SOME CLINICAL AND LABORATORY STUDIES OF LARGE PITUITARY TUMOURS TREATED WITH DOPAMINE AGONISTS

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A C K N O W L E D G E M E N T S

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D E C L A R A T I O N

This thesis is submitted for the Degree of Doctor of Medicine at the University of Edinburgh. It has not been previously submitted either wholly or in part for a degree at this or any other University. The experimental work reported in the main body of the thesis was performed by the author without technical assistance, and the thesis was composed by the author alone. The histological techniques were the work of others and are included as an appendix.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

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The patient with a large pituitary tumour presents a number of management problems and conventional treatment with surgery and radiotherapy is unsatisfactory. Reports between 1978 and 1982 showed that some tumours regress during dopamine agonist (DA) therapy, though this does not produce a permanent cure (Chapter 1). The aim of the present work was to clarify the effect of DA therapy on different types of large pituitary tumour and to characterise cells from both responsive and unresponsive tumours.

Three conclusions resulted from the clinical studies (Chapter 2): (1) Most macroprolactinomas shrink during DA therapy but the shrinkage is often asymmetrical and results in fibrosis during long-term therapy, both of which hamper subsequent surgery. (2) Disconnection hyperprolactinaemia may be considerable and lead to inappropriate DA therapy for non-adenomatous lesions. (3) Non-functioning tumours do not regress during DA therapy.

Tumour cells were characterised by tumour cell perifusion (methods described in Chapters 3 and 4), dopamine receptor measurement using [^{3}H]spiperone as radioligand (methods described in Chapter 5) and immunocytochemistry. Prolactin secretion rates from bromocriptine-treated macroprolactinomas were greatly reduced compared with untreated tumours. Most prolactinomas showed dose-related inhibition of prolactin secretion by dopamine and bromocriptine but one tumour was partially bromocriptine resistant in-vivo and in-vitro (Chapter 6). Fifty percent of the non-functioning tumours did not contain, secrete or immunostain for any known anterior pituitary peptide. The remainder secreted small amounts of gonadotrophins or alpha subunit, but the secretion was not inhibited by dopamine, immunostaining was confined to <10% of cells and tumour contents were low (Chapter 7).

Despite their failure to regress during bromocriptine treatment, non-functioning tumours were shown to possess similar dopamine receptors to prolactinomas and normal anterior pituitary (Chapter 8). Using a novel immunoassay, bromocriptine was shown to be bound to non-functioning tumour dopamine receptors in-vivo. In contrast, two TSH-secreting adenomas were also unresponsive to dopaminergic manipulation but lacked membrane-bound dopamine receptors (Chapter 9).

From these results, future clinical practice demands greater care in selecting patients for dopamine agonist or surgical treatments. The finding of dopamine receptors in non-functioning tumours suggests future research on these enigmatic tumours aimed at defining the cell type represented and post-dopamine receptor mechanisms (Chapter 10).

CHAPTER 1. INTRODUCTION

A. HISTORICAL ASPECTS OF PROLACTIN AND BROMOCRIPTINE

1. Prolactin: identification and measurement

"A galactorrhoeic non-pregnant woman is expected to have amenorrhoea"

[Hippocrates (460-375 BC) Aphorisms, V39]

The frequent association of galactorrhoea with amenorrhoea has been rediscovered since Hippocrates. In the mid-nineteenth century Chiari described the post-partum amenorrhoea-galactorrhoea syndrome and one hundred years later Argonz and del Castillo (1953) reported "a syndrome characterised by oestrogen deficiency, galactorrhoea, amenorrhoea and decreased urinary gonadotrophins". Forbes et al (1954) later attributed the condition to a pituitary adenoma but the full characterisation of prolactin, the anterior pituitary hormone responsible for the above syndromes, has only been possible over the past fifteen years.

During the 1950s and 60s a variety of bioassay studies demonstrated the importance of the central nervous system in the control of the "mammotroph hormone" and its central role in lactation was established; Meites et al (1963) showed that prolactin secretion increased when the pituitary gland was removed from intimate contact with the hypothalamus.

The existence of prolactin as a human pituitary hormone distinct from growth hormone (which is also lactogenic) was surmised from histological (Herbert and Hayashida, 1970), biochemical (Lewis et al, 1971) and
Forsyth et al., 1971
biological (Frantz and Kleinberg, 1970) evidence. Final confirmation awaited its isolation and purification from human pituitary glands (Hwang et al., 1972). The development of sensitive radioimmunoassays for prolactin (Hwang et al., 1971; McNeilly, 1973) promoted extensive investigation of prolactin secretion in normal and pathological states.

In 1977 human prolactin was fully sequenced and shown to be a single chain polypeptide of 198 amino acid residues, molecular weight 22554 daltons (Shome and Parlow, 1977). Despite its bioassay similarities to growth hormone, the two hormones exhibit only 16% amino acid sequence homology.

Fractionation of immunoreactive prolactin has revealed heterogeneity both in pituitary extracts (Sinha and Gilligan, 1981; Meuris et al., 1983) and in some peripheral blood samples (Farkouh et al., 1979). "Big" prolactin is probably a dimer and "big-big" prolactin possibly an aggregated form. However there is still controversy regarding the analytical and biological significance of these different forms (Franks, 1983). Most of the prolactin bioactivity in serum samples from prolactinoma patients appears to be due to monomeric prolactin (Rennie et al., 1985).

The human decidua also synthesises and secretes prolactin, apparently identical to the pituitary hormone, and the large amounts in amniotic fluid are derived from this source (reviewed by Franks, 1983). The mRNA coding for human prolactin has been recently identified in this tissue (Taii et al., 1984). The advent of human prolactin cDNA probes, first produced from RNA extracted from prolactin-secreting adenomas (Cooke et al., 1981), heralds a new era of investigation into prolactin gene regulation. The human prolactin gene is about 10 kilobases in length, contains 4 introns and is located on chromosome 6 as a single copy per haploid genome (Martial et al., 1984).

2. Prolactin: secretory mechanisms in the lactotroph

The morphology of the normal lactotroph and the intracellular processing and transport of secretory products have been recently reviewed by

Pelletier et al (1984). The normal human lactotroph contains abundant endoplasmic reticulum, a prominent Golgi apparatus and membrane-bound secretory granules of a diameter ranging between 150-250 nm. Pulse chase experiments and high resolution autoradiography using tritiated amino acids have shown that prolactin is rapidly synthesised (within 30 minutes) by ribosomes attached to the outer surface of the endoplasmic reticulum. Soon after synthesis commences (within 10 minutes) the radiolabelled prolactin is transported to the Golgi apparatus, by a transport mechanism that remains unclear. In the Golgi small immature granules are formed which then mature into the final secretory granules. Pelletier and Labrie (1982) found that 30 minutes after the beginning of incorporation of radiolabelled leucine into rat lactotrophs, about 25% of the radioactivity was associated with mature secretory granules.

The mature secretory granules must migrate to the plasma membrane for prolactin secretion to occur and little is known of the factors that influence this granule movement. A microtubule system seems to be involved since inhibitors such as colchicine decrease secretion of prolactin from rat lactotrophs (Antakly et al., 1979). Final prolactin release occurs when the granule membrane fuses with plasma membrane and hormone is extruded into the perivascular space by exocytosis. Increased exocytosis can be observed at the electron microscopic level when lactotrophs in-vitro are exposed to secretagogues such as cAMP (Pelletier et al., 1972). Horseradish peroxidase membrane-labelling experiments suggest that granule membranes are recycled from the plasma membrane back to the Golgi apparatus where they are reused in the formation of new granules (Pelletier et al., 1984).

3. Prolactin: neuroendocrine control of secretion

It has been known for some years that prolactin secretion is under tonic

inhibitory control from the hypothalamus. The major body of evidence which suggests that most of this inhibition is exerted by release of hypothalamic dopamine has been extensively reviewed (Macleod, 1970,1976; Ben-Jonathan, 1980; Leong et al., 1983; de Greef and van der Schoot, 1985). In brief, spontaneous prolactin secretion is inhibited by dopamine (Ben-Jonathan, 1980) acting in small amounts (Foord et al., 1983); dopamine receptors are found on pituitary membranes (Cronin and Weiner, 1979), in particular membranes of lactotrophs (Goldsmith et al., 1979); and dopamine is found in hypophysial stalk plasma in amounts sufficient to inhibit prolactin release (Neill et al., 1981; Bethea, 1985). There is some evidence that dopamine is not the only prolactin inhibiting factor mediating tonic hypothalamic inhibition (de Greef and van der Schoot, 1985). GABA (gamma-amino-butyric acid) can also inhibit prolactin release but controversy remains as to whether this is a physiological or a pharmacological effect (Mulchahey and Neill, 1982; Franks, 1983). Overall, there would seem little doubt that dopamine dominates physiological prolactin inhibitory activity.

Some purified hypothalamic extracts contain prolactin releasing activity and a wide variety of substances have been proposed as hypothalamic prolactin releasing hormones (Leong et al., 1983). Of these TRH and vasoactive intestinal peptide (VIP) have been the most studied and both are present in stalk plasma in concentrations sufficient to provoke prolactin secretion in-vitro. The involvement of TRH in the prolactin responses to suckling will be mentioned again in Chapter 6. VIP-uptake in the pituitary appears exclusively localised to the lactotrophs (Morel et al., 1982) but its role in the physiological regulation of prolactin secretion remains unclear. In-vitro, VIP stimulates prolactin secretion from normal and tumourous lactotrophs (Spada et al., 1983) and the potential use of membrane-bound VIP receptors as a marker of lactotroph-

like cells will be returned to in Chapter 10.

An important aspect in the control of prolactin secretion is the "short-loop" feedback whereby prolactin (which is not dependent on feedback from a target endocrine organ) is able to regulate its own secretion. The work which led to the description of this phenomenon has been reviewed by Peters et al (1982) and suggests that prolactin release by the pituitary is limited by a direct action of prolactin on hypothalamic dopamine turnover, thereby increasing dopamine delivery to the pituitary (Perkins et al., 1979; Foreman and Porter, 1981). This topic is further discussed in Chapter 6.

4. Prolactin-secreting tumours

Following the development of prolactin radioimmunoassays in the early 1970s it became apparent that as many as 70% of pituitary tumours previously considered to be functionless were associated with elevated serum prolactin concentrations (Frantz et al., 1972; Franks et al, 1977). It is probable that a proportion of patients described in these studies had non-functioning tumours causing disconnection hyperprolactinaemia (Nabarro, 1982). Nevertheless human tumours containing prolactin were identified in early immunocytochemical studies (Kovacs et al., 1976).

Prolactinomas in women are not uncommon, but most are less than 10 mm in diameter and referred to as microprolactinomas. Although these patients frequently have normal plain skull radiographs, high resolution computerised tomography reveals small tumours in the majority (Jung et al., 1982). Apparent abnormalities within the pituitary on computerised tomography are not necessarily pathological however, and may represent small cysts, areas of necrosis or artefacts (Chambers et al., 1982). On

the other hand small clinically silent adenomas are a frequent finding at necropsy; Burrow et al (1981) found an overall incidence of 27%, though most were less than 2 mm in diameter, and 41% of the tumours immunostained for prolactin.

The clinical manifestations of hyperprolactinaemia have been extensively reviewed (Franks and Jacobs, 1983; Grossman and Besser, 1985). Hyperprolactinaemia produces hypogonadism in both sexes, though clinically evident prolactinomas are much less common in men, who characteristically present late with larger tumours causing pressure symptoms (Nabarro, 1982).

5. Bromocriptine: development and early clinical studies

In 1967, 2-bromo-alpha-ergocryptine mesylate (bromocriptine), a semisynthetic ergot alkaloid, was specifically developed as an inhibitor of prolactin secretion (Fluckiger and Wagner, 1968). Although introduced into clinical research in 1971, bromocriptine was only later shown to directly stimulate dopamine receptors and to compete with the specific binding of [^{3}H]dopamine to bovine anterior pituitary membranes (Calabro and MacLeod, 1978). Early clinical studies showed the high efficacy of this agent in the treatment of the galactorrhoea/amenorrhoea syndromes with suppression of serum prolactin concentrations resulting in greatly reduced galactorrhoea and reversal of hypogonadism (Besser et al., 1972; Del Pozo et al., 1974; Thorner et al., 1974). The dopaminergic agonist actions of the drug also proved useful in the suppression of puerperal lactation (Rolland and Schellekens, 1973), and as an adjunct to other treatments for acromegaly (Liuzzi et al., 1974; Wass et al., 1977; Martyn and Bevan, 1980) and Parkinson's disease (Calne et al., 1974). In women with microprolactinomas bromocriptine restored ovulatory cycles in 80-90% (Thorner and Besser, 1978), many of whom rapidly achieved

pregnancy (Thorner et al., 1979). The effects of bromocriptine on pituitary macroadenomas will be discussed in the next section.

The pharmacology of bromocriptine has been extensively reviewed (Mehta and Tolis, 1979; Schran et al., 1980; Maurer et al., 1983) and various aspects will be further discussed in the section on bromocriptine measurement (Chapter 3.D). Bromocriptine is rapidly absorbed following oral administration and peak plasma levels are achieved after 2-3 hours. It has a prolonged duration of action on the pituitary and serum prolactin concentrations remain suppressed up to 14 hours after a single dose (Schran et al., 1980; see also Chapter 3.D). The drug is usually administered twice or three times daily to achieve steady-state plasma concentrations in the low nanomolar range (Thorner et al., 1980).

Following the development of bromocriptine as the prototype drug, other ergot alkaloids with dopaminergic agonist activity have been introduced for clinical use. These compounds can be divided into two broad categories, the ergopeptines and the ergolines, both of which contain the tetracyclic ergoline nucleus which confers the biological activity. The ergopeptines, of which bromocriptine is an example, consist of d-lysergic acid linked by an amide bridge to a cyclic tripeptide which is extensively metabolised in-vivo (Rosenthaler et al., 1983). Ergolines are derivatives of the tetracyclic skeleton of lysergic acid. Not surprisingly these compounds have differing pharmacokinetic properties; lisuride is shorter acting than bromocriptine (Chiodini et al., 1981), while pergolide can be administered once daily (Franks et al., 1983). Occasionally a patient intolerant or resistant to one drug may be better suited by another compound (Grossman et al., 1985a; Ahmed and Shalet, 1986). However, bromocriptine remains the most widely prescribed and best characterised dopamine agonist in clinical use.

B. THE PROBLEM OF THE LARGE PITUITARY TUMOUR

The management of the patient with a large pituitary tumour presents a variety of problems. The most frequent complication is upward extension with compression of the visual pathways producing, classically, a bitemporal field loss, though visual loss may be markedly asymmetrical in up to one third of patients (Fahlbusch et al., 1981), and there is frequently a delay of many months before the diagnosis is made. The patient may present with a variety of other tumour mass effects; an extreme suprasellar extension may block the foramen of Munro and result in hydrocephalus, lateral extension into the temporal lobe may cause fits and anterior extension into the frontal lobes may produce personality change. Local invasion or compression of cavernous sinuses may lead to cranial nerve palsies, particularly of the third and sixth nerves, although this occurs mainly in the context of pituitary apoplexy (Wakai et al., 1981). Rarely, macroadenomas metastasize within the central nervous system (Martin et al., 1981; Gasser et al., 1985).

Two tumour types most commonly produce these clinical syndromes; non-functioning tumours in both sexes, and macroprolactinomas particularly in men (Carter et al., 1978; Teasdale, 1983; Esiri et al., 1983). ACTH and growth hormone secreting tumours less often grow to this size and are not considered further in this thesis. Women with prolactinomas tend to present earlier with symptoms of menstrual disturbance or galactorrhoea, and usually have smaller tumours.

Large pituitary tumours may compromise anterior pituitary function either by direct compression of pituitary tissue or by interference with hypothalamic control mechanisms. This is particularly common with the non-functioning tumours, probably reflecting the fact that, as a group, they comprise the largest pituitary tumours (Bevan et al., 1987b). The

hypogonadism frequently present has a number of interrelated components. Firstly there may be direct interference with gonadotrophin release due to pressure effects on hypothalamus or pituitary (Ambrosi et al., 1985). Secondly, hyperprolactinaemia, whether from hypothalamo-pituitary disconnection or tumour secretion, inhibits gonadotrophin pulsatility probably by an action on the hypothalamic GnRH pulse generator (Moult et al., 1982; Ambrosi et al., 1985). Thirdly, prolactin excess may inhibit ovarian (but probably not testicular) steroidogenesis though this remains controversial (McNeilly et al., 1983).

Tumour decompression is the most urgent need when a patient presents with a large pituitary tumour, particularly if vision is impaired. Until recently this was usually achieved by transcranial surgery which frequently produced a good visual result (Elkington and McKissock, 1967; Symon and Jakubowski, 1979). However the operation carries a significant morbidity and, particularly in earlier series, an appreciable mortality (10% overall in the series of Elkington and McKissock, 1967). Furthermore, large tumours are virtually never cured by transcranial surgery alone (Nabarro, 1982) making external radiotherapy essential with the possible long-term prospect of hypopituitarism. Even with this combination therapy the tumour recurrence rate was as high as 8% (Elkington and McKissock, 1967).

Over the past two decades it has become apparent that tumours with large suprasellar extensions can be satisfactorily decompressed via the transsphenoidal route (reviewed by Teasdale, 1983), although in fact this was well recognised by Harvey Cushing in the 1920s (Cushing, 1921). The historical background of the transition from transcranial to transsphenoidal surgery has been reviewed by Rosegay (1981). Transsphenoidal surgery alone, although less traumatic for the patient, seldom cures the large pituitary tumour (Nabarro, 1982; Randall et al.,

1983; Bevan et al., 1987b) and radiotherapy is frequently applied for long-term tumour control.

Most would regard radiotherapy to be unsuitable as sole therapy for the large pituitary tumour. Although tumour mass is reduced in the long-term (Johnston et al., 1986) the effect is too slow for the patient presenting with visual failure. With prolactinomas serum prolactin concentrations take many years to fall and rarely reach normal (Gomez et al., 1977; Kelly et al., 1978; Sheline, 1981; Grossman et al., 1984). Furthermore, radiation-induced hypothalamic damage may lead to increased prolactin secretion from the normal pituitary and make the serum prolactin a poor marker of residual tumour volume (Shalet, 1981; Johnston et al., 1986). Radiotherapy undoubtedly reduces the recurrence rate after surgery (Sheline, 1981) but the possibility of eventual hypopituitarism necessitates repeated endocrine evaluation (Shalet, 1981).

Conventional therapies have therefore a number of major disadvantages. The first suggestion that bromocriptine might cause tumour regression of prolactinomas as well as suppressing prolactin secretion came from a number of case reports in the late 1970s (Corenblum et al., 1975; Nillius et al., 1978, Landolt et al., 1979, McGregor et al., 1979a). These were followed by early prospective studies on larger groups of patients (McGregor et al., 1979b; Wass et al., 1979, 1982; Chiodini et al., 1981; Sobrinho et al., 1981; Prescott et al., 1982; Nissim et al., 1982; Spark et al., 1982) which showed clear radiological evidence of tumour shrinkage in over one half of macroprolactinoma patients treated with dopamine agonists, usually bromocriptine. In a proportion of these medically treated patients it was unclear whether hyperprolactinaemia was due to tumour secretion or to hypothalamo-pituitary disconnection

and a number of non-functioning tumours were probably misclassified as "bromocriptine-resistant prolactinomas".

In tumours that were clearly prolactin-secreting bromocriptine-induced tumour shrinkage frequently produced improvement in visual failure (Chiodini et al., 1981). In contrast to the deleterious effects of transcranial surgery and radiotherapy on anterior pituitary function, dopamine agonist therapy occasionally produced improvement in pituitary function (Prescott et al., 1982; Wass et al., 1982), probably due to relief of pressure on the normal pituitary and to restoration of hypothalamic control. In addition to bromocriptine a number of other dopamine agonist drugs were shown to have a similar effect on macroprolactinomas including lisuride (Chiodini et al., 1981), pergolide (Kendall-Taylor et al., 1982) and, more recently, mesulergine (Grossman et al., 1985a).

The effect of dopamine agonist therapy on the size of pituitary tumours other than prolactinomas was unclear from these early studies. Some reports suggested that a proportion of non-functioning tumours would shrink during such therapy (Johnston et al., 1981; Spark et al., 1982; Wolleson et al., 1982) whereas other workers described probable non-functioning tumours that had not regressed (McGregor et al., 1979b; Wass et al., 1982). Similar uncertainty existed regarding growth hormone-secreting tumours (Wass et al., 1982 and Spark et al., 1982 versus McGregor et al., 1979b).

In the early 1980s some authors were enthusiastically advocating dopamine agonist therapy as the primary management of most patients with large pituitary tumours (Spark et al., 1982; Lancet Editorial, 1982). However uncertainties remained. The most important was whether dopamine

agonist therapy alone provided a permanent cure or whether therapy, such as surgery or radiotherapy, was required for long-term tumour control. A related question was whether significant bromocriptine "resistance" could emerge during long-term medical therapy. Furthermore it was unclear whether tumour size reduction occurred in adenomas that were not prolactin-secreting.

Eversmann et al (1979) demonstrated persistent lowering of prolactin levels in some macroprolactinoma patients after withdrawal of long-term bromocriptine. However serum prolactin concentrations rose in each case, though not to pre-treatment levels, and the mean withdrawal period was only 3 months. Several authors demonstrated prolactinoma re-expansion after withdrawal of bromocriptine, in some cases given for as long as one year (Thorner et al 1981; Nissim et al., 1982). More recent evidence concerning the long-term efficacy of bromocriptine is discussed in Chapters 6 and 10 and suggests that tumourous lactotrophs remain even after seven years of bromocriptine treatment (Johnston et al., 1984).

Up to 30% of macroprolactinomas failed to shrink during dopamine agonist therapy (Chiodini et al., 1981; Franks and Jacobs, 1983). The mechanism of the failure was unclear since serum prolactin concentrations were frequently reduced during therapy and the resistance extended to more than one dopamine agonist (Chiodini et al., 1981). The occurrence of "tumour escape" after initial tumour shrinkage with a dopamine agonist drug appeared to be relatively unusual (Breidahl et al., 1983).

The balance of evidence suggested that additional therapy was required for long-term control of large pituitary tumours after initial dopamine agonist treatment. At the start of my work, there was little guidance on the best type and timing of subsequent treatment. Some suggested that radiotherapy was appropriate to avoid expensive and probably indefinite

treatment with bromocriptine (Edwards and Feek, 1981). However if bromocriptine produced compact tumour shrinkage then it seemed possible that the poor results of transsphenoidal surgery for larger tumours might be improved by dopamine agonist pre-treatment. However asymmetrical tumour shrinkage in some cases (Prescott et al., 1982) raised the possibility that dopamine agonist therapy might produce a more difficult surgical "target", though the literature provided no information on the expected frequency of such shrinkage. There were also few data on the time course of bromocriptine shrinkage; several reports suggested rapid initial tumour regression (Thorner et al., 1981; Chiodini et al., 1981; Nissim et al., 1982) but none described whether this was a continuous process or whether maximum tumour shrinkage occurred after a certain duration of dopamine agonist therapy. A preliminary report suggested that previous bromocriptine therapy might adversely influence surgery for prolactinomas (Landolt et al., 1982), though the tumours described had not been specifically pre-treated in an attempt to improve the surgical results.

The doses of bromocriptine in the early studies were highly variable and ranged from 7.5 mg (Thorner et al., 1981) to 20 mg per day (Prescott et al., 1982), and some workers used doses as high as 60 mg (Wolleson et al., 1982; Wass et al., 1982). These very high doses frequently produce side-effects if not taken during food, which may have affected patient compliance.

