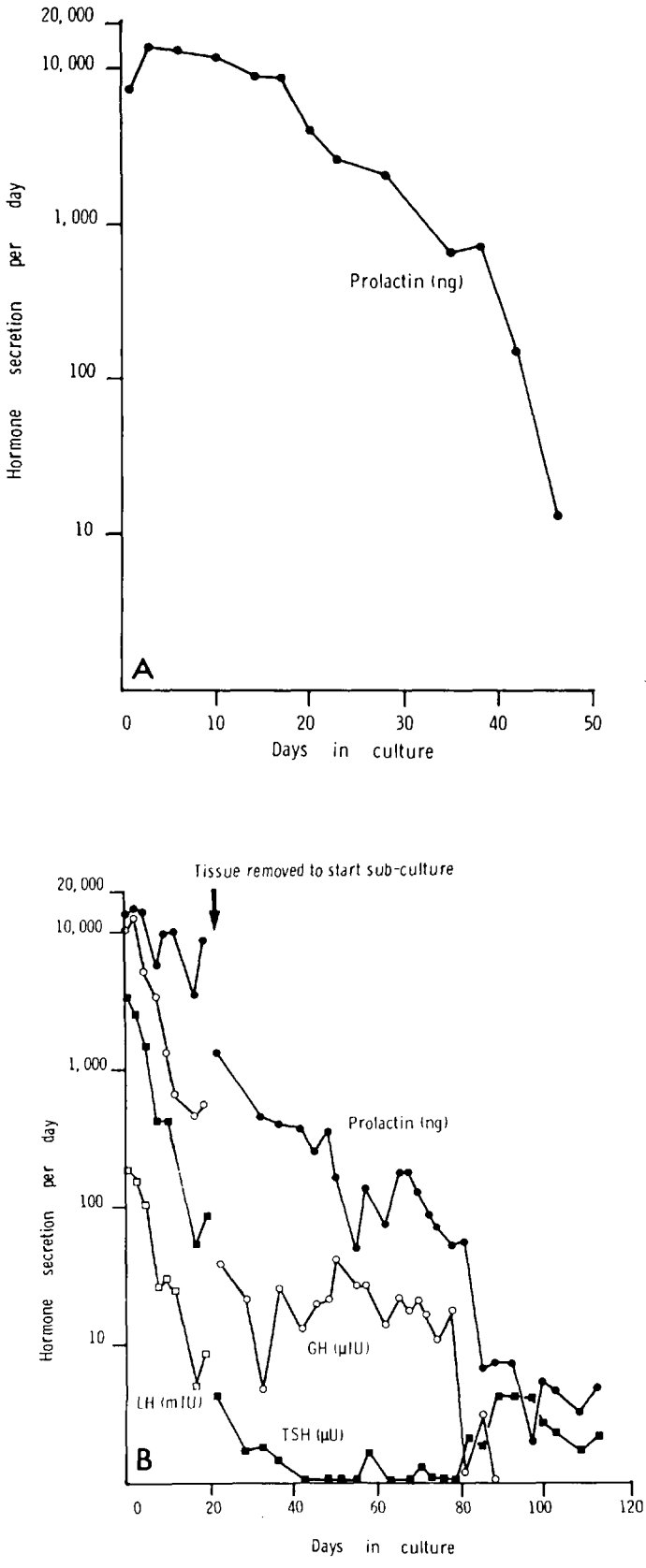

FIG. 3. A) PRL secretion in one of two cultures of the biopsy of the adenoma of case 1, a woman with hyperprolactinemic-amenorrhoea. Medium was changed every 3 or 4 days, and the radioimmunoassayable hormone secretion is represented as the mean output per 24 h. No other pituitary hormones were detected in either of the cultures at any time, and positive immunostaining of the original tissue was obtained only with PRL. B) Hormone secretion by one of two cultures of the adenoma obtained by surgical hypophysectomy from case 8, a woman with hyperprolactinemic-amenorrhoea. PRL, GH, LH, and TSH were secreted. Normal and neoplastic tissues were demonstrated morphologically, and positive immunostaining was obtained only for PRL in the adenoma.

observed above in the cultures from the biopsy specimens aspirated from the central areas of the tumors, the tissue obtained by a surgical approach secreted large amounts of GH, PRL, LH, FSH, and TSH in all three cases (Table 2). GH secretion predominated in the two acromegalics, whereas similar amounts of GH and PRL were secreted by the prolactinoma patient. The presence of both neoplastic and surrounding normal pituitary tissue in each case was confirmed by histological, immunocytochemical, and ultrastructural examination. In those areas where the tumor cells were identified, positive immunocytochemistry was obtained only for GH in the acromegalics and only for PRL in the prolactinoma.

The amount of each tumor hormone secreted in short term culture for the most part declined over the 6-day period of observation (Table 2), although PRL secretion increased in two of the seven cultures where it was found. Two prolactinomas were cultured for longer periods, one being obtained by biopsy (case 1) and the other surgically (case 8).The results are shown in Fig. 3. In both cultures, secretion remained relatively constant for a period of 20 days before declining. In the surgically removed specimen where there was secretion of the other anterior pituitary hormones, these declined more quickly so that PRL secretion predominated and was still detectable after 110 days.

Discussion

The use of immunocytochemical techniques to establish the existence of pituitary adenomas secreting solely GH and PRL and those secreting both hormones in differing proportions has been extensively documented (11–14). Zimmerman *et al.* (11) examined 21 pituitary adenomas and demonstrated a high degree of correlation between the elevation of hormones in plasma and their presence of tumor cells. Five of the 21 adenomas were of the mixed type and only 1 of these had a preponderance of PRL- over GH-staining cells. Corenblum *et al.* (12) reported on 6 mixed adenomas from patients with clinical acromegaly, but these authors were unable to determine whether one cell type showed preponderance. Halmi and Duello (13) examined 28 acidophilic pituitary tumors; 8 showed no immunostaining, 11 stained only for PRL, 3 stained only for GH, 5 contained mostly GH but some

PRL cells, and 1 contained predominantly PRL but some GH cells.

These findings are confirmed by the results of immunocytochemical staining in the present study which demonstrated two tumors containing solely PRL cells, one containing solely GH cells, and two mixed GH and PRL adenomas (one with a preponderance of GH and the other with a preponderance of PRL cells). Confirmation that these hormones are being secreted by the adenoma has been obtained by examination of the hormone secretory patterns in short term primary explant culture, a technique previously applied by Guyda *et al.* (14) to demonstrate active secretion of both GH and PRL from a single mixed pituitary adenoma. In each case in the present study, the pattern of hormone secretion into the medium was consistent with the classification arrived at by immunocytochemistry. This close correlation was particularly exemplified in case 3, who presented with amenorrhoea and galactorrhoea and a raised serum PRL. Large amounts of PRL together with very much smaller but significant quantities of GH were found in the culture medium, in agreement with the large number of PRL and very small number of separate GH cells found in the biopsy by immunostaining. Since random (morning) and glucose-suppressed serum GH levels were undetectable in this patient, it must be concluded that the activity of these few GH cells in the mixed adenoma was insufficient to raise serum GH levels or manifest itself clinically. Thus, the biopsy specimens obtained by needle aspiration during implant of ^{90}Yttrium appear to represent isolated human pituitary adenoma tissue. This was clearly not the case, however, for the surgically removed specimens, all of which secreted PRL, GH, LH, FSH, and TSH in significant quantities. These results confirm the earlier work of Kohler *et al.* (1) and illustrate the potential difficulties of using such heterogeneous preparations *in vitro* for interpretation of the hormonal activity of a tumor.

In the few previous studies where GH secretion from human pituitary adenomas in long term culture has been examined, levels have always been found to decline (1, 15–17). The present results are consistent with those findings and confirm that LH, FSH, and TSH decline in a similar manner (1). There have been few studies of PRL secretion by human pituitary tumors in culture. Since PRL secretion is considered to be under the dominant inhibitory regulation of the hypothalamus, an increase in PRL secretion might be anticipated and has been found to occur in studies using human fetal pituitaries (18, 19). In the present studies, PRL secretion generally remained stable for periods up to 20 days, although it did increase in two of seven short term cultures but again eventually declined. In a recent report appearing since initial submission of this paper, Peillon *et al.* (20) found that PRL secretion increased in some explant cultures of somatotrophic tumors, although in only one of four cultures was this a consistent finding and there was considerable variability in results from explants of the same adenoma. It is likely, therefore, that there is a variable degree of cell dedifferentation or tissue necrosis over the longer term, and further work will be required to establish optimal culture conditions if such preparations are to be used for investigation of the cellular mechanisms controlling hormone secretion by human pituitary tumors.

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