The mechanism whereby bromocriptine produces pituitary tumour shrinkage was unknown at the outset of these studies. The anti-mitotic action of the drug had been known for some time (Lloyd et al, 1975), but in slow growing human pituitary tumours it seemed an unlikely explanation for the rapid tumour regression. Bromocriptine-induced tumour infarction was another early explanation but patients treated with bromocriptine showed improvement without clinical evidence of pituitary apoplexy, and this

theory has not received support from subsequent histological studies (Barrow et al., 1984; Esiri et al., 1986). A number of reports in 1982 suggested that tumour regression might be due to lactotroph cell size reduction (Rengachary et al., 1982, Nissim et al., 1982, Tindall et al., 1982). A remarkable feature was the apparent reversibility of this cell shrinkage (Landolt et al., 1983) but the precise intracellular mechanisms remained unclear and these will be further discussed at various stages in the thesis.

C. THE ENIGMA OF THE NON-FUNCTIONING TUMOUR

Approximately 25% of surgically removed pituitary adenomas are unassociated with clinical or biochemical evidence of increased hormone production (Asa and Kovacs, 1983) and this figure rises to around 70% for large tumours causing visual failure (Teasdale, 1983). Some are "clinically silent" adenomas in which immunocytochemistry shows positive staining for one or more anterior pituitary hormones (Asa and Kovacs, 1983). A further group of tumours show significant oncocytic transformation with most cells containing large numbers of abnormal mitochondria (Roy, 1978). The significance of this phenomenon and the functional capacity of the mitochondria is unknown. Kovacs et al (1980) proposed the term null cell adenoma for tumours lacking immunostaining for specific pituitary peptides or hormones; fifty-six tumours were so classified out of a surgical series of 343 adenomas. Of these, 46 showed completely negative immunostaining for anterior pituitary hormones and their subunits. The remaining 10 showed scattered cells with positive immunostaining for the glycoprotein hormones, alpha subunit or prolactin.

Many null cell tumours may actually be poorly secreting rather than completely non-secreting. Electron microscopic examination demonstrates secretory granules in the majority, though these are rather smaller than those present in the classical functioning adenomas (Kovacs et al., 1980; Esiri et al., 1983). Furthermore, a secretory granule-specific protein, chromogranin, is frequently identified in non-functioning pituitary tumours (DeStephano et al., 1984). In cell culture, some secrete pituitary hormones, most commonly gonadotrophins. However the rate of secretion is low (Mashiter et al., 1981; Surmont et al., 1983) and 25-50% do not secrete any known anterior pituitary hormone (Adams and Mashiter, 1985).

However it is far from proven that all of these tumours are derived from gonadotroph cells. Firstly the gonadotrophin immunostaining, if present, is invariably confined to scattered or small groups of cells, leaving the identity of most of the cells unknown (Mashiter et al., 1981; Esiri et al., 1983). Secondly the gonadotrophin contents are much lower than those present in normal pituitary (De Marco et al., 1984b, Chapter 7 in this thesis). Furthermore serum gonadotrophin concentrations are usually within the normal range, although the differentiation of normal from abnormal secretion may be difficult in a post-menopausal woman (Surmont et al., 1983). Adenomas that are definitely gonadotrophin-secreting may be distinguished from null cell adenomas by the presence of elevated serum hormone concentrations and positive immunostaining in the majority of tumour cells (Whitaker et al., 1985), ideally supported by in-vitro secretion studies (Snyder et al., 1985).

The cell of origin of the non-functioning tumour remains an enigma. Ultrastructurally identical cells have been identified in both non-tumourous adenohypophysis and some functioning adenomas (Kovacs et al., 1980). Whether they represent undifferentiated stem cells is unknown. The presence of such cells in the normal human pituitary has yet to be proved but animal experiments seem to support their existence. For example, Shiino et al (1977) showed that Rathke pouches isolated from rat foetuses and maintained in tissue culture or grafted into the hypophysiotrophic area of the hypothalamus or under the kidney capsule were capable of cytodifferentiation and pituitary hormone secretion. Alternatively, null cells may be dedifferentiated mature cells in which hormone synthesis is halted and some specific cell markers lost. There is some experimental evidence which supports this phenomenon. Corenblum et al (1977) showed that growth hormone cells undergo dedifferentiation

in rats made hypothyroid with propylthiouracil. The GH cells lost their granules and become unrecognisable morphologically, an effect which was completely reversible when the antithyroid drug was withdrawn.

During pregnancy the percentage of lactotrophs in the anterior pituitary increases from around 20% to approximately 50% (Asa and Kovacs, 1982). It is unknown whether this occurs by lactotroph hyperplasia or by differentiation of pluripotent stem cells, possibly the null cells.

In summary, the non-functioning tumour is interesting because its tantalising resemblances to some normal anterior pituitary cells are combined with apparent secretory inertia or silence. The responses of cells from these tumours to hypothalamic releasing factors, dopamine and bromocriptine are described in subsequent chapters.

D. OBJECTIVES OF THE PRESENT STUDIES

1. Clinical objectives

The main clinical objectives were to investigate whether pre-operative dopamine agonist therapy could improve the surgical outcome for patients with large prolactin-secreting and non-functioning pituitary tumours and to improve the diagnostic distinction between the two. I intended to conduct a clinical trial of pre-operative bromocriptine in such patients, with detailed clinical, biochemical and radiological monitoring, in order to find the optimal dose and duration of therapy prior to surgical intervention. As reviewed above, the response of non-functioning tumours to bromocriptine was unclear at the outset of these studies so the usefulness of bromocriptine in patients with these tumours was an important objective. Furthermore, the literature suggested that perhaps 30% of prolactin-secreting macroadenomas exhibit some degree of bromocriptine "resistance" in terms of tumour shrinkage or prolactin suppression. I intended to examine the clinical characteristics of such patients, and to correlate pre-operative radiology and prolactin suppressibility with in-vitro tumour characteristics, as detailed below, in an attempt to define the mechanism of the "resistance". Eventual surgical treatment would ensure that each tumour was accurately classified and possible disconnection hyperprolactinaemia identified.

2. Laboratory objectives

The laboratory studies were linked with the clinical trial so that the in-vitro characteristics of bromocriptine-responsive and non-responsive tumours could be compared. A simple hypothesis to be tested was that deficient or defective membrane-bound dopamine receptors might account for the failure of some tumours to respond to dopamine agonist drugs. An

important tool was therefore the validation of a radioreceptor binding assay of sufficient sensitivity to measure dopamine receptors in the relatively small amounts of tumour tissue obtained at surgery.

Parallel hormone secretion studies on the same tumours were to be performed using a dynamic perifusion system. In the case of the non-functioning tumours the aim of such studies was to measure gonadotrophins and alpha subunit as candidates for the secretory product, if any, and to exclude prolactin secretion in patients with pre-operative disconnection hyperprolactinaemia. For the prolactin-secreting tumours, the objective was to compare dopamine and bromocriptine dose responses for tumours responsive or non-responsive to bromocriptine in-vivo. A comparison of in-vivo and in-vitro prolactin responses to TRH in prolactinomas was also planned.

Since little was known of the mechanism of bromocriptine-induced shrinkage, detailed immunocytochemical and electron microscopic studies of the bromocriptine-treated tumours were to be performed, with morphometric analysis of tumour cell size.

It was expected that the clinical studies would provide guidelines for the use of dopamine agonist therapy in patients with pituitary macroadenomas, and that recommendations could be made regarding the nature and timing of subsequent therapy. I anticipated that the laboratory studies would yield further insight into the cellular mechanisms involved in tumour responses to bromocriptine and hoped to gain at least a hint of the cell type represented in non-functioning tumours.

CHAPTER 2.

CLINICAL STUDIES OF PROLACTINOMAS AND NON-FUNCTIONING TUMOURS

This chapter describes the clinical investigations and is in three parts. The first is a comparison of serum prolactin concentration with eventual histopathological diagnosis in patients referred as pituitary adenomas, to find the diagnostic meaning of different prolactin levels. The second examines the effectiveness of transsphenoidal surgery used as primary treatment for patients with prolactinomas and non-functioning tumours. The third describes the macroadenoma patients given pre-operative bromocriptine in a specific attempt to facilitate transsphenoidal surgery.

The results of these studies have been recently published (Bevan et al., 1987a, 1987b).

A. SIGNIFICANCE OF THE SERUM PROLACTIN CONCENTRATION

1. Introduction

Any space occupying lesion in the pituitary region may disconnect lactotroph cells in the anterior pituitary from hypothalamic inhibitory control and lead to hyperprolactinaemia (Lundberg et al., 1981). Large non-functioning tumours may masquerade as prolactinomas in this way (Franks et al., 1977). Hence sellar enlargement associated with hyperprolactinaemia is not always due to a prolactin-secreting pituitary adenoma.

The distinction is of importance in view of the current widespread use of dopamine agonists in the primary treatment of macroprolactinomas. If the serum prolactin level is misinterpreted, inappropriate dopamine agonist therapy may be given to patients with non-adenomatous pathology. Furthermore, as will become apparent later in this thesis, dopamine

agonist therapy may be given to patients with pituitary adenomas that do not regress during such therapy.

This short section examines a series of 128 consecutive patients with a definitive pathological diagnosis referred for treatment of a presumed pituitary adenoma. It seeks to enable diagnostic interpretation of the serum prolactin concentration in patients with fossa enlargement.

2. Patients

All patients referred to Oxford between 1979 and 1985 for treatment of presumed pituitary tumour, without acromegaly or Cushing's syndrome, were reviewed. Those with drug-related hyperprolactinaemia, primary hypothyroidism or empty sellae diagnosed by computerised tomography were excluded, as were patients with surgically proven microprolactinomas but normal sellar radiology. This left 104 patients who had transsphenoidal surgery and 25 who had transfrontal craniotomy, and in these a definitive pathological diagnosis was made in all but one case. All had enlargement of the pituitary fossa on plain radiographs and/or extrasellar extension of the lesion.

3. Results

a. Non-adenomatous pathology

Fifteen of the 128 patients proved to have lesions other than pituitary adenomas (Table 2-1). The commonest "pseudo-prolactinoma" was the intrasellar craniopharyngioma. Serum prolactin concentration was raised in 10 of the 13 patients in whom it was measured but in all except one (of 5260) it was less than 2500 mU/L.

Table 2-1: Non adenomas

Case	Age	Sex	Pathology	Pretreatment Prolactin (mU/L)	Other Features
1	32	F	Empty sella	210	
2	9	F	Dysgerminoma	340	diabetes insipidus
3	22	F	Sterile abscess	550	
4	67	M	Adenocarcinoma (unknown primary)	610	
5	68	M	Arachnoid cyst	690	CT diagnostic
6	36	F	Empty sella	750	CT misleading
7	58	F	Carcinoid	790	bronchial carcinoid 10 years previously.
8	61	F	Lymphocyte infiltrate	840	virtually normal fossa size; anterior hypopituitarism and mild diabetes insipidus.
9	40	M	Intrasellar craniopharyngioma	1050	virtually normal fossa size; anterior hypopituitarism.
10	59	F	Aspergilloma	1200	
11	50	M	Meningioma	1500	
12	24	F	Intrasellar craniopharyngioma	2250	diabetes insipidus
13	29	M	"	5260	
14	30	M	"	ND	
15	36	M	Chondrosarcoma	ND	calcification on xray

Cases 8, 9, 11 and 12 received ineffective preoperative bromocriptine therapy elsewhere.

CT, computerised tomography
ND, not done

Plain fossa radiographs showed gross enlargement (>1500 mm^3) in 12 of the 15 patients but in cases 8 and 9 the combination of minimal fossa enlargement and severe anterior pituitary failure made it likely that the referral diagnosis of adenoma was incorrect. The dense calcification in case 15 and the radiological appearances in case 5 also made a diagnosis of pituitary adenoma unlikely. In the other 11 cases the plain radiographs and computerised tomography were consistent with pituitary adenoma, but in cases 2, 8 and 12 the presence of diabetes insipidus suggested hypothalamic dysfunction rather than adenoma.

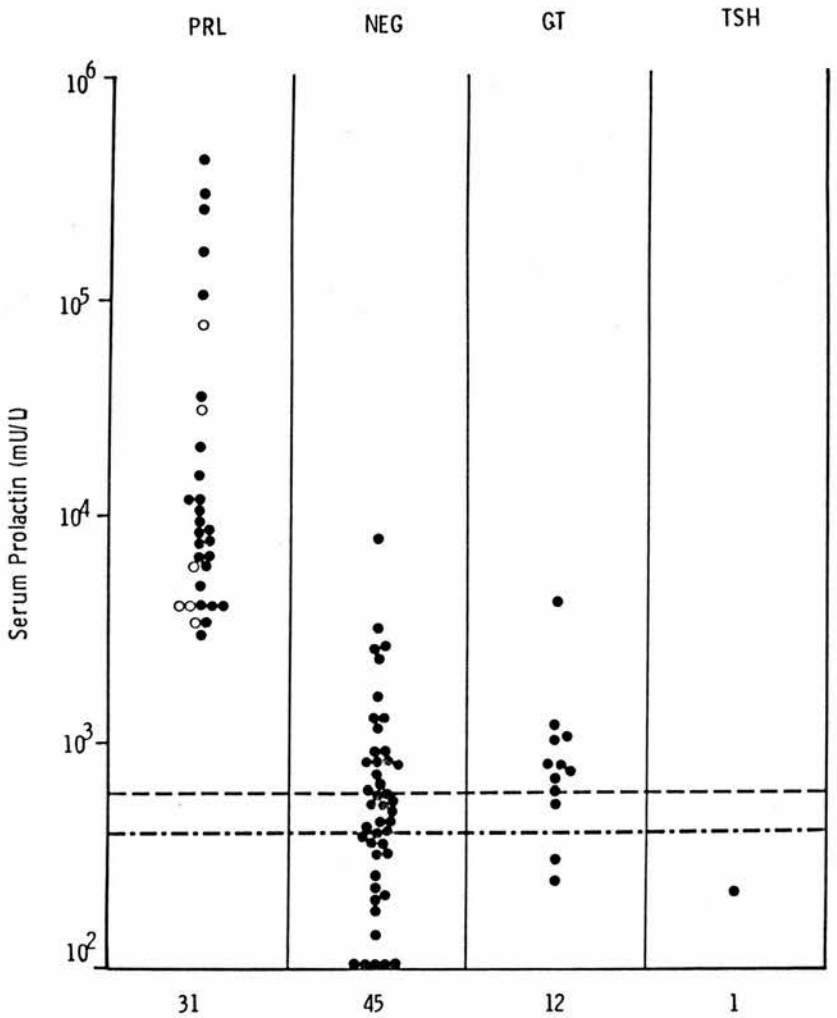
Four patients had received ineffective dopamine agonist therapy elsewhere before referral in an attempt to shrink the presumed adenoma. Visual failure had worsened in the patient with the meningioma during 4 months of pre-operative bromocriptine (Case 11). Since the above series was reviewed a patient with a pilocytic astrocytoma has been operated for deteriorating vision during inappropriate bromocriptine therapy given elsewhere.

b. Pituitary adenomas

One hundred and thirteen patients had histologically proven pituitary adenomas. Eighty-nine had preoperative serum prolactin concentrations measured and these are shown in Figure 2-1. The tumours were subdivided into four groups on the basis of immunocytochemistry as defined in the legend to Figure 2-1.

All the prolactinomas had prolactin levels above 3200 mU/L. Macro-prolactinomas (Group 3 as defined in section B.2) were associated with prolactin levels between 3280 and 394000 mU/L. Mesoadenomas (Group 2) produced prolactin levels between 3200 and 12180 mU/L.

Figure 2-1: Pretreatment serum prolactin concentrations in 89 pituitary adenomas



Serum prolactin concentrations are plotted on a logarithmic scale. Broken lines indicate upper limits of the normal laboratory range for women (600 mU/L) and men (450 mU/L).

Open circles indicate minimum prolactin levels in samples not fully diluted.

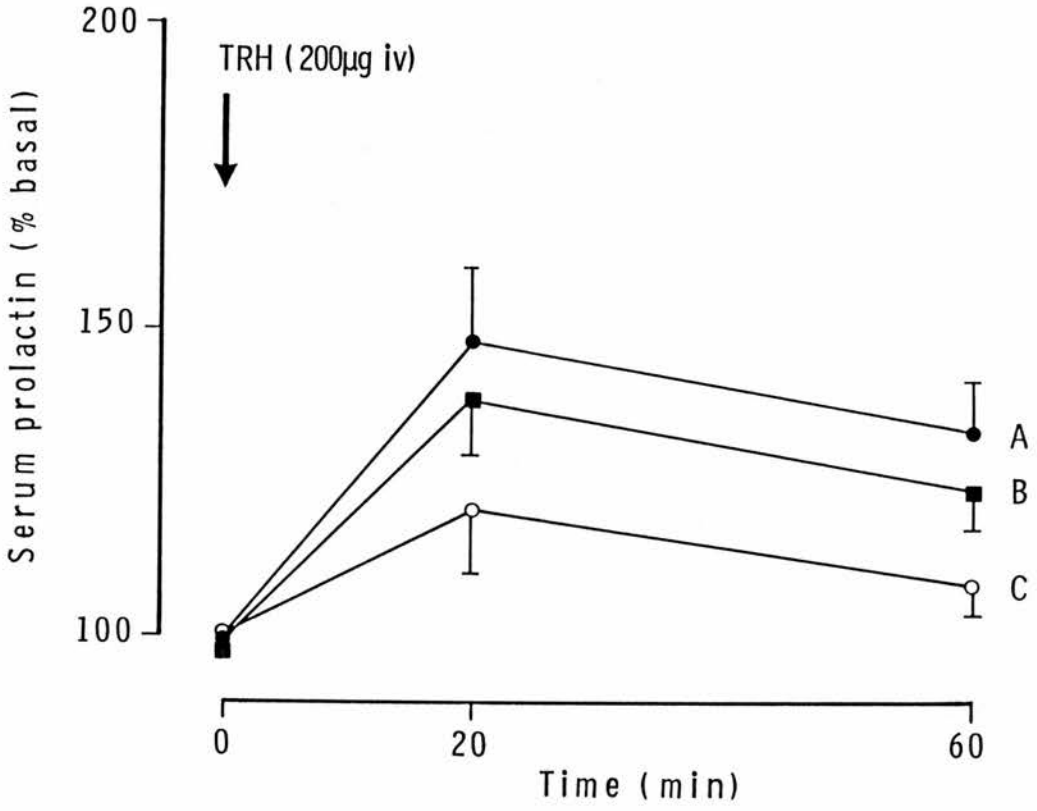
Immunostaining:

PRL = prolactin (positive in >90% cells)
NEG = immunostaining entirely negative
GT = gonadotrophin (positive for LH or FSH or both, but never in >10% cells)
TSH = thyrotrophin (positive for TSH in 40% cells)

In contrast, most tumours other than prolactinomas were associated with prolactin levels below 3000 mU/L. However three patients with these tumours had prolactin levels between 3000 and 8000 mU/L.

The magnitude of the prolactin responses to TRH did not distinguish tumourous and non-tumourous hyperprolactinaemia (Figure 2-2). Both groups of patients had grossly subnormal responses compared with normal subjects who achieve peak prolactin concentrations of 6-7 times the basal value following TRH (Cowden et al., 1979, Johnston et al., 1985). Only one patient represented in Figure 2-2 achieved a peak prolactin level of greater than 200% of the basal concentration. Furthermore, the pattern of the TSH response to TRH did not define the patients with disconnection hyperprolactinaemia, as recently suggested by Scanlon et al (1986) - data not shown.

Figure 2-2: Serum prolactin responses to TRH



Serum prolactin responses to 200 µg intravenous TRH in 38 patients with large pituitary tumours. Values shown are mean ± SEM.

A Non-functioning tumours with basal prolactin levels <600 mU/L (343 ± 35, mean ± SEM, n=16)

B Non-functioning tumours with basal prolactin levels >600 mU/L (1152 ± 327, mean ± SEM, n=11)

C Macroprolactinomas with basal prolactin levels in the range 3280-247000 mU/L (n=11)

The results were analysed using the Mann-Whitney U test (statistical significance was taken as $P < 0.05$).

There was no difference between A and B at 20 or 60 minutes. Neither A nor B was significantly different from C at 20 minutes, but each was significantly higher than C at 60 minutes.

4. Conclusions

These patients clearly illustrate how difficult it is to fully interpret a series of patients with sellar enlargement that have been medically treated, in the absence of definitive pathology. For instance, 6 of the 7 bromocriptine-resistant prolactinomas described by Wass et al (1982) may have been non-functioning tumours judging by the modest pre-treatment prolactin levels.

The results suggest that a serum prolactin concentration greater than 8000 mU/L is always due to a prolactinoma. Twenty-seven of the adenomas that did not immunostain for prolactin had elevated serum prolactin concentrations, although these were generally below 3000 mU/L. An adenoma of sufficient size to cause significant sellar enlargement (>1500 mm^3) but associated with a serum prolactin concentration of less than 3000 mU/L is therefore most unlikely to be prolactin-secreting.

A "grey area" of overlap exists with prolactin levels between 3000 and 8000; the pituitary lesion may or may not be an adenoma and may or may not be prolactin-secreting. Although modest fossa enlargement with a prolactin level in this range is most likely to be due to a mesoprolactinoma three patients with adenomas that did not immunostain for prolactin had prolactin levels in the same range.

The prolactin response to TRH was unhelpful diagnostically. This is consistent with the short loop positive feedback hypothesis whereby increased circulating prolactin concentrations, of whatever cause, are thought to increase hypothalamic dopamine release which, in turn, would reduce TRH-induced prolactin release (Rodriguez-Arnao et al., 1983). As will be seen in Chapter 6 most prolactinomas release prolactin in response to TRH in-vitro but this can invariably be blocked by dopamine.

The problem of the non-adenomatous lesion expanding the pituitary fossa and causing disconnection hyperprolactinaemia is a potentially serious one. These lesions are diverse as shown by Table 2-1 and primary surgical biopsy is obviously desirable. The serum prolactin concentration is usually less than 3000 mU/L though the occasional value may fall into the above "grey area". Additional factors are often important in the prediction of a non-adenomatous lesion. Computerised tomography may give important clues with dense calcification and marked cystic change being relative pointers to non adenomas. The presence of diabetes insipidus strongly suggests hypothalamic and not primary pituitary disease. Finally minor fossa enlargement associated with major hypopituitarism is an unusual combination for a pituitary adenoma.

B. RESULTS OF TRANSSPHENOIDAL SURGERY

1. Introduction

This section examines the Oxford experience in the use of transsphenoidal surgery for the treatment of prolactinomas and non-functioning tumours. Factors affecting the surgical outcome in a large series of 125 patients will be considered. Cure rates, relapses, effects on anterior pituitary function and surgical complications for various sub-groups of patients will be described and followed by a discussion of the current indications for surgical treatment of these tumours.

2. Patients and methods

The series included 67 patients operated for prolactinoma and 58 patients operated for non-functioning tumour. Details of clinical presentation are given below.

Pituitary fossa volumes were measured from plain skull radiographs by the method of Lusted and Keats (1967), and supplemented by computerised tomography (CT) and occasionally metrizamide cisternography. Invasion was defined as radiological or surgical evidence of tumour in bone, sphenoid sinus or cavernous sinus. When the surgical results were analysed they were found to differ greatly in patients with surgical or radiological evidence of invasion or if the fossa volume was greater than 1500 mm^3, and so three groups have been used.

Group 1 (normal plain radiographs, microadenomas) comprised tumours associated with fossa volumes of <900 mm^3, no evidence of invasion and a suprasellar extension (SSE), if present, of less than 10 mm.

Group 2 (mesoadenomas) included tumours with fossa volumes between 900 and 1500 mm^3, no evidence of invasion and a SSE, if present, of <10 mm.

Group 3 consisted of macroadenomas with fossa volumes >1500 mm^3 and/or evidence of invasion and/or >10 mm SSE.

All operations were performed by Mr CBT Adams (Radcliffe Infirmary, Oxford) using a sublabial paraseptal approach. The visible amount of normal pituitary remaining was carefully noted and biopsied if in doubt, one of the objectives being to determine the functional validity of this surgical assessment. If CSF was visible in the sella turcica or the diaphragma sellae appeared incomplete the fossa and sphenoid sinus were packed with fat and fascia taken from the thigh.

Tumour tissue was characterised by immunocytochemistry and electron microscopy (Esiri et al., 1983) and this was supported by in-vitro secretion studies on 30 tumours.

Pituitary function was assessed before and 3 to 7 weeks after surgery, clinically and by the following tests: ACTH and GH function by peak response to insulin hypoglycaemia (or glucagon if hypoglycaemia was contraindicated), normal being cortisol >550 nmol/L and GH >20 mU/L. TSH function: normal serum total or free T4, irrespective of the TSH response to TRH. Prolactin was considered normal if the mean of two basal cannula samples was less than 700 mU/L in females (the highest level at which menses and natural pregnancy occurred after operation, and corresponding to the widened range of Davis et al., 1984), and 500 mU/L in males (the highest level shown by a patient with normal testosterone and reproductive function after operation). Gonadotrophin function in post-menopausal females was defined as normal if serum LH was greater than 8 U/L irrespective of serum prolactin; in males it was normal if plasma testosterone was normal (>14 nmol/L); and in premenopausal females it was normal if there were regular menses or pregnancy. Low testosterone in men, or amenorrhoea in women, associated

with hyperprolactinaemia was recorded as unassessable gonadotrophin function. The results of LHRH tests in all patients were not used as evidence of gonadotrophin function.

3. Tumour presentation

The presenting features in the 125 patients are summarised in Table 2-2. Group 1 and 2 prolactinoma patients were usually young women presenting with menstrual disturbance associated with infertility. None of these patients had significant impairment of anterior pituitary function at presentation and there were only two men with small tumours.

However the largest prolactinomas occurred in men and frequently caused visual failure. It was notable that even large prolactinomas were rarely associated with significant impairment of ACTH or TSH function (7% of Group 3 tumours).

Non-functioning tumours occurred in men and women with equal frequency and patients were mostly in the sixth decade of life. Over 50% presented with large tumours causing visual failure and one quarter were associated with major pituitary hypofunction. In this older group of patients infertility was seldom an issue though impotence in men was a frequent clinical problem.

Nine patients with non-functioning tumours presented in more unusual ways. Five presented with third or sixth nerve palsies and in four this was caused by major tumour haemorrhage (Wakai et al., 1981). Two patients with non-functioning tumours presented with pituitary abscesses caused by bacteria presumed to have originated in air sinuses and upper respiratory tract. Two women presented with galactorrhoea secondary to disconnection hyperprolactinaemia.

Table 2-2: Presenting symptoms in patients with prolactinomas and non-functioning tumours

		Number	Mean age (yr)	Reproductive symptoms			Symptoms of ACTH/TSH deficiency	Pressure symptoms	
				Amen	Gal	Infert		HA	Visual failure
PROLACTINOMAS									
Women	Group 1	24	31	85%	42%	83%	0	0	0
	Group 2	13[a]	28	100%	38%	54%	0	0	0
	Group 3	11[b]	29	91%	36%	18%	0	18%	9%
				Impot	Gal	Infert			
Men	Group 1	0							
	Group 2	2	42				0	0	0
	Group 3	17[c]	39	47%	6%	0	12%	41%	35%
				Amenorrhoea/ Impotence					
NON-FUNCTIONING TUMOURS									
Women		26[d]	51	23%			15%	19%	65%
Men		32[d]	56	50%			34%	16%	50%

Amen = amenorrhoea
Gal = galactorrhoea
Infert = infertility
Impot = impotence
HA = major headache

a = 2 previous radiotherapy
b = 1 previous radiotherapy
c = 2 previous transfrontal surgery
d = 2 previous transfrontal surgery and radiotherapy

4. Tumour cure rate

a. Cure of prolactinomas

Overall, serum PRL was reduced to normal ("biochemical cure") in 51% of patients but this conceals large differences between subsets of patients. Because there was strong correlation between fossa volume over 1500 mm^3, invasion and suprasellar extension over 10 mm Group 3 was defined as possessing any of these features (including one tumour with small sella volume and suprasellar extension of >10 mm), and in this group the cure rate was 8 of 28 (29%). In patients with modest fossa enlargement (900-1500 mm^3) and no invasion (Group 2) the cure rate was 11 of 15 (73%) and in Group 1 (patients with fossa volumes less than 900 mm^3) 16 of 24 (67%) were cured. Analysis of individual variables showed the following. Twenty-seven of 40 patients (67%) with **fossa volume** less than 1500 mm^3 were so cured, in contrast to 7 of 19 (37%) with larger volumes without tumour invasion. Eight patients showed radiological or surgical evidence of **invasion** and none was cured. These were all large tumours, no evidence of invasion being found in the 40 tumours of fossa volume below 1500 mm^3. **Suprasellar extensions** over 10 mm were present in 12 patients, 11 of them with fossa volumes over 1500 mm^3.

Surgical procedure: apparently complete but selective adenoma removal leaving visible normal pituitary was possible in 22 patients with 16 (72%) being cured; and 20 of the 22 had fossa volumes below 1500 mm^3. More radical surgery in 45 diffuse or invasive tumours of all sizes, however, cured as many as 18 (40%).

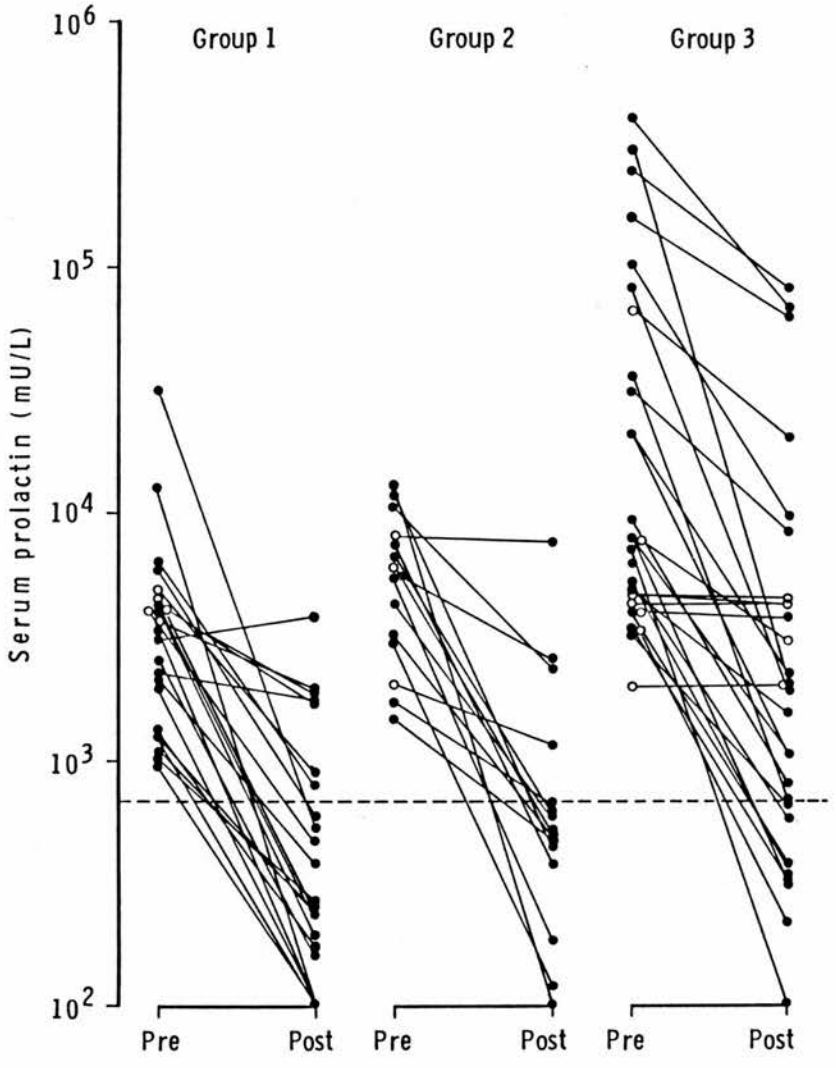
Two patients showed later **relapse.** Of 11 Group 2 patients initially cured and followed for 28.2 patient years (median 0.9, range 0.5-8.2) one developed amenorrhoea and galactorrhoea at 3.1 years with a serum

prolactin of 2400 mU/L. Of the 8 Group 3 patients initially cured and followed for 6 patient years (median 1.1, range 0.3-2.6) one relapsed in the third year (serum prolactin 1920 mU/L). Recurrent tumour was not visible on CT scan in either of the two relapses. None of the 16 Group 1 patients initially cured has relapsed over 59.8 patient years of follow-up (median 4.0, range 0.3-8.9).

The results for the three groups are shown in detail in Figure 2-3 and summarised in Figure 2-4.

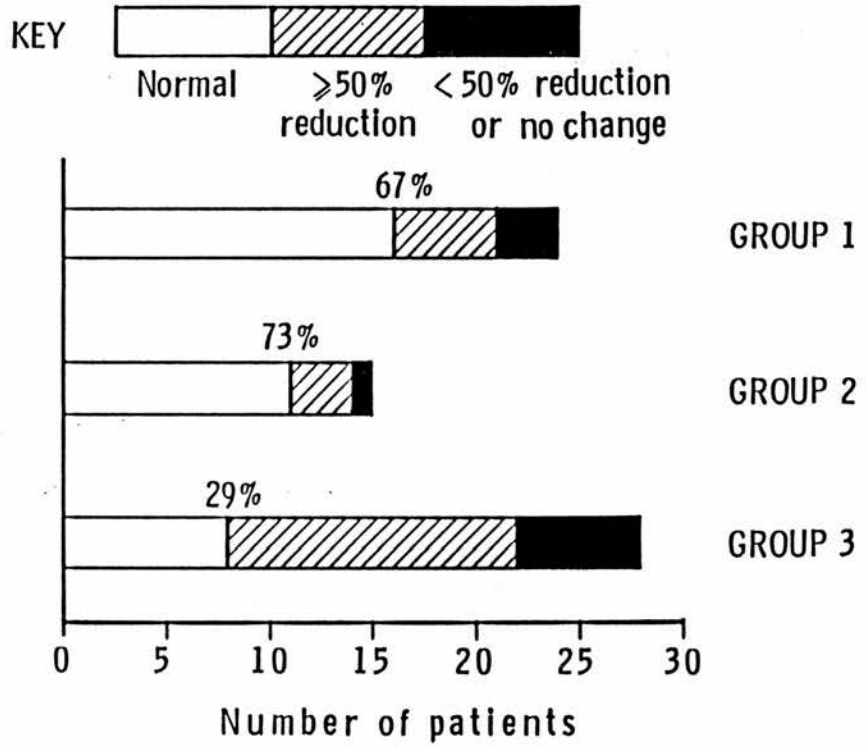
However biochemical cure of prolactin excess takes no account of **reproductive function.** Of 19 men with prolactinomas only 1 was biochemically cured and testosterone was subnormal post-operatively in 14. Of the 48 pre-menopausal women, two had been hysterectomised, 2 had previous pituitary radiotherapy elsewhere, and one developed premature menopause: in 6 others follow-up was incomplete. Twenty of the remaining 37 wished to become pregnant, and the results are shown in Figure 2-5. Of the 17 not wishing pregnancy, 11 were cured of the hyperprolactinaemia and 10 resumed normal menses.

Figure 2-3: Serum prolactin concentrations before and after surgery in 67 prolactinoma patients



Pre-operative and early post-operative serum prolactin concentrations (logarithmic scale) in 67 patients operated for prolactinoma. Groups 1-3 defined in section B.2. Open circles indicate minimum prolactin levels in samples not fully diluted. Broken line indicates 700 mU/L.

Figure 2-4: Reduction in serum prolactin concentrations in the three prolactinoma groups

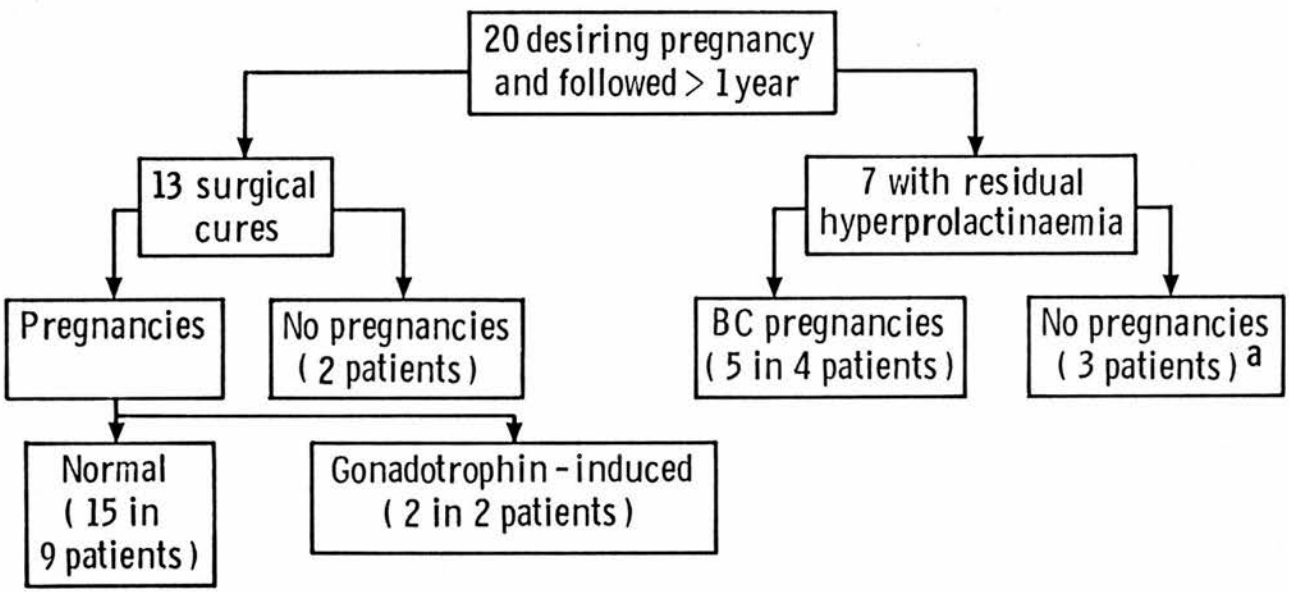


Normal prolactin ranges defined on p.30:

Females <700 mU/L.

Males <500 mU/L.

Figure 2-5: Pregnancies in women operated for prolactinoma



Of the 20 women desiring pregnancy and followed for >1 year 14 were in Group 1, 4 in Group 2 and 2 in Group 3.

a Marked BC intolerance (2)
Unknown cause for infertility (1)
[Normal prolactin and menses during bromocriptine.
Normal LH responses to LHRH and clomiphene]

b. Surgical results in non-functioning tumours

The lack of a chemical marker makes assessment of cure of non-functioning tumours a matter of recurrence rate on follow-up. Fifty-four of the 58 were in Group 3; over half presented with visual failure and severe headache was common (Table 2-2). Surgery improved visual failure in 29 of the 33 who presented with it, to normal in 9. Headache was relieved in all 10 patients in whom it was a major presenting symptom. All 5 cranial nerve palsies resolved after tumour decompression.

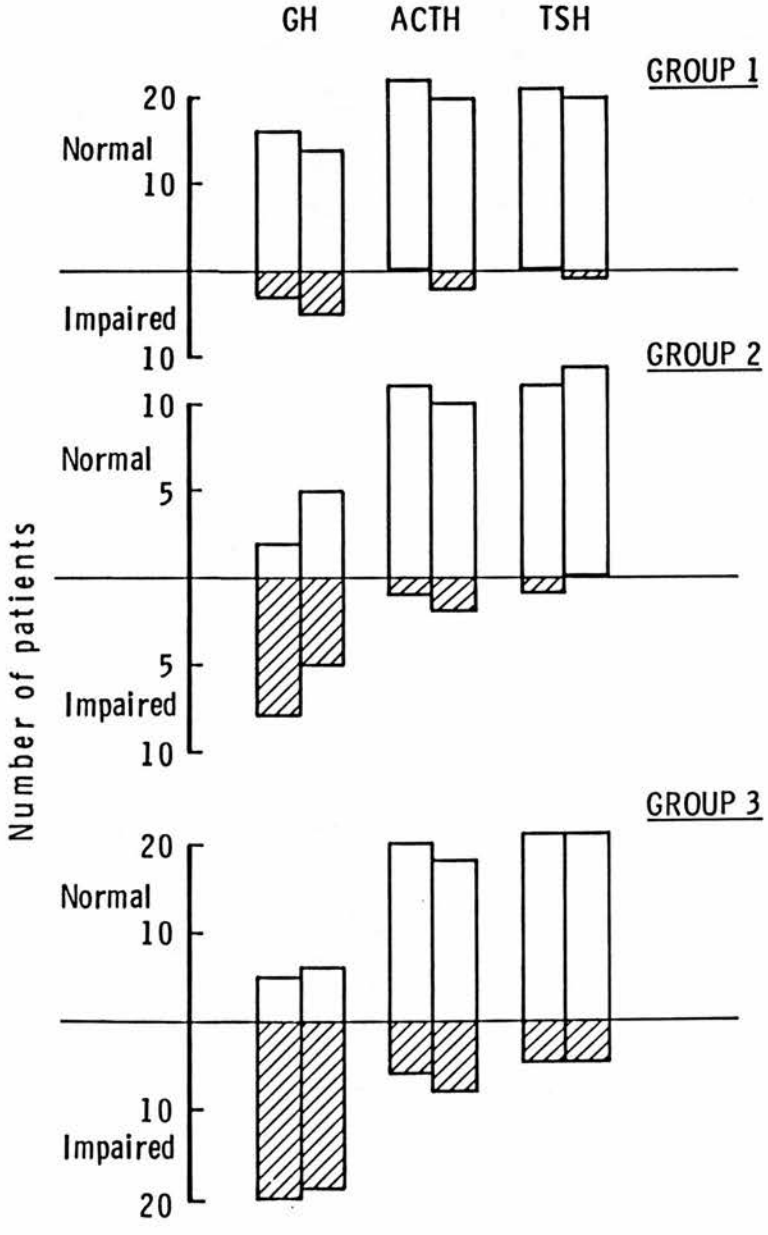
Twenty-one patients were given radiotherapy to prevent recurrence, especially if they were hypopituitary post-operatively. Thirty-two patients, mostly with normal post-operative pituitary function, chose biennial CT follow-up instead of radiotherapy, and 10 of these have been followed in Oxford. Post-operatively all had sellae that were at least 30% empty on CT scan, and have been followed for 29.1 patient years (median 2.1, range 1.0-6.4). The sellar contents have enlarged in two, though remaining within the sella, and radiotherapy has been applied at 1.8 and 6.4 years respectively.

5. Effects of surgery on anterior pituitary function

a. Prolactinomas

ACTH, TSH and GH function before and after surgery for the three prolactinoma groups is shown in Figure 2-6. Four patients required corticosteroid replacement following surgery (one in Group 1, one in Group 2 and two in Group 3) and two patients required thyroxine replacement (one in Group 1 and one in Group 3). The reason for this was obscure in the Group 1 and 2 patients since normal anterior pituitary tissue was seen and preserved at the time of surgery in each case.

Figure 2-6: Anterior pituitary function before and after surgery in patients operated for prolactinoma



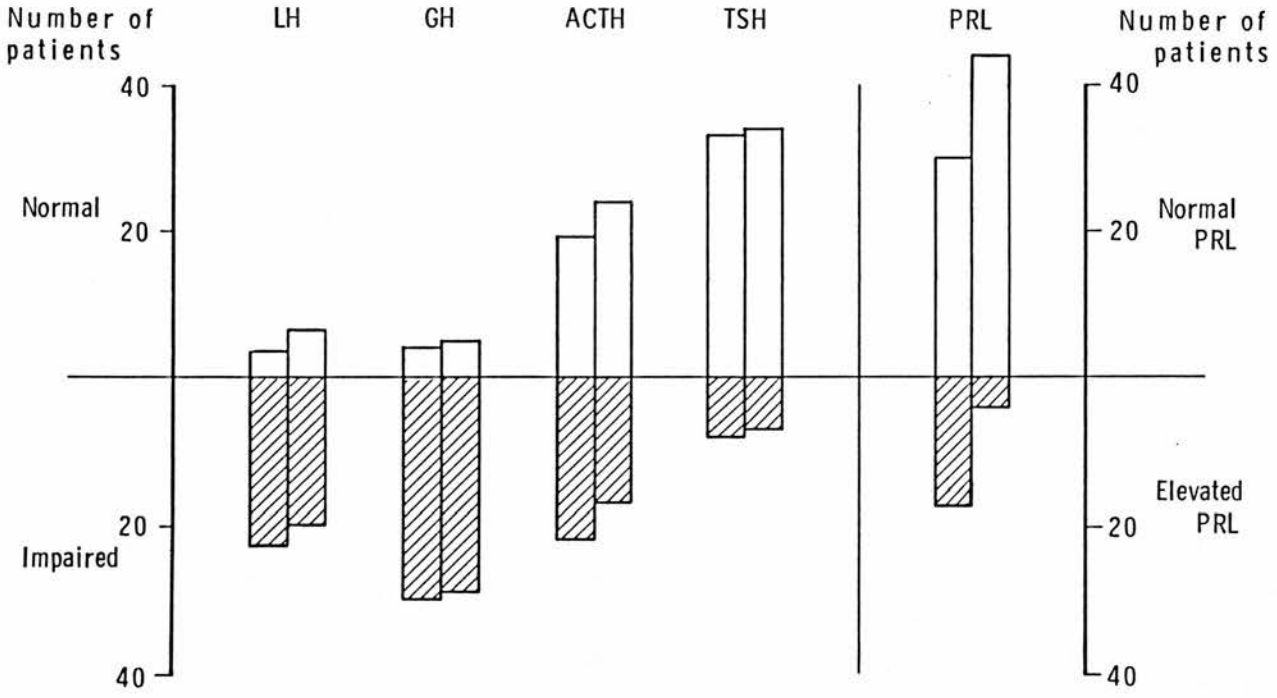
For each hormone the left hand bar indicates the number of patients with normal and impaired function preoperatively and the right hand bar the corresponding number post-operatively. Only patients with pre- and post-operative data are included. Normal values are defined in section B.2.

b. Non-functioning tumours

LH, GH, ACTH and TSH function before and after surgery is shown in Figure 2-7. In contrast to the patients with prolactinomas no patient operated for non-functioning tumour required corticosteroid or thyroxine replacement as a consequence of the surgical intervention. However 25% of patients with non-functioning tumours presented with ACTH or TSH deficiency or both (Table 2-2). Several pre-operative hormone defects were normalised by surgery; LH (4), GH (1), ACTH (5) and TSH (1).

Seventeen patients had elevated serum prolactin concentrations before surgery presumably on a hypothalamo-pituitary disconnection basis (Figure 2-7). Values up to 4350 mU/L were present in 14 of the 48 patients with non-functioning tumours and suprasellar extension over 10 mm, and was corrected by surgery in 10. Hyperprolactinaemia was present in 3 of the 10 patients with suprasellar extension of less than 10 mm and corrected by surgery in all. No patient acquired disconnection hyperprolactinaemia as a result of surgery. The correction was not due to complete restoration of normal control mechanisms since the post-operative prolactin response to TRH varied from absent to a 2.9 fold increase.

Figure 2-7: Anterior pituitary function before and after surgery in patients operated for non-functioning tumour



Same format as Figure 2-6

6. Surgical complications

The surgical complications for both tumour groups are shown in Table 2-3. The table includes the complications in bromocriptine-treated macroprolactinomas and this will be referred to in section C.4.
Twelve of the 125 patients experienced transient diabetes insipidus; this appeared rather commoner in the small tumours, and usually lasted 12-36 hours.

In two non-functioning tumour patients vision deteriorated after surgery: one developed an intrasellar haematoma the day after surgery, which was successfully evacuated transsphenoidally. In the second patient, vision worsened 8 days post-operatively. There was no haematoma and the (permanent) visual deterioration was presumed to be due to vascular neural damage.

If the patients prospectively treated with bromocriptine and those with temporary diabetes insipidus are excluded, 11 complications ensued in 108 patients of whom 71 had large tumours. The permanent sequelae of surgery in non bromocriptine-treated patients comprised 3 cases of permanent diabetes insipidus and the patient with visual loss described above.

Table 2-3: Surgical complications in patients operated for prolactinoma and non-functioning tumour

	Diabetes insipidus		CSF rhinorrhoea		Miscellaneous
	Trans[a]	Permanent	Trans[b]	Operative correction	
PROLACTINOMAS					
Group 1 (24)	5	1			Nasal septum repair(1) Damage to apical vessels of incisor tooth (1)
Group 2 (15)	2		1		
Group 3 (21) (Not on bromocriptine)	2		2		Buccal incision infection (1)
Group 3 (7) (Bromocriptine pre-treatment)	1	1		1	Transient 3rd nerve palsy (1) Severe postop headache (1) Brain swelling (1)
NON-FUNCTIONING TUMOURS					
(n=58)	2	1	1		Visual deterioration (2)[c]

a Transient diabetes insipidus requiring 1-3 µg DDAVP i.m.

b CSF rhinorrhoea requiring treatment with lumbar puncture-all resolved within 6 days.

c Both cases described in text.

7. Conclusions

In common with other reports these data show that tumour size is a predictor of surgical outcome for prolactinomas (Randall et al., 1983, Fahlbusch et al., 1984, Scanlon et al., 1985, Thomson et al., 1985). The tumour groupings used in the above analysis, derived from fossa measurements and computerised tomography, were useful in predicting the likelihood of success from surgery alone. The early cure rate of 67% for the Group 1 microprolactinomas was comparable to that reported in other large transsphenoidal series (Fahlbusch et al., 1984, Thomson et al., 1985). Group 3 were all macroadenomas of greater than 10 mm diameter and surgery alone seldom resulted in normoprolactinaemia. The role of surgery in the management of large prolactinomas will be considered in greater detail in the next section.

An intermediate mesoadenoma grouping was useful (Group 2) because although this group contained some tumours of greater than 10 mm diameter it was associated with a 73% surgical cure rate. These results confirm the findings of Scanlon et al (1985) who, although using a different microsurgical approach, reported a high cure rate for similar tumours between 10 and 19 mm in diameter.

What is the the current role of surgery for smaller prolactinomas ? Management policies for patients with microprolactinomas have changed as the benign natural history of this condition has become apparent. Long-term follow-up indicates that presumed microprolactinomas seldom enlarge and that serum prolactin concentrations remain constant over several years or even decline (March et al., 1981; Rjosk et al., 1982; Martin et al., 1985). Prolactin levels may remain suppressed for up to 5 years after treatment of presumed microprolactinomas with bromocriptine, particularly if pregnancy results (Hancock et al., 1985; Moriondo et

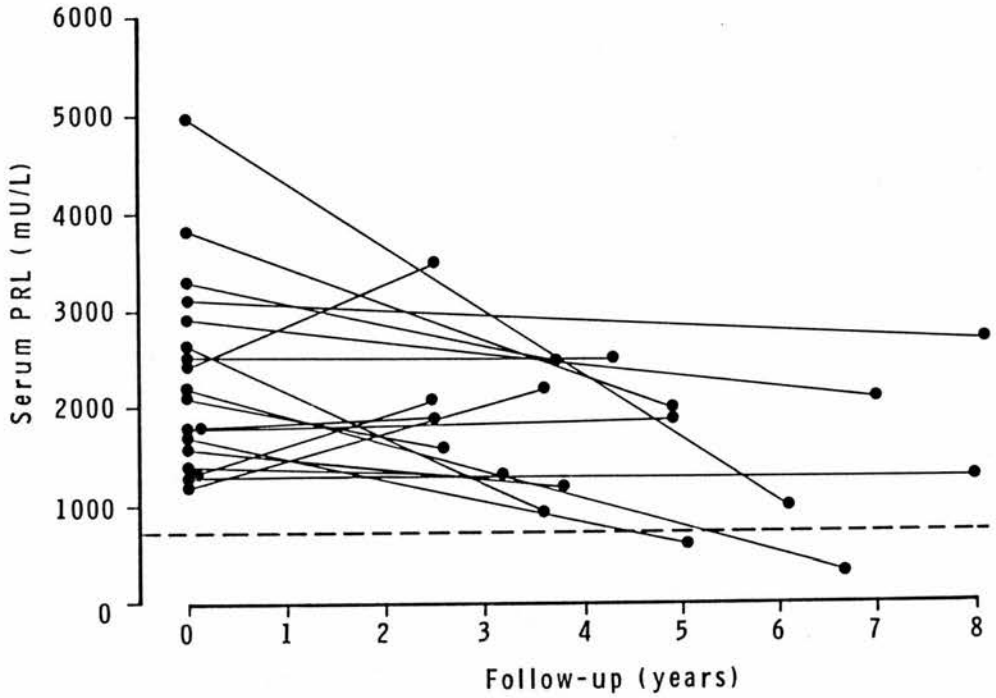
al., 1985).

Figure 2-8 shows similar prolactin data obtained from 18 women with presumed microprolactinomas followed in Oxford for longer than two years (median 4.3 years, range 2.4-8.1 years). Thirteen women received interim treatment with bromocriptine to restore reproductive function and achieve pregnancy. No patient received bromocriptine for longer than 6 months and all but one patient, who had marked bromocriptine intolerance, achieved pregnancy. During bromocriptine-induced pregnancies serum prolactin concentrations rose no higher than 10,000 mU/L, though a similar prolactin increase during pregnancy may occur in normal women (Woodhouse et al., 1985). On long-term follow-up serial prolactin levels remained constant in 12 women and fell significantly in 6. Two women developed normoprolactinaemia following bromocriptine-induced pregnancies.

The use of surgery for microprolactinomas has therefore declined. However there are still patients with bromocriptine resistance or intolerance who have a good chance of cure by selective adenomectomy. The above results show that there is a small risk of endocrine substitution therapy being required after such surgery, but no cured patient has relapsed to date, the longest patient follow-up being 9.8 years.

The mesoadenoma is a more contentious issue. Presumably these are tumours which, if left, would enlarge and develop into macroadenomas (von Werder et al., 1983). The surgical results demonstrate that they can be successfully treated by surgery, with little endocrine loss, and a relapse rate similar to that found by Serri et al (1983) of up to 80% at 5 years has not occurred (so far) in the Oxford series. Some would advocate that women with tumours in this category should receive surgery

Figure 2-8: Serum prolactin concentrations in 18 women with presumed microprolactinomas



Serum prolactin concentrations at diagnosis and at latest follow-up in 18 women with presumed microprolactinomas. No sample was taken within 2 months of bromocriptine therapy.

or radiotherapy prior to pregnancy, although the risk of tumour expansion is low (Nillius and Bergh, 1984).

The results demonstrate that the management of non-functioning tumours by transsphenoidal surgery was, in many ways, extremely satisfactory. Tumour pressure effects were substantially improved without significant endocrine penalty or peri-operative morbidity. This, together with the frequent improvement in impaired anterior pituitary function, enabled several patients to be followed without immediate post-operative radiotherapy. Subsequent CT scans suggest the majority may eventually need radiotherapy but its postponement in younger patients with fertility needs has been advantageous. Other workers have reported a similar improvement in impaired anterior pituitary function after surgical decompression of non-functioning tumours (Arafah, 1986).

C. MACROADENOMAS GIVEN PRE-OPERATIVE BROMOCRIPTINE

1. Introduction

It has been shown in the preceding section that transsphenoidal surgery alone seldom cures large prolactinomas. The tumour-shrinking properties of bromocriptine and other dopamine agonists have been reviewed in Chapter 1. This section describes 15 patients with macroadenomas who were given pre-operative bromocriptine in an attempt to facilitate transsphenoidal surgery. In addition the study was designed to examine the time course of tumour shrinkage and to provide guidelines on the dose and duration of bromocriptine required. At the outset of this work it was unclear whether non-functioning tumours regressed during dopamine agonist therapy and although the management of non-functioning tumours by primary transsphenoidal surgery was, in some respects, satisfactory (section B) the effect of bromocriptine treatment on subsequent surgery was investigated.

2. Patients and clinical methods

Fifteen patients with large pituitary tumours (7 prolactinomas and 8 non-functioning tumours) were studied. Anterior pituitary function testing was performed as described in section B. Visual assessment included Goldmann perimetry and was performed by an independent optician. Neuroradiology involved 4th generation computerised tomography with intravenous contrast.

Patients were randomised to either 10 or 20 mg bromocriptine daily and commenced treatment with 2.5 mg daily, increasing the dose in 2.5 mg increments every 2 days until the final dose was achieved. Goldmann perimetry and computerised tomography were performed at intervals during treatment and tumour volumes were calculated by planimetry (Breiman et

al., 1982). Serial prolactin concentrations were measured during treatment and pituitary function testing was repeated on at least one occasion. Surgery was performed at various times during bromocriptine therapy and the drug was continued to within 4 days of surgery in all cases. Each patient gave informed consent to the bromocriptine treatment and the study was approved by the Central Oxford Research Ethics Committee.

Table 2-4 gives the clinical, biochemical, radiological and bromocriptine treatment details for each patient.

3. Clinical, biochemical and anatomical responses to bromocriptine

Serum prolactin concentrations and visual changes during bromocriptine treatment in 5 patients with prolactinomas and 5 patients with non-functioning tumours presenting with visual failure are shown in Figure 2-9. Impaired vision in all 5 patients with prolactinomas started to improve within one week of commencing bromocriptine and became clinically normal within one month of treatment in 4 patients. Visual improvement was associated with marked suppression of the greatly elevated prolactin concentrations; four of the 5 patients attained levels of less than 1000 mU/L. In one patient (P6 in Table 2-4) the prolactin level remained above 10,000 mU/L throughout 20 weeks of treatment. None of the 5 patients with non-functioning tumours showed improvement in visual field loss during bromocriptine treatment. Serum prolactin suppression in these patients presumably represented inhibition of prolactin secretion from normal pituitary.

Table 2-4: Details of macroadenomas given pre-operative bromocriptine

	Sex/ Age	BC-daily dose and duration	Pretreatment serum PRL (mU/L)	+ hormone deficiency	Height of SSE (mm)	Visual status and duration (weeks)
PROLACTINOMAS						
P1	M 54	10mg (3 weeks)	9100	GH	10	N
P2	M 27	10mg (3 weeks)	394000	GH, TSH ACTH	26	CS (6)
P3	M 31	10mg (12 weeks)	300000	GH	28	HH (16)
P4	M 25	10mg (12 weeks)	101000	GH	22	BH (4)
P5	M 66	10mg (8 weeks) 20mg (12 weeks)[a]	182000	Nil	0	N
P6	M 45	10mg (20 weeks)	247000	GH	20	BH (4)
P7	M 45	20mg (36 weeks)	8880	GH	20	BH (12)
NON-FUNCTIONING TUMOURS						
N1	F 43	10mg (3 weeks)	2820	GH, ACTH	15	BH (10)
N2	F 81	7.5mg (4 weeks)[b]	770	GH	10	BH (6)
N3	F 54	10mg (6 weeks)	1030	GH, TSH	10	BH (12)
N4	F 37	10mg (12 weeks)	680	Nil	5-10	TH (32)
N5	F 39	20mg (24 weeks)	160	GH, TSH	16	BH (16)
N6	M 63	20mg (28 weeks)	400	GH, ACTH	5-10	N
N7	M 55	20mg (48 weeks)	260	GH	15	N
N8	M 60	20mg (24 weeks) 40mg (24 weeks)[c]	220	GH, TSH ACTH	14	N

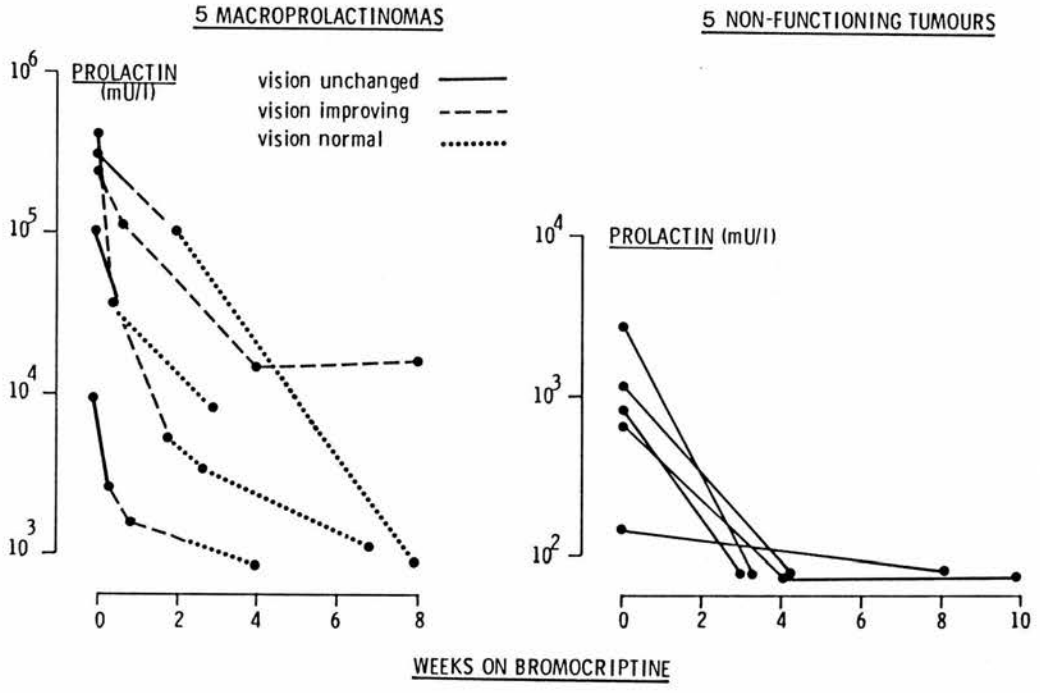
BC = bromocriptine, PRL = prolactin, SSE = suprasellar extension.

a Dose doubled since headache was not abolished on 10 mg daily.
b Failed to reach 10 mg because of bromocriptine intolerance.
c Dose doubled because there was no tumour regression on lower dose.

Anterior pituitary hormone deficiencies - see definitions and discussion on p.30.

Visual status: N = normal, CS = central scotoma,
BH = bitemporal hemianopia, TH = temporal hemianopia,
HH = homonymous hemianopia

Figure 2-9: Changes in serum prolactin and vision during bromocriptine



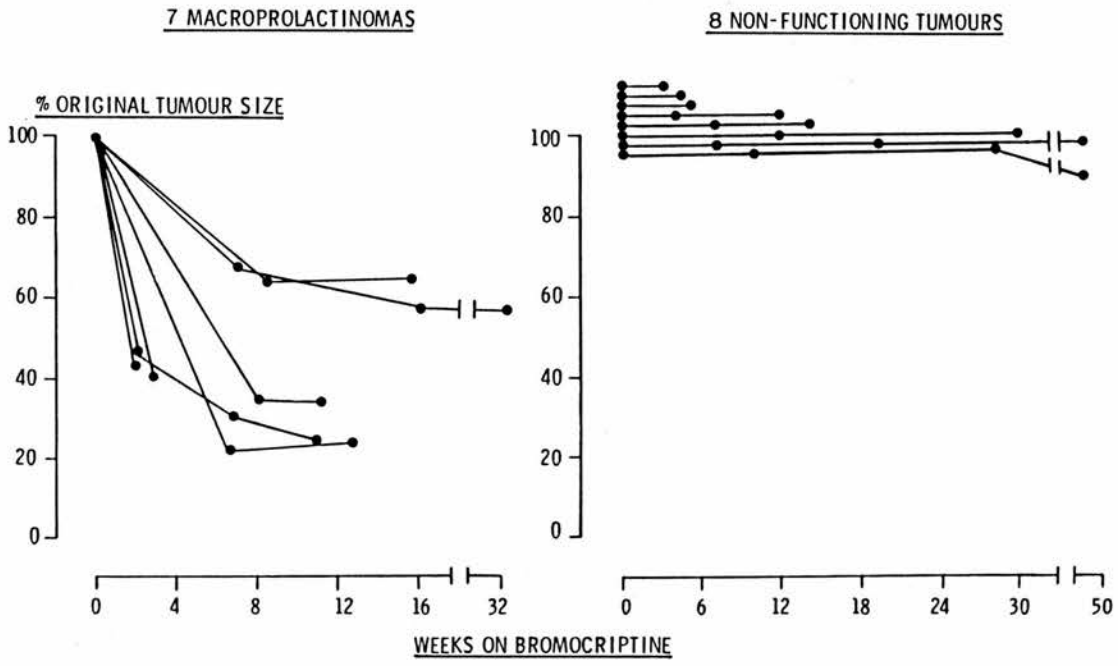
Changes in serum prolactin concentration (logarithmic scale) and vision during bromocriptine therapy in ten patients with large tumours causing visual failure: 5 prolactinomas (left) and 5 non-functioning tumours (right). Key to visual changes shown in figure. Normal vision indicates normal Goldmann perimetry and visual acuities.

Only the prolactinomas shrank during bromocriptine therapy. Tumour regression to approximately 50% of the original tumour volume occurred in these tumours and maximum tumour shrinkage had taken place after 6 weeks of bromocriptine (Figure 2-10). In contrast none of the non-functioning tumours showed tumour regression. Three of the patients with non-functioning tumours, but without visual failure, were treated with 20-40 mg bromocriptine daily for over 6 months without effect. In these 3 patients "therapeutic" steady-state plasma bromocriptine concentrations of 2-5 nmol/L were demonstrated using the assay described in Chapter 3.D.

Testosterone concentrations in two men with prolactinomas increased to within the normal range but otherwise there was no improvement in impaired anterior pituitary function in the 15 patients during bromocriptine therapy.

The serum prolactin responses to 200 μg TRH or 2.5 mg bromocriptine yielded no additional diagnostic information to the basal prolactin concentration, and were unhelpful in predicting the likelihood of tumour regression during bromocriptine treatment. The TRH responses for the 15 patients are included in Figure 2-2. In the prolactinoma patients serum prolactin concentrations fell to between 14 and 44 % of the pre-treatment values within 6 hours of the first 2.5 mg tablet of bromocriptine (See Chapter 3.D). The serum prolactin concentrations in the non-functioning tumour patients fell to less than 100 mU/L within 6-12 hours of the same bromocriptine challenge.

Figure 2-10: Changes in tumour volume during bromocriptine



Tumour volume changes during bromocriptine therapy in 15 patients with large tumours: 7 prolactinomas (left) and 8 non-functioning tumours (right).

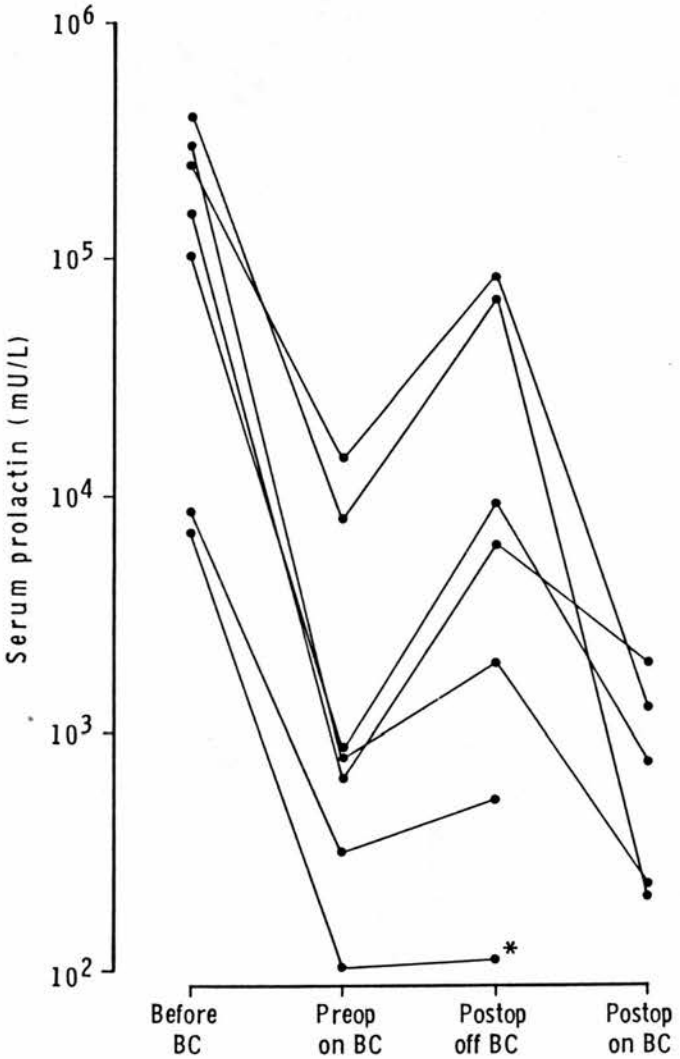
4. Effects of bromocriptine on subsequent surgery

Bromocriptine therapy neither facilitated nor hindered surgery in non-functioning tumours. No alteration in tumour consistency was apparent at operation and in 4 out of 5 patients visual failure was substantially improved by surgery. There were no peri-operative complications in these patients.

In contrast, bromocriptine therapy of more than 10 weeks duration severely hampered subsequent surgery of macroprolactinomas. There were two main problems: asymmetrical tumour shrinkage and tumour fibrosis. Asymmetrical shrinkage occurred in 4 of the 7 bromocriptine-treated prolactinomas and in the most extreme case resulted in a virtually empty sella, but with residual suprasellar tumour on one side which was inaccessible via the transsphenoidal route. The irregular shrinkage of these tumours therefore presented a much more difficult surgical "target".

Bromocriptine-related fibrosis produced a tough tumour consistency which made surgical removal extremely difficult. The degree of fibrosis and the surgical difficulty increased with the duration of bromocriptine treatment and was particularly severe in patients P3-P7 (see p.59). Normoprolactinaemia was restored in only 1 prolactinoma patient (P1) by subsequent transsphenoidal surgery and this patient had received bromocriptine for only 3 weeks (Figure 2-11). The prolactinoma fibrosis was responsible for increased peri-operative morbidity in these patients, 3 of the 7 suffering 5 major complications (Table 2-3). The worst example was patient P7 who had received the highest bromocriptine exposure pre-operatively (20 mg daily for 9 months). Despite considerable tumour shrinkage there was still 15 mm suprasellar extension and surgery was advised prior to radiotherapy. The fibrous

Figure 2-11: Prolactin levels during management of seven macroprolactinomas



Serum prolactin concentrations in 7 patients with macroprolactinomas. (1) At diagnosis and prior to bromocriptine, (2) Lowest value on bromocriptine and prior to surgery, (3) Post-operation and off bromocriptine, (4) Post-operation and during further bromocriptine therapy (2.5-7.5 mg daily).

* = P7

suprasellar extension could not be delivered into the fossa and the patient developed fixed dilated pupils one hour later. Computerised tomography showed diffuse tumour swelling and he underwent transfrontal tumour removal. The tumour and surrounding brain were swollen and removal of fibrous tumour was difficult. Following this the patient was rendered panhypopituitary, developed a CSF leak requiring reoperation and had severe permanent visual failure and dementia.

5. Pathology of bromocriptine-treated tumours

a. Introduction

At the outset of this work there were few reports of the pathological changes in pituitary tumours exposed to bromocriptine (Rengachary et al., 1982, Tindall et al., 1982). This sub-section describes the immunocytochemical and electron microscopic appearances of the above tumours. It also includes a quantitative study of the amount of fibrous tissue present in bromocriptine-treated prolactinomas and non-functioning tumours. Two control groups of macroadenomas, matched for tumour size, that had not been exposed to bromocriptine (8 prolactinomas and 7 non-functioning tumours) were studied for comparison. A brief discussion setting these findings in the context of the current literature concludes this sub-section. The work has been recently published (Esiri et al., 1986) and the pathology methods are included as an Appendix.

b. Light microscopy and immunocytochemistry

Prolactinomas that had not been exposed to bromocriptine all showed uniform immunostaining for prolactin in greater than 90% of tumour cells. By contrast prolactinomas from bromocriptine-treated patients showed greatly reduced prolactin staining which was present in less than

20% of cells.

All non-functioning tumours were entirely negative for prolactin immunostaining. Three of the non-functioning tumours from bromocriptine-treated patients showed immunostaining for LH, FSH or both, though this was confined to less than 10% of cells in each case. Gonadotrophin staining was accompanied by immunostaining for alpha sub-unit in the same cells. One tumour showed a small number of cells staining for alpha sub-unit alone. Two of the control non-functioning tumours stained in similar fashion for gonadotrophins. Bromocriptine treatment did not appear to alter any of the light microscopic features in non-functioning tumours.

c. Electron microscopy

Prolactinomas from bromocriptine-treated patients showed clear evidence of cell size reduction at the electron microscopic level compared with control tumours. There was a 54% reduction in total cell area after bromocriptine, with decreases in both cytoplasmic and nuclear components, particularly the former (Table 2-5). The cytoplasmic shrinkage was mostly due to marked involution of the rough endoplasmic reticulum and Golgi apparatus.

Bromocriptine produced no changes in non-functioning tumour cell morphology at the ultrastructural level although this was not formally quantified. Four control and five bromocriptine-treated non-functioning tumours had significant oncocytic change (>10% tumour cells containing an abundance of mitochondria).

Table 2-5: Cell morphometry of macroprolactinomas from bromocriptine-treated and untreated patients

	Total cell area	Nucleus area	Cytoplasmic area
BC-treated (n=5)	0.1558±0.0106	0.0674±0.0051	0.0884±0.0072
Untreated (n=4)	0.3355±0.0423	0.1182±0.0131	0.2173±0.0307
P value for difference	0.008	0.008	0.008

Values are mean ± SEM. Cell areas are given in mm^2
Statistical analysis was with the Mann-Whitney U test.

d. Tumour fibrous tissue content

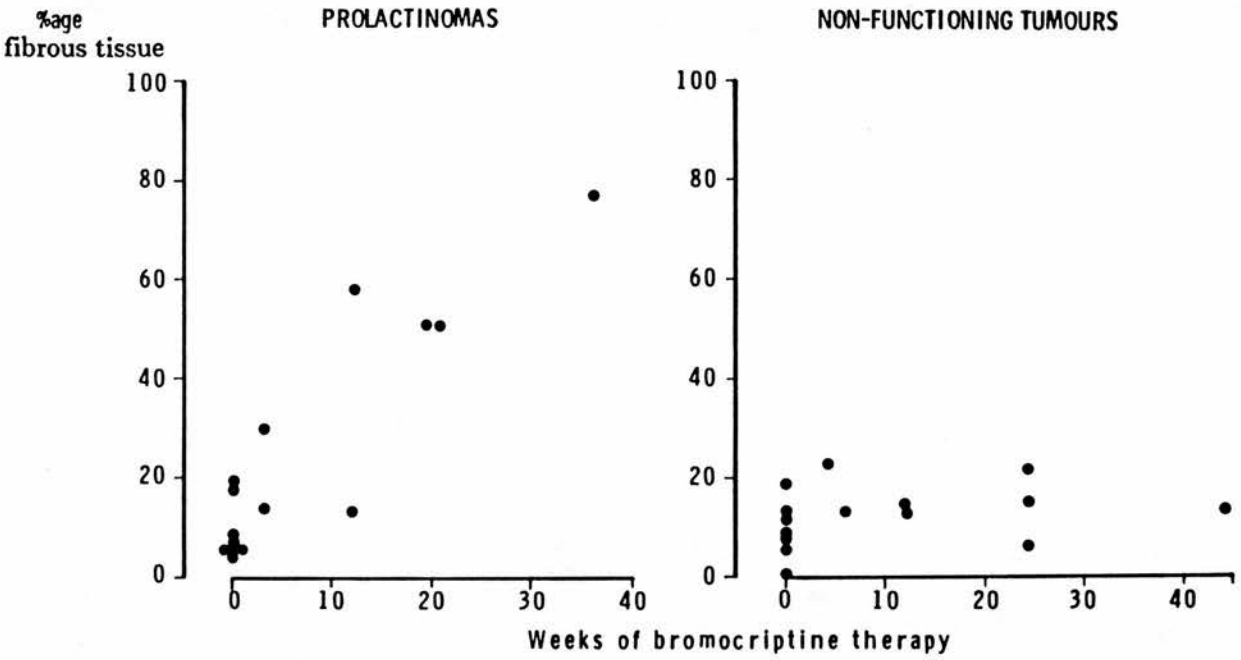
In view of the tough fibrous consistency of bromocriptine-treated macroprolactinomas at operation the effect of bromocriptine exposure on fibrous tissue content of prolactinomas and non-functioning tumours was studied. Fibrous tissue content was measured using a point-counting technique on tumour sections stained for reticulin (see Appendix). The results are shown in Figure 2-12. Most untreated prolactinomas contained very little fibrous tissue and the tumour area occupied by fibrous tissue varied from 4-19%. Prolactinomas exposed to bromocriptine contained a clear excess of fibrous tissue and after 12 weeks of bromocriptine therapy all values for the proportion of tumour area occupied by fibrous tissue lay above the range for untreated patients. Furthermore there was a significant tendency for tumour fibrous tissue content to increase with the duration of bromocriptine therapy ($r = 0.82$, $P < 0.005$, Figure 2-12 [left]).

Non-functioning tumours from untreated patients contained similar amounts of fibrous tissue to the control prolactinomas and the tumour area occupied by fibrous tissue varied from 1-19%. In contrast to the prolactinomas however there was no increase in fibrous tissue following bromocriptine (Figure 2-12 [right]).

e. Discussion

The finding of markedly reduced prolactin immunostaining in cells from bromocriptine-treated macroprolactinomas was in agreement with other reports (Rengachary et al., 1982, Barrow et al., 1984, Gen et al., 1984). However Bassetti et al (1984) found that prolactin immunostaining was increased in some treated prolactinomas and speculated that in these tumours bromocriptine was exerting a different net effect on prolactin

Figure 2-12: Fibrous tissue and duration of bromocriptine treatment



The relationship of fibrous tissue to duration of pre-operative bromocriptine treatment in patients with prolactinomas and non-functioning tumours.

release and synthesis. These observations are of some relevance to the mechanism of action of bromocriptine on the tumourous lactotroph and will be discussed in more detail in Chapter 10.

The lactotroph cell size reduction described above confirms the findings of other workers (Barrow et al., 1984, Bassetti et al., 1984). It seems likely that this explains the striking shrinkage of macroprolactinomas during bromocriptine therapy. It was intriguing that the size reduction of individual lactotrophs of approximately 50% was similar to the average percentage reduction in overall tumour volume (Figure 2-10). There was certainly no gross cell necrosis in the bromocriptine-treated tumours and this has also been the experience of most others (Tindall et al., 1982, Barrow et al., 1984, Bassetti et al., 1984), although Gen et al (1984) described some cell necrosis in macroprolactinomas treated with bromocriptine for 14-36 weeks.

Macroprolactinomas treated with bromocriptine for more than 3 months and to within 4 days of surgery contained a clear excess of fibrous tissue. These light microscopic findings complement the observations of Landolt and Osterwalder (1984) who carried out a quantitative electron microscopic study and found a significant increase in perivascular fibrous tissue in 21 prolactinomas treated with bromocriptine for more than 3 months. The tumours they studied were considerably smaller than those described above, and bromocriptine was withdrawn for a considerable time before surgery (several weeks to 5 years), which may explain why they were unable to relate severity of fibrosis to duration of treatment. In contrast, there was a strong correlation between fibrosis and treatment duration in the macroprolactinomas presented here. The findings of Landolt and Osterwalder (1984) suggest that there is increased deposition of connective tissue, not simply collapse of the

connective tissue stroma already present, to explain the increased proportion of tumour occupied by connective tissue. This, they suggest, is secondary to bromocriptine-induced cell size reduction. Certainly the increase in fibrous tissue after bromocriptine treatment occurred only in the macroprolactinomas and not in the non-functioning tumours, none of which shrank during treatment.

6. Pre-operative bromocriptine: general conclusions

There have been few reports of specific bromocriptine pre-treatment of macroprolactinomas in an attempt to facilitate subsequent surgery. Weiss et al (1984) claimed that prior bromocriptine treatment of macroprolactinomas improved the surgical results. However a proportion of their 19 tumours given bromocriptine for 8-14 weeks probably fell into my mesoadenoma grouping; only 3 of the 9 cured tumours had initial serum prolactin levels greater than 10,000 mU/L although all had some degree of extrasellar extension. There was no mention of adverse tumour consistency in prolactinomas treated with bromocriptine for up to 14 weeks. Barrow et al (1984) reported that macroprolactinomas pre-treated with bromocriptine for 6 weeks had a softer and more fluid tumour consistency which facilitated removal by suction. In contrast, Fahlbusch et al (1984) found that pre-operative bromocriptine for 2-3 weeks did not improve the cure rate for macroprolactinomas and felt that even this short treatment period led to a firm tumour consistency which increased the surgical difficulty.

There is also controversy regarding the time course of prolactinoma shrinkage during bromocriptine therapy. Weiss et al (1983) and Barrow et al (1984) found that most tumour shrinkage had occurred after 6 weeks of treatment. Others have demonstrated further tumour regression over longer periods of time (Gen et al., 1984; Clayton et al., 1985; Molitch

et al., 1985).

The above results provide clarification of these two issues. It is quite clear that bromocriptine treatment of macroprolactinomas for longer than 10 weeks severely hampers subsequent surgery due to tumour fibrosis producing a tough tumour consistency. The degree of fibrosis and surgical difficulty were directly proportional to the duration of therapy. In fact the bromocriptine trial in its original form was discontinued after relatively few patients because it became rapidly clear that prolonged bromocriptine treatment was so hampering to later surgery as to be unethical. Furthermore sequential computerised tomography showed that most tumour regression had occurred after 6-8 weeks of bromocriptine and little further anatomical gain accrued during treatment for up to 32 weeks.

These results are important for the management of patients with macroprolactinomas. At present there is still no convincing evidence that bromocriptine therapy alone is curative. The infrequent occurrence of major tumour re-expansion after short-term withdrawal of long-term bromocriptine therapy (Johnston et al., 1984) is presumably due to bromocriptine-related tumour fibrosis. The frequent reappearance of significant hyperprolactinaemia under these circumstances suggests the continuing presence of functioning tumourous lactotrophs and one would expect tumour enlargement in the long-term. Most centres would therefore advise additional therapy to bromocriptine in the form of radiotherapy, surgery or both. The results presented in this chapter would suggest that if surgery is to be performed on a bromocriptine-treated macroprolactinoma then it is prudent to limit the duration of pre-operative bromocriptine therapy to 2-4 weeks; most anatomical advantage has been gained and, even after this short period of treatment, early tumour fibrosis may be present. If surgery is not wished, or if the

tumour is clearly invasive, bromocriptine provides excellent decompression of most macroprolactinomas as a prelude to definitive radiotherapy.

The adverse effect of bromocriptine on surgery for prolactinoma appears to be confined to the large tumours. Ten Group 1 and six Group 2 prolactinomas described in section B had been previously exposed to bromocriptine, although this had been generally stopped more than 3 months prior to surgery. The surgical cure rates in these two sub-groups of patients were 60% and 67% respectively, not significantly different from the overall results for Groups 1 and 2.

Early reports suggested that a proportion of non-functioning tumours would regress during long-term bromocriptine therapy (Johnston et al., 1981, Wolleson et al., 1982). The above results, and those of others (Verde et al., 1985; Zarate et al., 1985; Grossman et al., 1985b; Pullan et al., 1985), have shown that the major shrinkage commonly encountered with prolactinomas is unlikely to occur with non-functioning tumours even during prolonged therapy. Bromocriptine had no clinical or pathological effects on these tumours and, at present, surgery remains the treatment of choice.

D. GENERAL CONCLUSIONS FROM THE CLINICAL STUDIES

The implications of these results for patient management are discussed fully in the final chapter. This section presents the conclusions in summary form before the thesis moves on to the laboratory studies.

1. Macroprolactinomas

A large pituitary lesion associated with a serum prolactin concentration of greater than 5000 mU/L is likely to be a macroprolactinoma and transsphenoidal surgery, although it relieves pressure effects, seldom results in cure. Bromocriptine causes tumour regression of most macroprolactinomas by reducing lactotroph cell size. Such shrinkage does not however improve the poor surgical results because the regression is frequently asymmetrical and prolonged treatment (>10 weeks) causes tumour fibrosis, both of which make surgery more difficult. Maximum shrinkage occurs after about six weeks of treatment. In most patients, tumour decompression with bromocriptine is best followed by radiotherapy. If surgery is required the duration of pre-operative bromocriptine should probably be limited to one month.

2. Disconnection hyperprolactinaemia

A large variety of lesions in the pituitary region may disconnect the anterior pituitary from hypothalamic inhibition and result in hyperprolactinaemia, though the serum prolactin concentration is usually less than 3000 mU/L. However four patients described above had higher levels than this (maximum 8000 mU/L). Large pituitary lesions associated with prolactin levels of less than 5000 mU/L are unlikely to be prolactinomas and require surgery for diagnosis and decompression. The prolactin responses to TRH do not differentiate between tumourous and non-tumourous hyperprolactinaemia. Other features, such as diabetes

insipidus, may point to a diagnosis other than pituitary adenoma.

3. Non-functioning tumours

Large non-functioning tumours may be associated with disconnection hyperprolactinaemia up to 3000 mU/L (and exceptionally as high as 8000 mU/L). These tumours do not regress during dopamine agonist therapy, even when high doses are used for several months. Bromocriptine does not cause fibrosis of such tumours and neither hampers nor facilitates surgery. The treatment of choice is transsphenoidal surgery which relieves pressure effects, with little peri-operative morbidity, and often produces some improvement in impaired anterior pituitary function. Radiotherapy is indicated as an adjunct to surgery in most cases although this may be delayed in selected patients who retain normal pituitary function.

The studies raised a number of fundamental questions. Why do prolactinomas and non-functioning tumours respond differently to dopamine agonist therapy ? What is the mechanism of cell size reduction in bromocriptine-treated macroprolactinomas ? What pituitary cell-type is represented in the non-functioning tumour ? These and other questions will be addressed in the following chapters which deal with laboratory studies of bromocriptine-treated and untreated prolactinomas and non-functioning tumours.

CHAPTER 3. LABORATORY METHODS I: IMMUNOASSAYS

This chapter describes the validation of the immunoassays. Firstly, the prolactin radioimmunoassay is considered in some detail because of its central importance to both the clinical studies in Chapter 2 and the perifusion studies in Chapters 6 and 7. This is followed by a short section summarising the methods for measuring growth hormone, TSH, FSH and LH. Alpha subunit radioimmunoassay is then described in greater detail to highlight some of the special problems of this assay. Lastly there is a section on the development of a novel ^{125}I-based radioimmunoassay for bromocriptine.

A. RADIOIMMUNOASSAY OF HUMAN PROLACTIN

1. Introduction

The relatively recent characterisation of this hormone and its measurement by radioimmunoassay have been reviewed in Chapter 1. Monomer prolactin is a single polypeptide of 198 amino acid residues and is relatively easily radioiodinated. The radioimmunoassay described below features a particularly robust separation step involving a solid phase second antibody.

2. Materials

1. Buffer: Stock phosphate buffer (0.5 M) contained 57.4 g anhydrous disodium hydrogen orthophosphate and 13.07 g anhydrous potassium dihydrogen orthophosphate dissolved in 1 litre of deionised water. Working buffer comprised 100 mL stock buffer, 5 g bovine serum albumin (Fraction V, Sigma) and 1 g sodium azide made up to 1 litre with deionised water (pH 7.4).

2. Standards: Human prolactin standard prepared from amniotic fluid and

calibrated against IRP 75/504 was obtained from the Chelsea Hospital for Women (London). The addition of 500 µL working buffer to each ampoule produced a standard of 5600 mU/L. For the routine assay this was serially diluted to give standards over the range 90 to 5600 mU/L.

3. Controls: Lyophilised human control sera (Diagnostic Products Corporation, Los Angeles) were used in all assays since there was no protein matrix effect in the prolactin assay when serum volumes of 100-200 µL were used.

4. Antisera: Rabbit anti-human prolactin antiserum (PRL-001, Steranti Research, St Albans, vial designated 500 tubes) was reconstituted in 0.5 mL deionised water and stored at -20°C. This was further diluted 1:60 with working buffer to produce a working antiserum dilution of 1:450,000. This solution was stable for at least 4 weeks at 4°C. Donkey anti-rabbit antiserum coupled to cellulose beads (Sac-Cel, Wellcome Diagnostics, Dartford) was used to separate antibody-bound label.

3. Prolactin iodination

A glass column (1.0 cm i.d.) was packed to a height of 14 cm with Sephadex G-150 Superfine (Pharmacia, Sweden) and equilibrated with 0.05 M phosphate buffer (pH 7.4). The column was pre-run with 2.0 mL 10% bovine serum albumin in 0.05 M phosphate buffer.

A 1 mg/mL solution of Iodo-Gen (Pierce and Warriner (UK) Ltd., Chester) in dichloromethane was diluted 1:25 in dichloromethane. A 30 µL volume was pipetted into a small glass vial and the solvent evaporated under a stream of nitrogen. To the reaction vessel were added 10 µL 0.5 M phosphate buffer, 10 µL $Na^{125}I$ (1mCi, Amersham International, Amersham, Bucks) and 5 µg human prolactin (National Hormone and Pituitary Program, Maryland, USA). The contents were mixed by gentle swirling and the

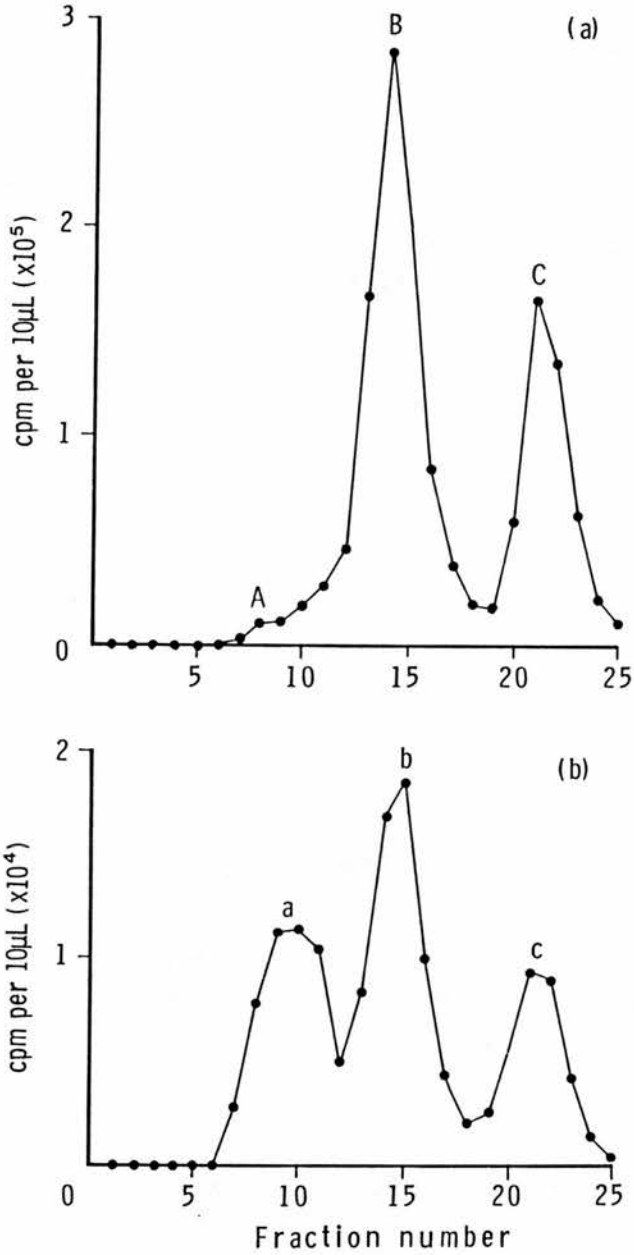
reaction allowed to proceed at room temperature for 10 minutes, when 200 µL of 0.05 M phosphate buffer were added.

The total contents of the vial were then transferred to the Sephadex G-150 column and eluted with 0.05 M phosphate buffer. Fractions of eight drops were collected into plastic tubes (LP4, Luckhams, Sussex) containing 100 µL 2% bovine serum albumin in 0.05 M phosphate buffer containing a trace of thiomersal (BDH, Poole). The radioactivity in a 10 µL aliquot of each 500 µL fraction was counted. A typical elution profile is shown in Figure 3-1a. Fractions around peak B were pooled and stored in aliquots at -20°C. For use in the assay radiolabel was diluted in working buffer to produce about 200,000 cpm/mL (20,000 cpm per assay tube). This dilute radiolabel was stable for at least 2 weeks at 4°C. Each batch of prolactin radiolabel could be used for up to 3 months during which time binding ($[B_o-NSB]/T$) fell from 50% to around 20%. A typical repurification profile is shown in Figure 3-1b; binding using peak b radiolabel was restored to around 50%. The shoulder (A) to peak B following an iodination possibly represented prolactin aggregates; this radiolabel bound more avidly to the prolactin antiserum than peak B radiolabel but was not easily displaceable by non-radioactive prolactin standard. The proportion of prolactin radiolabel in high molecular weight form increased during storage.

4. Assay procedure

Volumes of standards, controls and samples (100 µL) were pipetted, in duplicate, into small plastic tubes (LP3, Luckhams). This was followed by 100 µL radiolabel (20,000 cpm) and 100 µL of antiserum. Tubes were vortex-mixed and incubated overnight at room temperature. The following morning anti-rabbit Sac-Cel (100 µL) was added to each tube except those for total counts and tubes were vortex-mixed and incubated at room

Figure 3-1: Iodination of prolactin and repurification of radiolabel



Sephadex G-150 (Superfine) chromatography
Column: 14 x 1.0 cm. Eluting buffer: 0.05 M phosphate (pH 7.4)

(a) A typical elution profile following prolactin iodination. Percent binding: A (prolactin aggregate ?) 73%, B (prolactin monomer) 45%, C (free iodide) 0%.

(b) Repurification of prolactin radiolabel following storage at -20°C for 13 weeks.

temperature for 15 minutes. Finally 1.5 mL of 0.1% (v/v) Triton X-100 (BDH, Poole) in deionised water was added and the tubes centrifuged at 2000 g for 5 minutes. Supernatant fluid was aspirated and the Sac-Cel pellet was counted for 1 minute on a multihead gamma counter (Multigamma, LKB Ltd., Selsdon, Surrey). Percentage binding [100(B-NSB)/(B_o-NSB)] of standard samples was calculated and fitted to a spline function; results of unknown samples were calculated automatically.

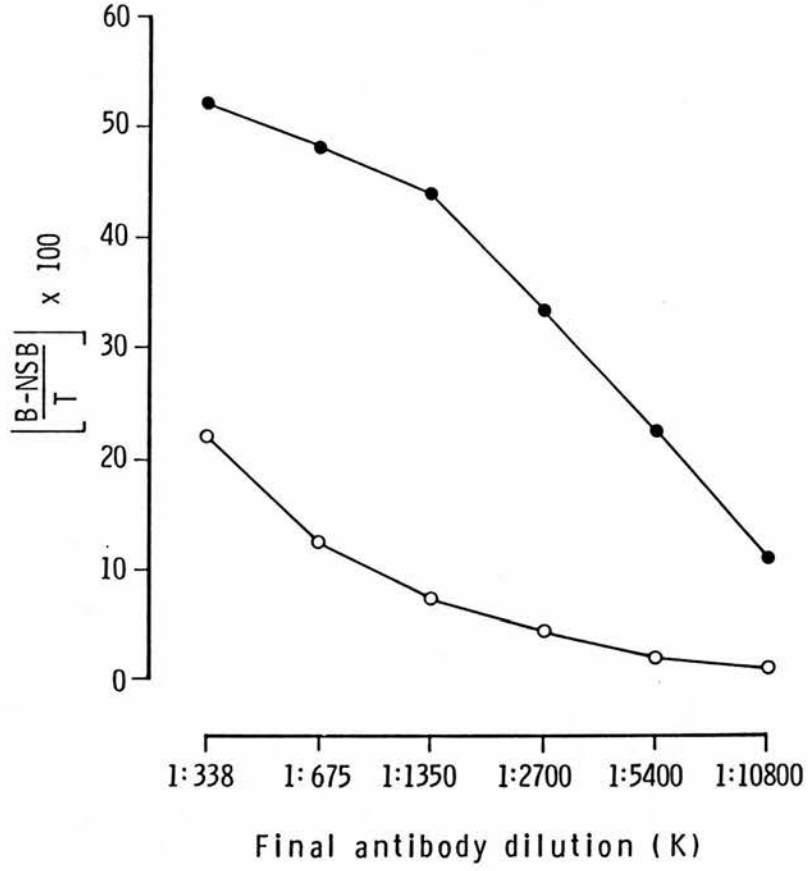
5. Assay validation

An antibody dilution curve of the anti-prolactin antiserum showed that a final dilution of 1:1,350,000 gave 45% binding of tracer at zero dose (Fig 3-2) and this dilution was used in all routine assays. Maximum first antibody binding required overnight incubation at room temperature (Fig 3-3a).

The volume of solid phase second antibody required to precipitate the anti-prolactin antibody completely was investigated by the addition of different volumes of undiluted Sac-Cel to zero dose and non-specific binding tubes. Incubations were at room temperature, and overnight for the first incubation between tracer and anti-prolactin antiserum and for 15 minutes with the Sac-Cel. For routine use a 100 µL volume of undiluted Sac-Cel was used to precipitate the first antibody (Figure 3-3b).

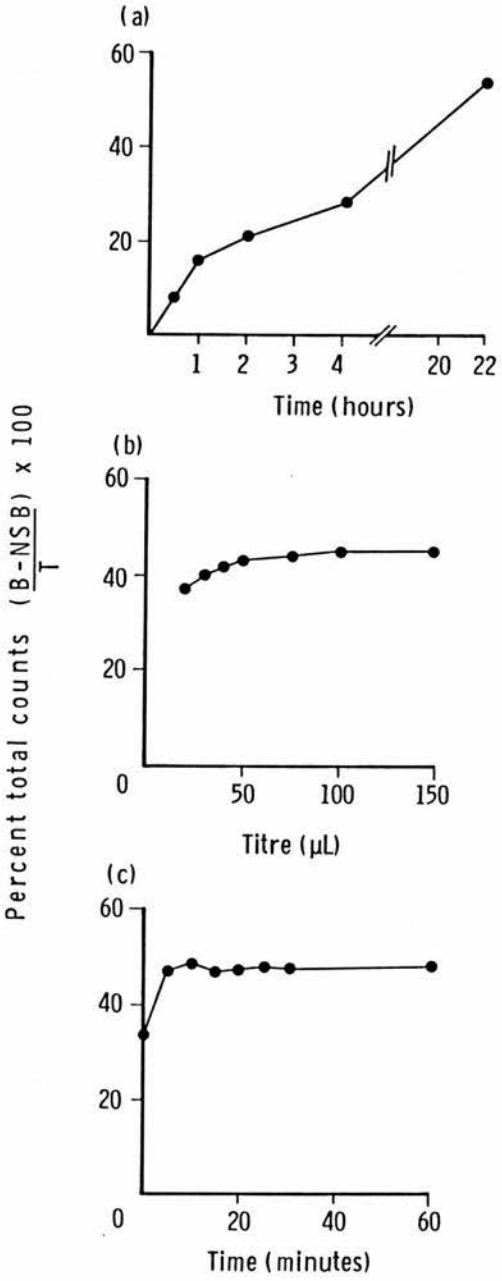
The time required to attain equilibrium between anti-prolactin antiserum and Sac-Cel (100 µL) at room temperature was about 5 minutes and 15 minutes was the time used in all assays (Figure 3-3c).

Figure 3-2: Antibody dilution curve for prolactin



Solid circles indicate binding at zero dose and open circles the binding in the presence of non-radiolabelled prolactin (16,000 mU/L). The final antibody dilution was that present in a reaction volume of 300 µL.

Figure 3-3: First and second antibody reactions in the prolactin radioimmunoassay



The effect of incubation time on binding of prolactin tracer to anti-prolactin antiserum at zero dose (a); the effect of Sac-Cel volume on zero dose binding (b); the effect of incubation time on second antibody reaction at zero dose (c).

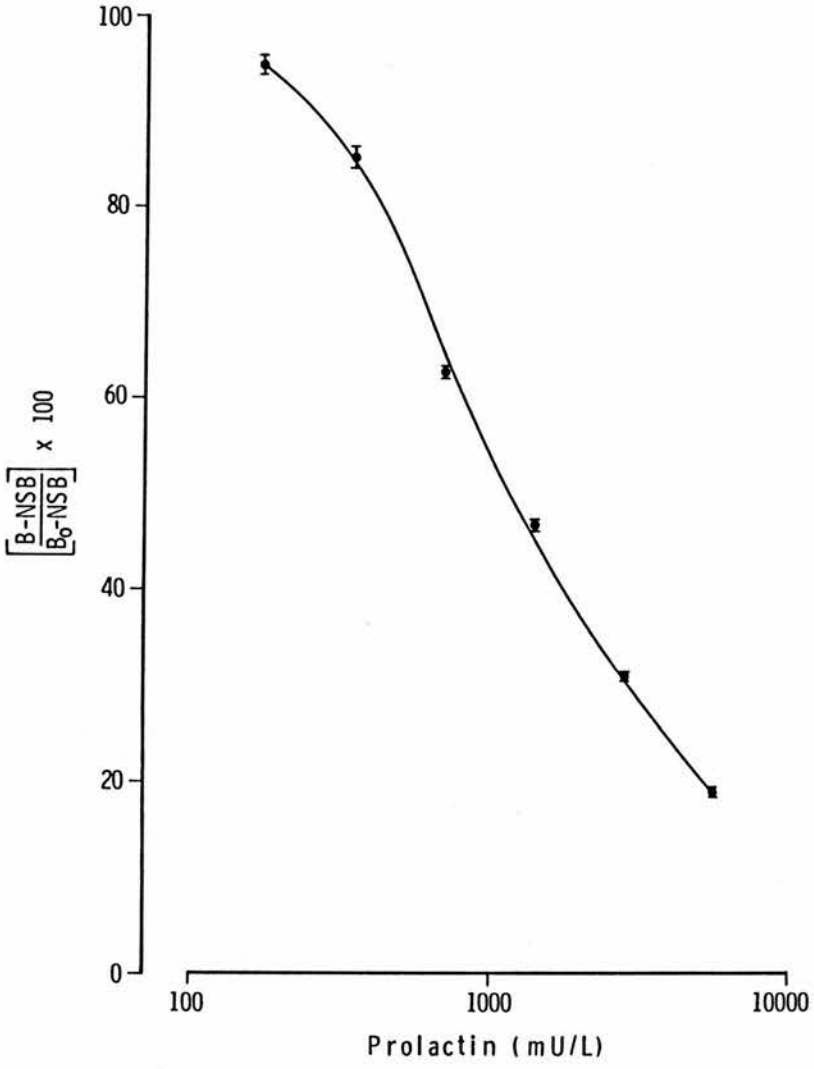
Non-specific binding was less than 2.5% for Sac-Cel volumes of 100µL or less.

The standard curves were highly reproducible and between batch coefficients of variation for serum controls were always less than 10%. The within batch coefficient of variation was 3.8% at a prolactin concentration of 500 mU/L. A set of consecutive assays performed over a period of 8 weeks is represented in Figure 3-4.

The standard assay (section A.4) was satisfactory for measuring serum prolactin concentrations and for most of the perifusion analyses. However, as will be seen in Chapter 6, prolactinomas that had received pre-operative bromocriptine secreted relatively little prolactin in-vitro and a more sensitive assay was required. This was achieved by increasing the sample volume and adding the radiolabelled prolactin after an initial overnight incubation with first antibody alone. These experiments are illustrated by Figure 3-5; a 200 µL sample volume combined with a sequential incubation lowered the detection limit to 15 mU/L.

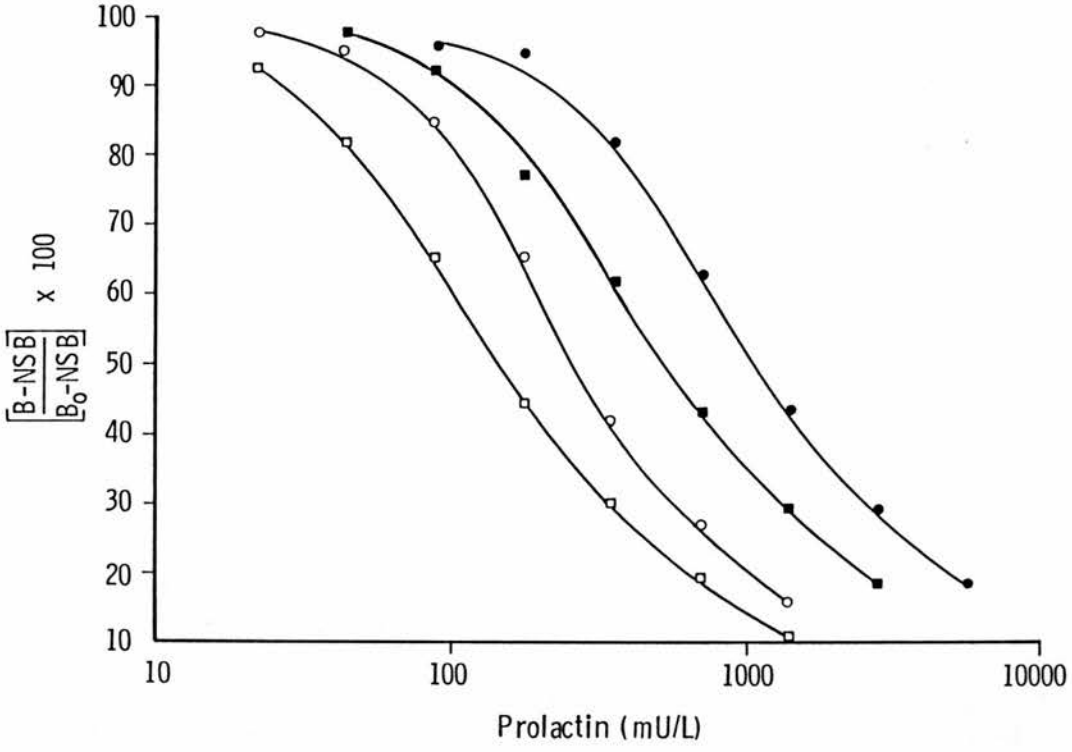
There was no protein matrix effect and serum samples or samples in phosphate buffer or perifusion medium diluted parallel to the standard curve. Crossreactivity studies were performed with other anterior pituitary hormones and there was negligible antibody crossreactivity (less than 5% displacement of prolactin tracer) with GH (200 mU/L, acromegalic serum sample measured against the 4th UK working standard), TSH (200 mU/L, MRC 68/38) and LH (50 IU/L, IRP 68/40).

Figure 3-4: Standard displacement curve for prolactin



Eight consecutive prolactin assays performed with the same batch of tracer over a period of 8 weeks. Binding of tracer ($[B_o$-NSB]/T) was 48% in the first assay and had fallen to 27% at 8 weeks. Values shown are mean $\pm$ SEM.

Figure 3-5: Increasing the sensitivity of the prolactin assay



1.Sample volume: 100 µL
Incubation: Overnight anti-prolactin antiserum and tracer (●)

Overnight anti-prolactin antiserum alone followed by a further 5 hours with tracer present (O)

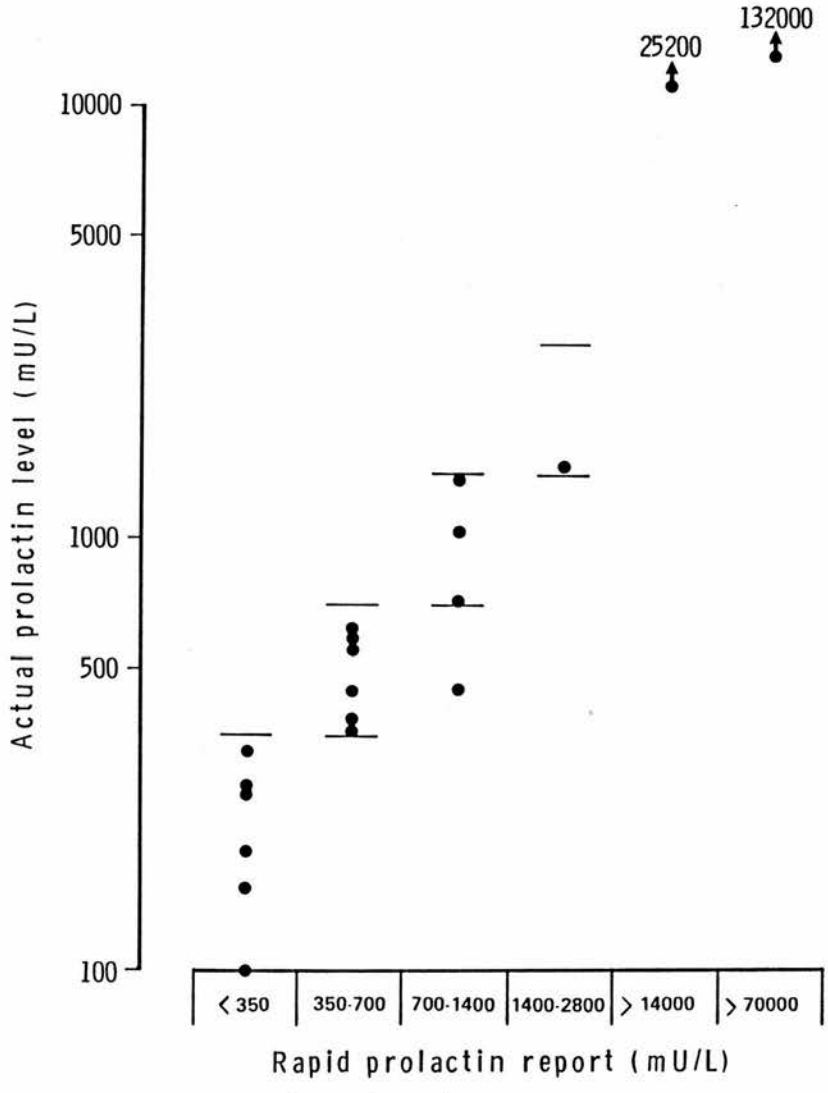
2.Sample volume: 200 µL
Incubation: Overnight anti-prolactin antiserum and tracer (■)

Overnight anti-prolactin antiserum alone followed by a further 5 hours with tracer present (□)

6. Rapid prolactin assay

It will be obvious from Chapter 2 that the most crucial investigation in determining the management of a patient with a large pituitary tumour is the basal serum prolactin concentration. A simple assay was therefore established to provide a rapid estimation of the serum prolactin level in urgent patients with visual failure. The method was identical to that described for the routine assay in section A.4 except that the incubation was for 60 minutes at 37°C. Tubes were prepared in singlicate; standards contained 0, 350, 700, 1400 and 2800 mU/L prolactin and the unknown sample was assayed neat and at 1:10 and 1:100 dilutions. Tracer binding using label less than 6 weeks old averaged 21% of total counts. Rapid prolactin reports were issued in the ranges shown in Figure 3-6 which illustrates the excellent correlation with the formal reports eventually provided by the routine immunoassay laboratory. The rapid prolactin assay accurately predicted the 2 prolactinomas and prevented unnecessary bromocriptine therapy being given to the remainder.

Figure 3-6: Rapid prolactin assay



Comparison of rapid prolactin report with the actual prolactin value eventually assigned to serum samples from 19 newly-presenting patients with large pituitary tumours, 12 of whom had visual failure.

B. RADIOIMMUNOASSAY OF OTHER ANTERIOR PITUITARY HORMONES

This section describes the materials and methods used for the radioimmunoassay of growth hormone, TSH, FSH and LH in serum, perifusion medium and tumour extracts. The methods have several similarities to the prolactin assay and these common aspects will not be described in detail.

Each assay used the working phosphate buffer described in the prolactin method. None of the assays exhibited significant matrix effects except that for growth hormone; the inclusion of 0.05% (v/v) Triton X-100 in the buffer completely overcame this phenomenon. Each assay used a 100 µL sample volume except for growth hormone where 50 µL was chosen. Lyophilised human control sera (Diagnostic Products Corporation, Los Angeles) were used in all assays. Each assay involved the addition of 100 µL diluted antiserum and 100 µL diluted radiolabel (20,000 cpm per assay tube).

All the primary antisera had been raised in rabbits and the separation step already described for the prolactin assay, employing solid phase donkey anti-rabbit antiserum (Sac-Cel), was used in each method. All between assay coefficients of variation were less than 10% and all within assay coefficients of variation were less than 6%, at hormone concentrations which produced approximately 50% displacement of tracer.

The following paragraphs give details of standard source, assay working range, primary antiserum, radiolabel and incubation procedure for each assay.

1. Growth hormone

1. Standard: 4th UK Working Standard (EQAS, Edinburgh)
2. Working range: 0.6-40 mU/L

3. Primary antiserum: Rabbit anti-HGH (6B) was supplied by R.Bacon (EQAS, Edinburgh). Final dilution 1:125,000.
4. Radiolabel: ^{125}I-HGH was produced in the laboratory using identical methodology to that described for prolactin (section A.3). Five µg of highly purified HGH (Boehringer Mannheim, Lewes) was the iodination substrate.
5. Incubation: Overnight with primary antiserum followed by 3 hours with label.

2. TSH

1. Standard: MRC 68/38 (NIBSC)
2. Working range: 1.6-100 mU/L
3. Primary antiserum: Rabbit anti-TSH (Lot No.1112) was obtained from Radioimmunoassay Ltd (Cardiff). Final dilution 1:45,000.
4. Radiolabel: ^{125}I-TSH was produced in the laboratory using the methodology of section A.3. Five µg of highly purified TSH (Flow Laboratories Ltd, Rickmansworth) was the iodination substrate.
5. Incubation: Four hours with primary antiserum followed by overnight incubation with label.

3. FSH

1. Standard: A lyophilised preparation of IRP 78/549 (40 mIU/vial) was obtained from the Chelsea Hospital for Women (London)
2. Working range: 0.6-40 IU/L
3. Primary antiserum: Rabbit anti-human FSH was obtained from Cambridge Medical Diagnostics (CMD) Inc. (Mass, USA). Final dilution was 1:30,000.
4. Radiolabel: ^{125}I-FSH was obtained from CMD.
5. Incubation: Overnight with primary antiserum followed by 6 hours with

label.

4. LH

1. Standard: A lyophilised preparation of IRP 68/40 (50 mIU/vial) was obtained from the Chelsea Hospital for Women (London).
2. Working range: 1.6-50 IU/L
3. Primary antiserum: Rabbit anti-human LH was obtained from CMD. Final dilution was 1:300,000.
4. Radiolabel: ^{125}I-LH was obtained from CMD.
5. Incubation: Overnight with primary antiserum followed by 6 hours with label.

5. Crossreactivity studies

These studies were hampered by the relative impurity of some of the international reference preparations of pituitary hormones, notably the glycoprotein hormones TSH, FSH and, to a lesser extent, LH. IRP 78/549 (FSH) also contains LH, TSH and HGH, and MRC 68/38 (TSH) contains significant amounts of FSH and LH. Patient samples containing pathologically high levels of pituitary hormones were therefore used in some experiments to determine crossreactivity.

Table 3-1 summarises the results of the crossreactivity studies.

Table 3-1: Crossreactivity of pituitary hormone antisera

(A) Crossreactivities in the PRL, HGH, TSH and FSH assays

Test Hormone		Hormone assay PRL	HGH	TSH	FSH
PRL[a]	(mU/L)	-	50,000	100,000	50,000
HGH[b]	(mU/L)	200	-	100	60
TSH[c]	(mU/L)	200	600	-	25[d]
FSH[e]	(ng/mL)	ND	ND	6.3	-
LH[f]	(IU/L)	50	200	200	100
Alpha subunit[g]	(ng/mL)	ND	ND	80	22

Table (A) shows the maximum hormone concentration producing negligible crossreactivity (<5% displacement of tracer binding).

(B) Crossreactivities in the alpha subunit and LH assays

Test hormone	Hormone assay Alpha subunit	LH
Alpha subunit[g]	100	34.8
FSH[e]	6.6	7.4
LH[f]	10	100
TSH[c]	0.8	0.2

Table (B) shows the percentage crossreactivities calculated on a weight basis.

KEY

a = Prolactinoma patient sample (186,000 mU/L against IRP 75/504)
b = Acromegalic patient sample (204 mU/L against 4th Working Std)
c = MRC 68/38
d = Sample from hypothyroid prepubertal boy (180 mU/L against MRC 68/38)
e = NIBSC research preparation of highly purified FSH (83/575)
f = IRP 68/40
g = NIADDK standard (National Hormone and Pituitary Program, USA)
ND = not done

C. RADIOIMMUNOASSAY OF ALPHA SUBUNIT

1. Introduction

The glycoprotein hormones TSH, FSH, LH and chorionic gonadotrophin comprise an alpha and beta subunit (Saxena and Rathnam., 1971). The beta subunit is unique to each hormone and confers specificity of biological action. In contrast, the alpha subunits of the four hormones are virtually identical (Vaitukaitis et al., 1976). Alpha subunit consists of 89 amino acid residues together with polysaccharide side-chains and has an approximate molecular weight of 14700 daltons (Keutmann et al., 1978)

Alpha subunit has been demonstrated in a number of different types of pituitary adenoma. Those which secrete gonadotrophins usually secrete alpha subunit in addition (Snyder et al., 1980) and some apparently functionless pituitary adenomas produce alpha subunit alone (Ridgeway et al., 1981). The presence of an increased serum alpha subunit/TSH molar ratio in a patient with inappropriate secretion of TSH favours a diagnosis of TSH-secreting pituitary tumour rather than thyrotroph hyperplasia (Faglia, 1984). Beck-Peccoz et al (1986) showed that alpha subunit was secreted by a sub-group of growth hormone-secreting pituitary adenomas.

The following section describes the validation of a radioimmunoassay for alpha subunit used for measurement in serum, perifusion medium and tumour extracts.

2. Alpha subunit iodination

A glass column (1.0 cm i.d.) was packed to a height of 27 cm with Sephadex G-100 Superfine (Pharmacia, Sweden) and equilibrated with 0.05 M phosphate buffer (pH 7.4). The column was pre-run with 2.0 mL 10%

bovine serum albumin in 0.05 M phosphate buffer.

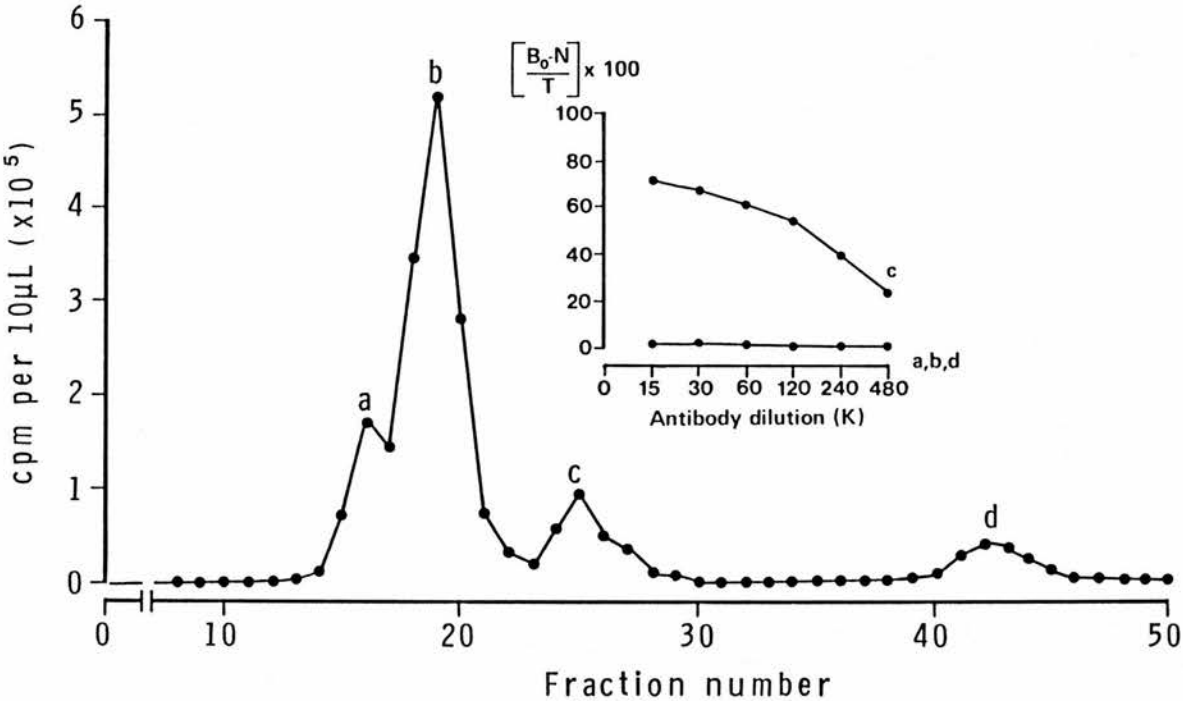
Twenty µL 0.5 M phosphate buffer and 2 µg (10 µL) FSH alpha subunit (Batch 2, NIADDK, National Hormone and Pituitary Program, USA) were dispensed into a small polythene vial and mixed. 10 µL of $Na^{125}I$ (1 mCi, Amersham International) was then added. The reaction was started with 10 µL of Chloramine T (2.0 mg/mL, Sigma) and allowed to proceed for 20 s at room temperature. The iodination was terminated with a 100 µL volume of sodium metabisulphite solution (1.0 mg/mL, BDH, Poole).

The total contents of the vial were then transferred to the Sephadex G-100 column and eluted with 0.05 M phosphate buffer. Fractions of eight drops were collected in plastic tubes containing 100 µL 2% bovine serum albumin in 0.05 M phosphate buffer containing a trace of thiomersal. A typical elution profile is shown in Figure 3-7. Peak c presumably represented monomeric alpha subunit since this was the only peak which showed radiolabel binding to the alpha subunit antiserum (Figure 3-7, inset). Peaks a and b probably represented aggregates of alpha subunit and peak d free iodide. Peak c radiolabel was stored in aliquots at -20°C and could be used for up to 3 months. A gel filtration column of at least 20 cm in length was required to reveal peak c containing the monomeric ^{125}I-alpha subunit.

3. Assay validation

The antibody dilution curve of anti-alpha subunit antiserum showed that a final dilution of 1:120,000 gave 55% binding of tracer at zero dose (Figure 3-7) and final dilutions of 1:120,000-1:160,000 were used in all assays. The separation step with solid phase second antibody was identical to that described for the prolactin method. The buffer used was the working phosphate buffer previously described supplemented with

Figure 3-7: Iodination of alpha subunit and antibody dilution curves



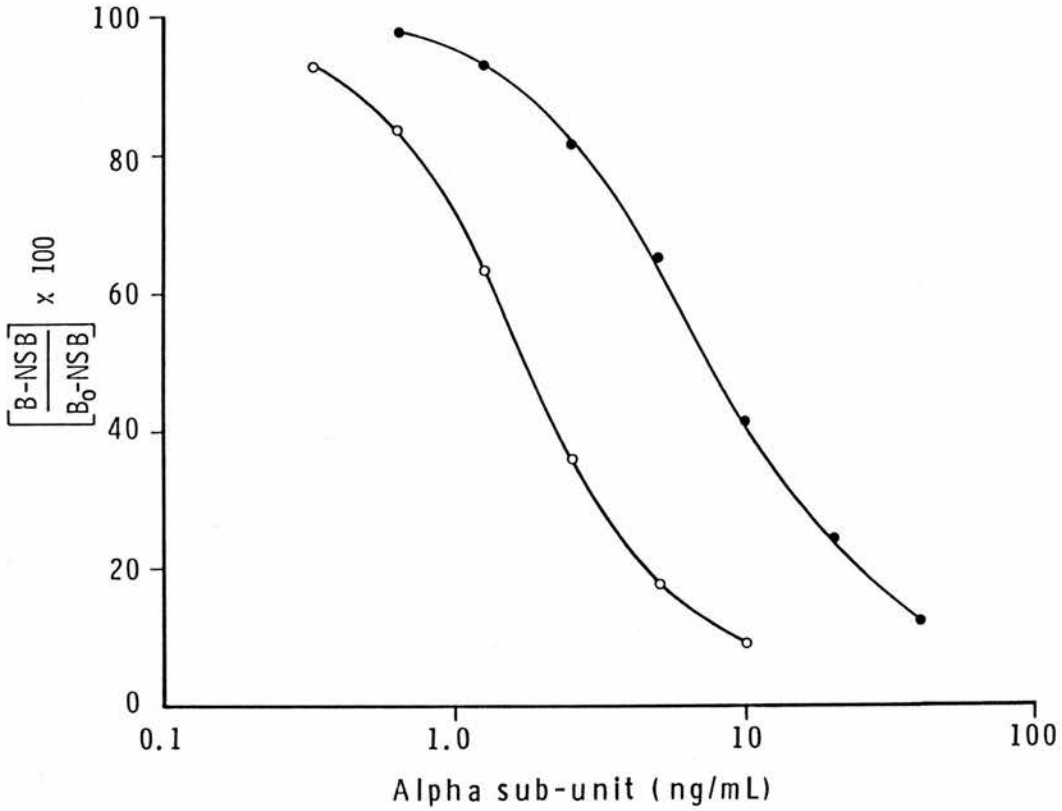
Sephadex G-100 (Superfine) chromatography following alpha subunit iodination. Column 27 x 1.0 cm. Eluting buffer: 0.05 M phosphate (pH 7.4)

Inset figure shows antibody dilution curves for the four iodination peaks. 100 µL diluted rabbit anti-FSH alpha subunit (Batch 1, NIADDK) was incubated overnight with 100 µL diluted radiolabel (20,000 cpm).

0.05% (v/v) Triton X-100. The surfactant was necessary to overcome the matrix effects observed when high alpha subunit concentrations in tissue extracts were measured. This was probably due to alpha subunit aggregation, a phenomenon possibly suggested by the gel filtration profile following an iodination (Figure 3-7).

The effects of sample volume and incubation conditions on the standard displacement curve were investigated and Figure 3-8 illustrates two sets of conditions, one optimised for serum measurements and the other for tissue extract measurements. Crossreactivity studies were performed with other pituitary glycoprotein hormones and on a weight basis the following crossreactivities were obtained; TSH (0.8%, MRC 68/38), FSH (6.6%, NIBSC 83/575), and LH (10%, IRP 68/40). These values were similar to those reported by other authors using alpha subunit assays based on NIADDK materials (Ridgeway et al., 1981; Surmont et al., 1983).

Figure 3-8: Standard displacement curves for alpha subunit



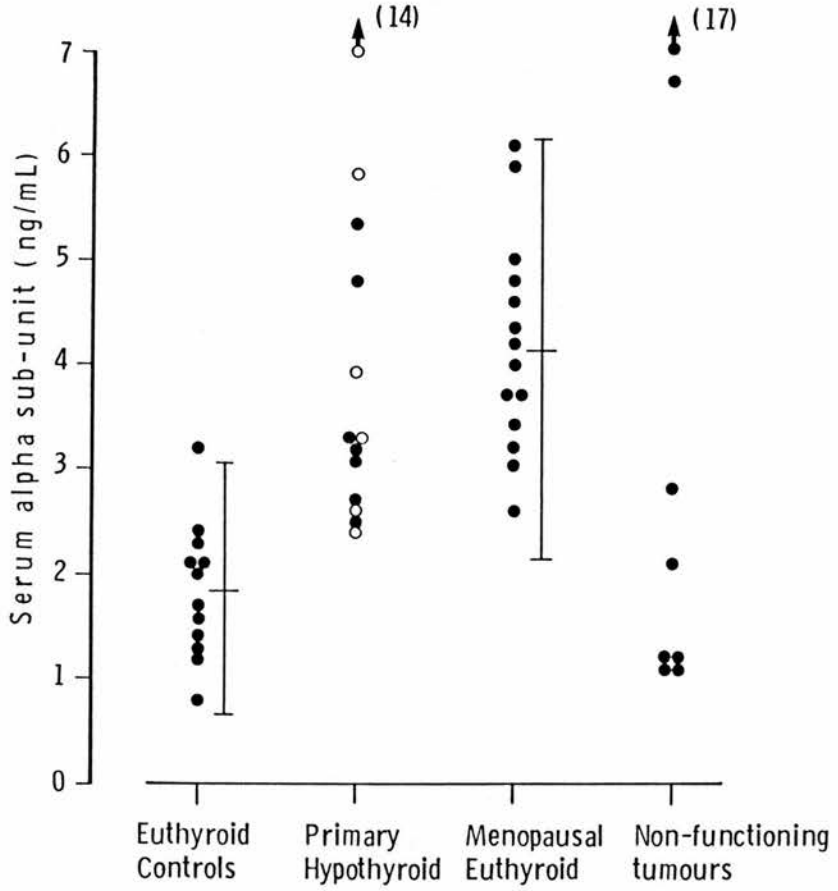
(●) Sample volume 100 µL.
Overnight incubation with primary antiserum and tracer.
Working range 0.6-40 ng/mL.
Suitable for tissue content measurement.

(O) Sample volume 200 µL.
Overnight incubation with primary antiserum alone followed by a further 5 hours with tracer present.
Working range 0.2-10 ng/mL.
Suitable for serum measurement.

4. Serum alpha subunit concentrations

Serum alpha subunit concentrations were measured in a group of euthyroid control subjects and compared with those in postmenopausal women and patients with primary hypothyroidism to establish normal and pathological reference ranges. Several patients with non-functioning pituitary tumours were also investigated. The results are shown in Figure 3-9 and were in good agreement with previously published data (Ridgeway et al., 1981, Faglia et al., 1984). The non-functioning tumours from the two patients with serum levels above 6 ng/mL contained a number of cells which immunostained for both alpha subunit and FSH. Three non-functioning tumours in which a minority of cells immunostained for alpha subunit alone were removed from patients with normal serum alpha subunit levels. In these patients alpha subunit was not a reliable tumour marker. Two patients with TSH-secreting macroadenomas had elevated alpha subunit levels (4.5 and 10.4 ng/mL) as will be discussed in Chapter 9.

Figure 3-9: Serum alpha subunit concentrations



Confidence limits, where marked, indicate the 95% level.

Euthyroid controls (n=13) TSH <5 mU/L
(8 women, 5 men, age 38.1 ± 5.4 years [Mean ± SEM])

Primary hypothyroidism (n=14) TSH >40 mU/L
(10 women, 4 men, age 69.9 ± 4.8 years [Mean ± SEM])
Open circles indicate 6 women who were, in addition, post-menopausal.

Post-menopausal euthyroid women (n=14) FSH >40 IU/L
(age 50.4 ± 2.3 years [Mean ± SEM])

D. RADIOIMMUNOASSAY OF BROMOCRIPTINE

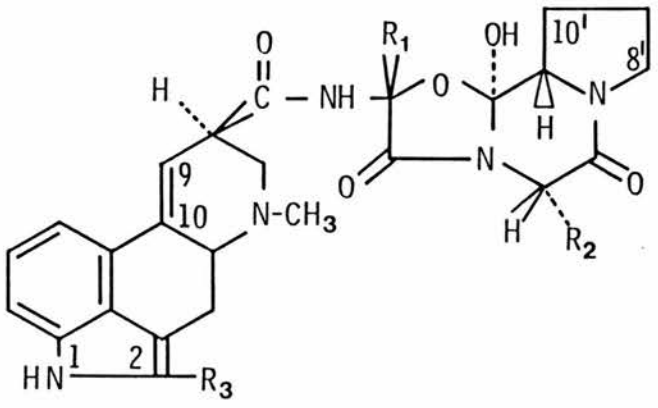
1. Introduction

The opportunity to develop a radioimmunoassay for bromocriptine arose from my attempts to produce an ^{125}I-labelled radioligand to improve the sensitivity of the pituitary membrane dopamine receptor assay as will be further discussed in Chapter 5. Some aspects of the chemistry of bromocriptine and its relation to the compound's pharmacological properties have been covered in Chapter 1. The structural formulae of bromocriptine and two other dopamine agonists relevant to this section are shown in Figure 3-10.

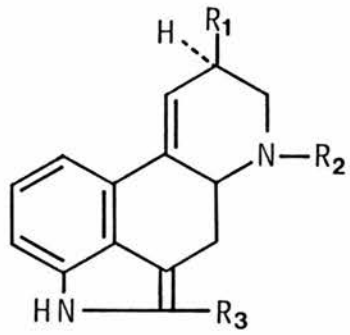
An assay capable of measuring therapeutic concentrations of bromocriptine in plasma has to be exquisitely sensitive. Plasma concentrations of bromocriptine are low due to the small therapeutic dose required and the high hepatic extraction ratio and extensive metabolism of the drug (Maurer et al., 1983). Early pharmacokinetic studies on the drug were limited to sensitive but nonspecific methods involving the administration of radiolabelled drug (Aellig et al., 1977). The development of antibodies which recognized the intact, and metabolically labile, tripeptide portion of the molecule led to the development of specific radioimmunoassays for bromocriptine (Schran et al., 1979, Rosenthaler et al., 1983). All radioimmunoassays described to date have employed ergopeptines labelled with tritium and as a consequence have lacked the convenience, precision and sensitivity of the method to be described which employs ^{125}iodine.

This section describes the production of a ^{125}I-labelled derivative of dihydroergocriptine and its use in a rapid and sensitive radioimmunoassay which employed a specific antiserum directed against the tripeptide moiety of bromocriptine. The method was used to measure

Figure 3-10: Structural formulae of bromocriptine, dihydroergocriptine and pergolide



Ergopeptine skeleton



Ergoline skeleton

Ergopeptines	R_1	R_2	R_3
Bromocriptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	Br
Dihydroergocriptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	H
Ergoline			
Pergolide	CH_2SCH_3	$CH_2CH_2CH_3$	H

Dihydroergocriptine and pergolide are reduced at the 9-10 double bond.

plasma bromocriptine concentrations following the oral administration of 2.5 mg bromocriptine to patients with pituitary tumours. Chapter 8 describes the further use of this assay in the measurement of bromocriptine in pituitary tumour extracts. The method has been recently published (Bevan et al., 1986).

2. Materials

1.Buffer: Assay buffer comprised 13.23 g citric acid and 10.51 g anhydrous disodium hydrogen orthophosphate per litre, pH 3.9, and contained 0.1% (w/v) sodium azide as preservative.

2.Standards: 2-bromo-α-ergocriptine methane sulphonate (bromocriptine) was a gift from Sandoz Ltd, Basle, Switzerland. Bromocriptine was initially dissolved in acidified ethanol and subsequently diluted in pooled normal plasma to produce a stock standard of 100 nmol/L which was stored in aliquots at -20°C for up to 2 months. For each assay an aliquot was diluted in plasma to provide standard solutions over the range 0.08-5.0 nmol/L.

3.Controls: Controls were prepared in drug free plasma in the range 0.2-2.5 nmol/L and stored at -20°C in aliquots of 0.25 mL.

4.Antisera: Sheep anti-bromocriptine antiserum was a gift from Dr.J.Rosenthaler of Sandoz Ltd, Basle, Switzerland. The conjugation schedule involved linking bromocriptine to bovine serum albumin via the indole nitrogen at position 1 (Figure 3-10). Lyophilised antiserum was reconstituted in assay buffer to give a stock dilution of 1:1,600 which showed no loss of binding after at least 5 freeze-thaw cycles. Stock antiserum was further diluted in assay buffer to give a working dilution of 1:40,000 which was stable for at least 2 weeks at 4°C.

Donkey anti-sheep/goat antiserum coupled to cellulose beads (Sac-Cel, Wellcome Diagnostics, Dartford) was used to separate antibody-bound label.

3. Iodination and radiolabel

Dihydro-α-ergocriptine methane sulphonate [DHE] (gift from Sandoz Ltd, Basle, Switzerland) was used as the substrate for this reaction. Its structure is identical to bromocriptine except for the presence of a hydrogen atom in place of the bromine at position 2 in the ergoline moiety, and reduction of the 9-10 double bond (Figure 3-10).

Deionised water (70 µL) and 5 µg DHE (10 µL of a 500 µg/mL solution in ethanol/water [1:9 v/v]) were dispensed into a small polythene vial and mixed. 10 µL of $Na^{125}I$ (1 mCi, Amersham International, Amersham, Bucks) was then added. The reaction was started with 10 µL of Chloramine T (0.5 mg/mL) and allowed to proceed for 2 minutes at room temperature. The iodination was terminated with a 50 µL volume of sodium metabisulphite solution (0.5 mg/mL). Experiments to optimise the iodination procedure showed that no further ^{125}I was incorporated by incubating with Chloramine T for longer than 2 minutes. Various concentrations of Chloramine T were investigated and 5 µg was found to be optimal for the iodination of 5 µg DHE and thin layer chromatography showed that approximately 20% DHE remained unchanged following the iodination procedure.

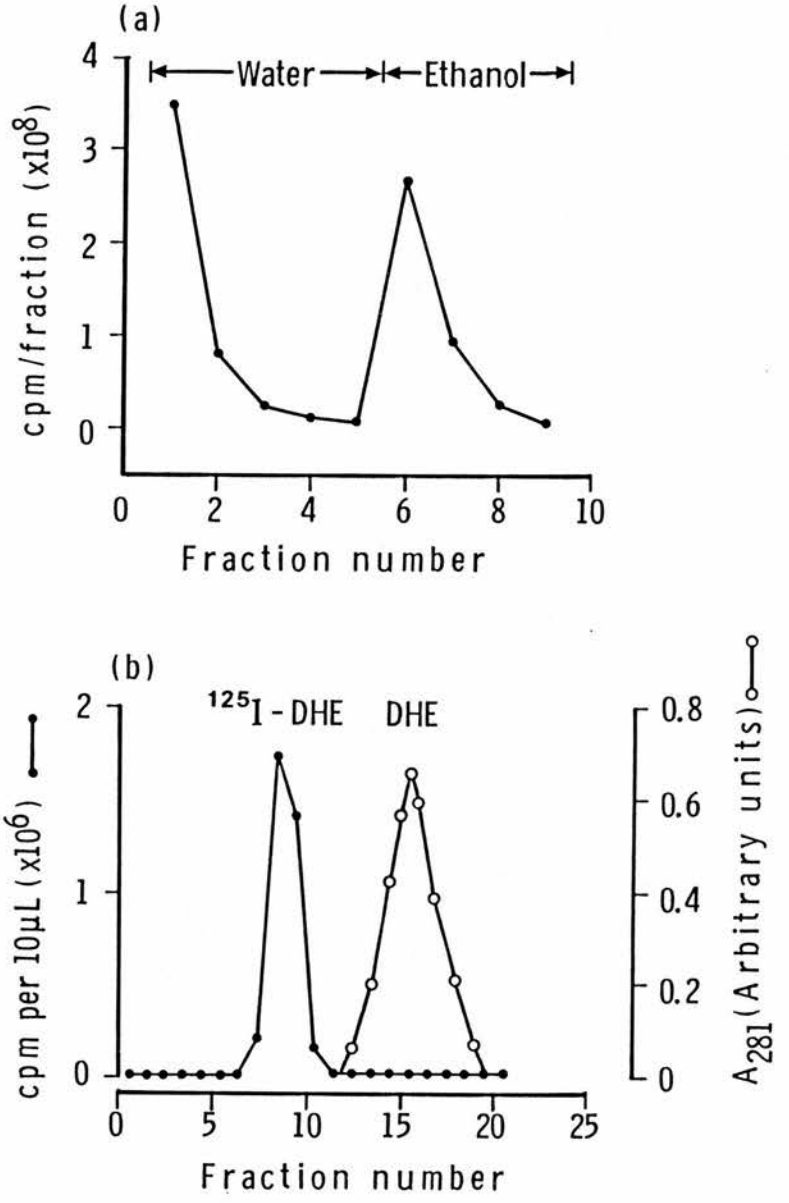
In the early experiments ^{125}I-radiolabelled dihydroergocriptine (^{125}I-DHE) was separated from free iodide using Bio-Gel P2 (200-400 mesh, Bio-Rad Laboratories, Richmond, California) gel filtration. The eluting buffer was 0.05 M phosphate buffer (pH 7.4) and the inclusion of 0.5% (v/v) Triton X-100 was essential to prevent non-specific adsorption of the ^{125}I-DHE to the column. ^{125}I-DHE was thus obtained for initial

evaluation of a suitable bonded silica extraction column for the direct and convenient separation of the iodination reaction products. These studies will not be described in detail but in summary 90% of an ^{125}I-DHE load could be recovered from a phenyl-substituted silica Bond-Elut cartridge (Analytichem International, California) using 2 mL ethanol. ^{125}I-DHE bound strongly to a C-18 substituted Bond-Elut and equivalent recovery into ethanol was only 39%.

The following procedure was used to separate the products of DHE iodination. A phenyl-substituted Bond-Elut was primed with 2 mL methanol followed by 4 mL deionised water. The contents of the iodination vial were then transferred to the top of the cartridge, together with two reaction vial washings with 0.5 mL deionised water. This volume of approximately 1.2 mL was forced through the cartridge under positive pressure (fraction 1). Four washes each of 1 mL of deionised water were similarly passed through and collected as separate fractions. Finally four washes of 1 mL ethanol were applied and collected as separate fractions. Figure 3-11a shows the radioactivity recovered in each fraction during the initial Bond-Elut extraction to remove unreacted iodide. Iodine incorporation of 45% suggested a minimum specific activity for the radiolabel of 250 Ci/mmol.

A 10 µL aliquot of each fraction was diluted into 1 mL of assay buffer and the radioactivity counted. The first ethanol fraction (fraction 6) was evaporated to dryness under a stream of nitrogen at a temperature of 60°C. The unreacted DHE and ^{125}I-DHE were then taken up into 100 µL of a 9:1 mixture of chloroform and methanol and transferred to a bonded silica column (Sepralyte, 40 µm particle size, Analytichem) for separation of the ^{125}I-DHE. The column was 14 cm in length (bed volume 2.8 mL) and equilibrated with 9:1 chloroform/methanol. Fractions were

Figure 3-11: Iodination of dihydroergocriptine



(a) Typical Bond-Elut (phenyl) separation of unreacted iodide (aqueous) from ^{125}I-DHE (ethanol).

(b) Silica gel chromatogram: solid circles show ^{125}I-DHE elution profile following an iodination, open circles show a separate experiment, under identical conditions, with eluted cold DHE detected by UV spectroscopy.

collected every 1.5 minutes (250 µL approximately) and the eluted radioactivity counted. Clear separation of DHE from the ^{125}I-DHE using silica column chromatography is shown in Figure 3-11b.

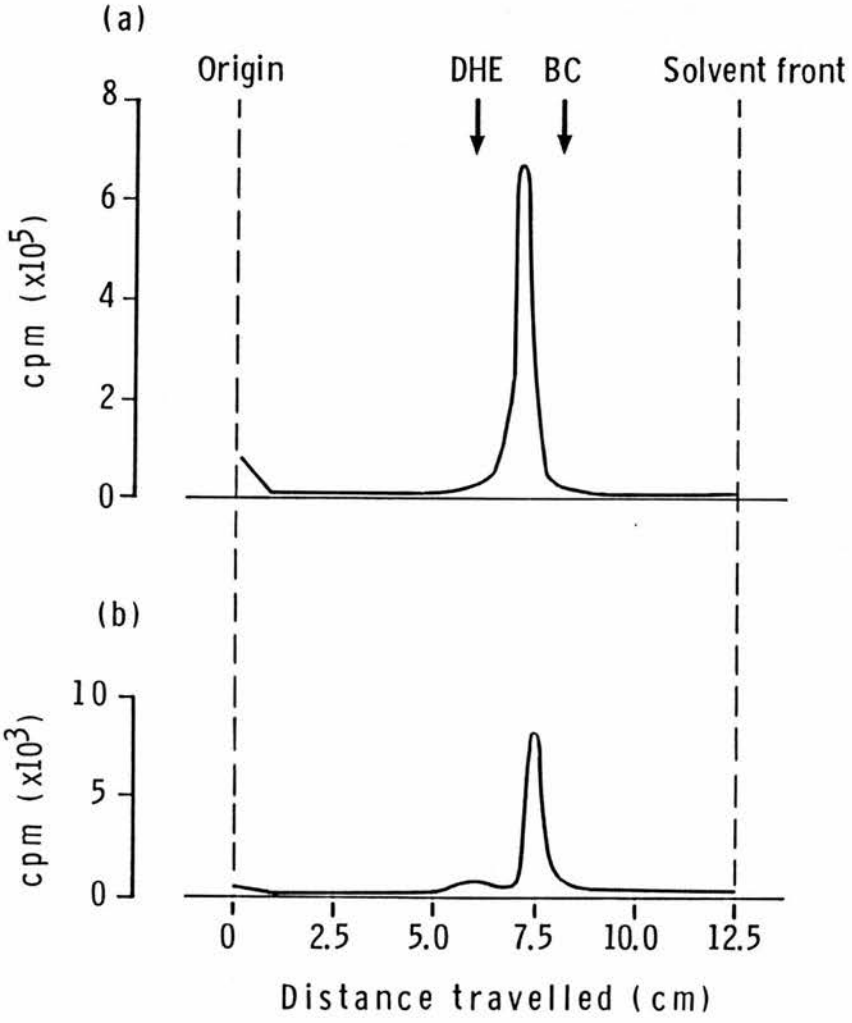
Peak fractions were pooled and dried down under a stream of nitrogen at room temperature. The radiolabel was then taken up in 250 µL ethanol and stored protected from light and under nitrogen, in a silanised glass vial at -20°C. The complete iodination procedure took approximately one hour.

For use in the radioimmunoassay radiolabel was diluted in assay buffer to produce about 300,000 cpm/mL (30,000 cpm/assay tube). The diluted radiolabel was placed in a silanised vial protected from light.

Thin layer radiochromatography of the radiolabel was performed on silica gel plates (silica gel 60, Merck, Darmstadt) using two different solvent systems: (a) chloroform:methanol (9:1, v/v) (b) chloroform:ethanol: glacial acetic acid (9:5:1, by volume). Marker spots of bromocriptine and dihydroergocriptine were visualised using 4-dimethylaminobenzaldehyde (HCl).

The ^{125}I-DHE proved to be stable when stored as described above; no breakdown could be demonstrated using thin layer radiochromatography after 8 months storage at -20°C (Figure 3-12). In each solvent system it travelled as a single peak of radioactivity, close to bromocriptine. In chloroform/methanol (9:1) the ^{125}I-DHE peak had an R_f of 0.59, ahead of DHE (R_f 0.48) and just behind bromocriptine (R_f 0.65). Radiolabel performance in the radioimmunoassay was limited only by the half-life of ^{125}I and it could be used for at least 3 months without significant deterioration in the radioimmunoassay.

Figure 3-12: Thin layer radiochromatography of ^{125}I-DHE



Thin layer radiochromatography of ^{125}I-DHE (2 µL stock) stored for 1 day (a) and 8 months (b).

Solvent: Chloroform/methanol (9:1).
Run time: 45 minutes

DHE and BC denote marker spots of dihydroergocriptine and bromocriptine respectively.

In common with bromocriptine and dihydroergocriptine, the ^{125}I-DHE showed a marked tendency to adsorb onto glass and plastic surfaces. Aqueous solutions could, however, be diluted without losses if silanised glass was used. Furthermore there were no losses on dilution in glass and plastic containers if plasma was present. Identical standard curves were generated in plastic, glass or silanised glass tubes provided 100 uL of plasma per tube was present.

A second potential problem was photosensitivity of the ^{125}I-DHE; if diluted radiolabel were exposed to light at room temperature its antibody-binding ability declined at a rate of 1.8 % per hour. Protection from light reduced this to 0.4 % per hour. In practice, provided tracer solutions were made up fresh for each assay, photosensitivity was not a problem.

4. Assay procedure

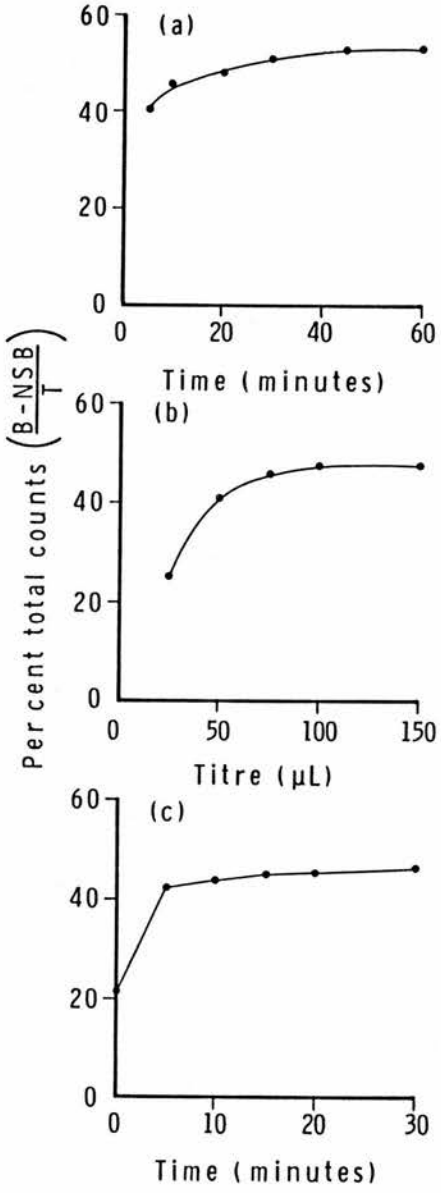
Volumes of standards, controls and samples (100 µL) were pipetted, in duplicate, into small plastic tubes (LP3, Luckhams) followed by 100 µL of antiserum (1:40,000 dilution). Tubes were vortex-mixed and incubated at room temperature for 30 minutes. Then 100 µL of radiolabel was added and the tubes were vortex-mixed and incubated at room temperature for a further 30 minutes. Anti-sheep/goat Sac-Cel (100µL) was added to each tube except those for total counts and tubes were mixed and incubated at room temperature for 15 minutes. Finally 1.5 mL of 0.1% (v/v) Triton X-100 in deionised water was added and the tubes centrifuged at 2000 g for 5 minutes. Supernatant fluid was aspirated and the Sac-Cel pellet was counted for 1 minute on a multihead gamma counter. Percentage binding $[100(B-NSB)/(B_o-NSB)]$ of standard samples was calculated and fitted to a spline function; results of unknown samples were calculated automatically.

5. Assay validation

An antibody dilution curve of the anti-bromocriptine antiserum showed that a working dilution of 1:40,000 gave 50% binding of the radiolabel at zero dose, in the presence of 100 µL of plasma. This was equivalent to a final 1:120,000 dilution in the assay. First antibody equilibrium was reached after 20-40 minutes incubation at room temperature (Figure 3-13a)

The volume of solid phase second antibody required to precipitate the anti-bromocriptine antiserum completely was investigated by the addition of different volumes of undiluted Sac-Cel to zero dose and non-specific binding tubes, in the presence of 100 µL of plasma. Incubations were at room temperature, and for 30 minutes for the first incubation between radiolabel and anti-bromocriptine antiserum and for 15 minutes with the Sac-Cel. For routine use a 100 µL volume of undiluted Sac-Cel was used to precipitate the first antibody (Figure 3-13b). The time required to attain equilibrium between anti-bromocriptine antiserum and Sac-Cel (100 µL) at room temperature was about 5 minutes (Figure 3-13c)

Figure 3-13: First and second antibody reactions in the bromocriptine radioimmunoassay



The effect of incubation time on binding of ^{125}I-DHE to bromocriptine antiserum at zero dose (a); the effect of Sac-Cel volume on zero dose binding (b); the effect of incubation time on second antibody reaction at zero dose (c).

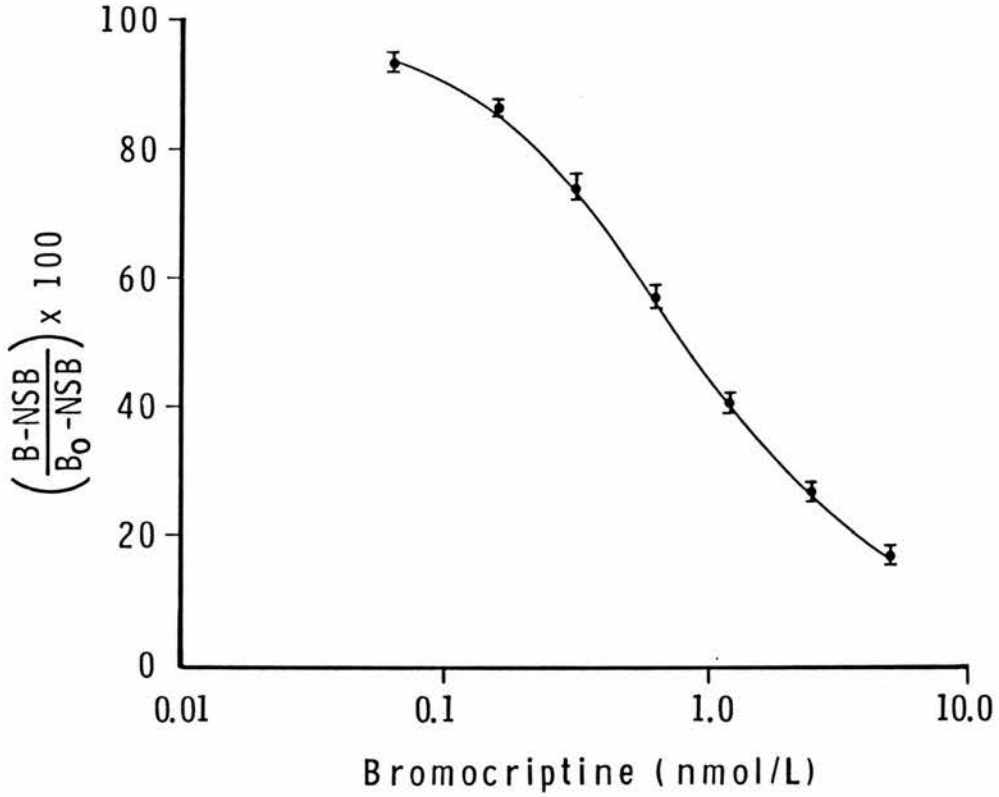
Non-specific binding was less than 4% for Sac-Cel volumes of 100 µL or less.

The working range of the assay was established by altering the sample volume and 100 µL was chosen for routine use, giving 50% displacement at approximately 1.0 nmol/L bromocriptine (Figure 3-14). The detection limit with this sample volume was 0.05 nmol/L, the bromocriptine concentration corresponding to 2.5 times the standard deviation of percent antibody-binding in the zero standard. Recovery of bromocriptine added to plasma ranged between 93 and 113% (104.9 $\pm$ 2.4, mean $\pm$ SEM, n=8).

When neutral buffers were used, direct addition of plasma resulted in falsely elevated apparent bromocriptine concentrations, which did not dilute parallel to the standard curve. This plasma effect was greatly reduced by using acid buffer but standard curves in buffer and plasma were still not identical. Attempts to remove this residual matrix effect using low concentrations of surfactant (0.01% Triton X-100), methanol or a variety of blocking agents, including the ergoline pergolide, were unsuccessful and denatured the anti-bromocriptine antibody. For these reasons standard curves were generated in normal pooled plasma. Inter-patient variation in non-specific binding was small (range 4.5-6.0%) relative to total binding, so it was unnecessary to use a patient's pre-treatment plasma to construct the standard curve for that patient's samples as has proved necessary in most of the tritium based assays (Thorner et al., 1980).

The standard curves were highly reproducible and between batch coefficients of variation for bromocriptine controls were less than 10%. The within batch coefficient of variation was 3.4% at a bromocriptine concentration of 1 nmol/L.

Figure 3-14: Standard displacement curve for bromocriptine



Sample volume: 100 µL

Eight consecutive bromocriptine assays performed with the same batch of tracer over a period of 4 weeks. Binding of tracer was 55% in the first assay and 44% in the final assay.

Values shown are mean ± SEM.

6. Crossreactivity

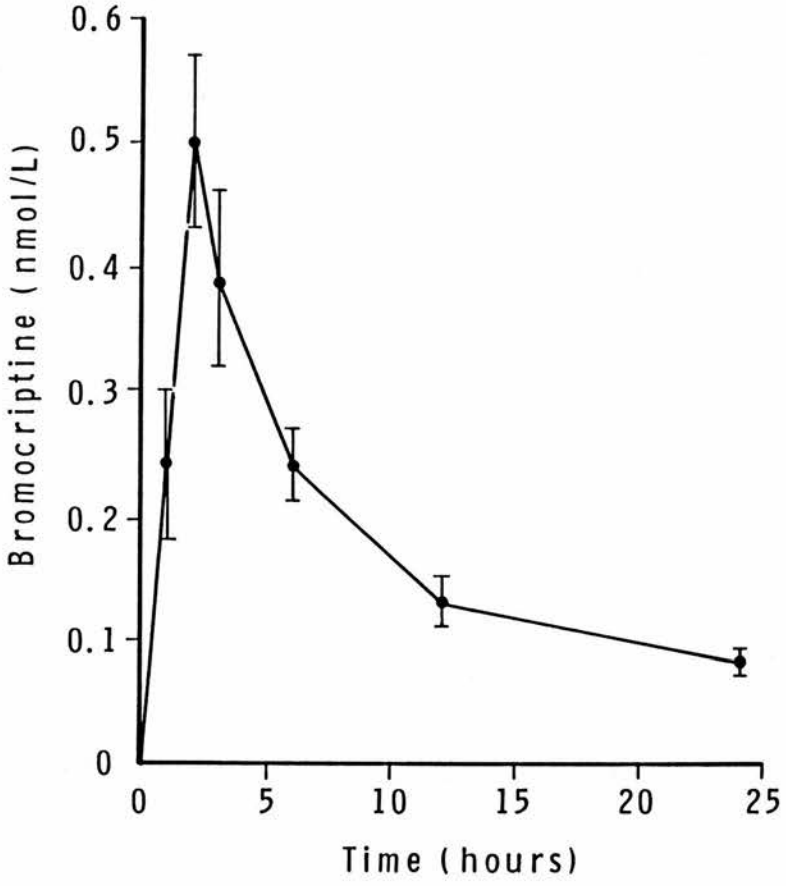
The specificity of the anti-bromocriptine antibody has been extensively investigated by Sandoz Ltd and it is directed against the intact tricyclic peptide moiety of the bromocriptine molecule (Rosenthaler et al., 1983). In a limited number of experiments using the present assay DHE produced a displacement curve identical to bromocriptine itself. Pergolide (Lilly, Surrey), an ergoline of similar structure to the lysergic acid moiety of bromocriptine but without the tricyclic peptide (Figure 3-10) showed no crossreactivity at concentrations up to 10 µmol/L.

7. Measurement of bromocriptine in plasma

Twelve patients with large pituitary tumours were studied and immunocytochemistry of the resected adenomas showed that 8 were prolactin-secreting, 1 was thyrotrophin-secreting, and 3 were non-functioning. An indwelling intravenous cannula was inserted into a forearm vein and, after basal samples were taken at 0900 hours, one 2.5 mg tablet of bromocriptine was administered with a light snack of 2 sandwiches and a glass of milk. Blood was collected for bromocriptine and prolactin estimation at 0, 1, 2, 3, 6, 12 and 24 hours. Samples for bromocriptine estimation were placed into lithium heparin tubes and the plasma separated within 2 hours and stored at $-20^{o}C$ until analysis. This acute bromocriptine suppression test was the prelude to a course of pre-operative bromocriptine therapy in 7 of the 12 patients.

Plasma bromocriptine concentrations in the 12 patients following 2.5 mg orally are shown in Figure 3-15. The mean peak concentration was 0.51 $\pm$ 0.07 (SEM) nmol/L and mean time to peak concentration was 2.3 $\pm$ 0.2 (SEM) hours.

Figure 3-15: Plasma bromocriptine concentrations



Plasma bromocriptine concentrations in 12 patients with large pituitary tumours given 2.5 mg bromocriptine orally.

Values shown are mean ± SEM

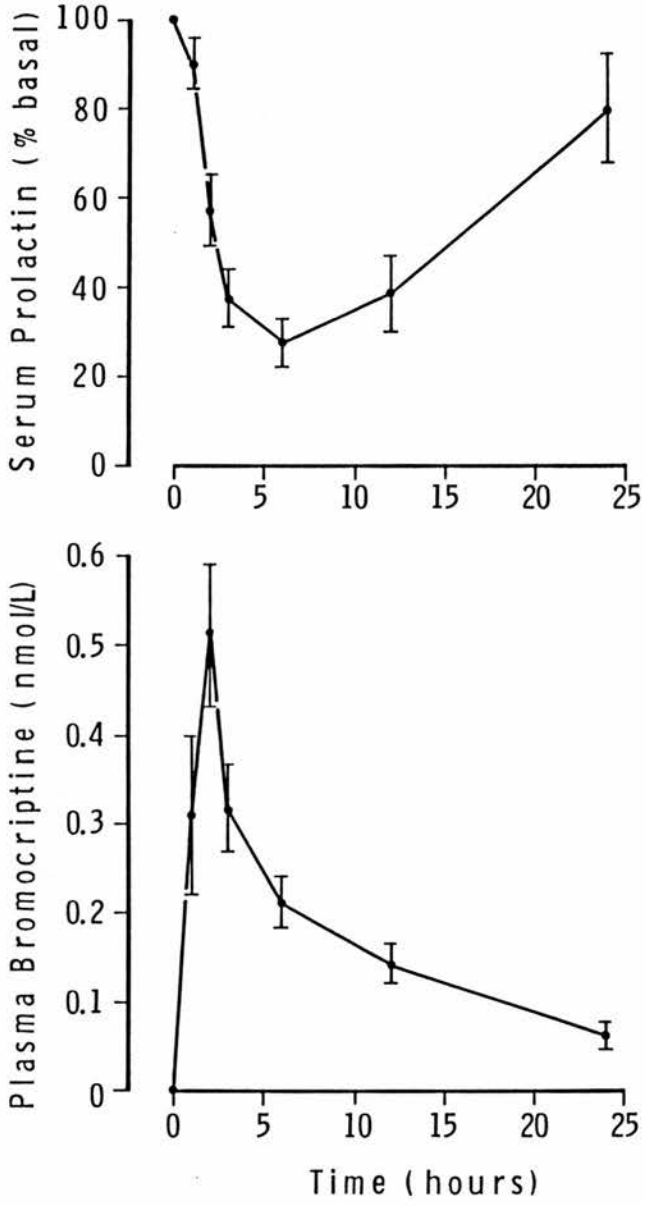
In prolactinoma patients serum prolactin concentrations fell dramatically at, or just after, the time of the bromocriptine peak (Figure 3-16). Lowest prolactin levels were obtained 6 hours after bromocriptine was given. Prolactin levels rose towards pre-treatment values again at 12 to 24 hours, when bromocriptine concentrations fell below 0.15 nmol/L.

8. Discussion

The combination of iodine radiolabel and specific bromocriptine antiserum produced a specific radioimmunoassay for measuring plasma bromocriptine concentrations of greater simplicity, precision and sensitivity than previous radioimmunoassays employing tritium.

Ergot peptide alkaloids are extensively metabolised in the liver with hydroxylation at the 8′-position (Figure 3-10) forming the probable first metabolic step (Rosenthaler et al., 1983). This is followed by further hydroxylation at the 9′-position, eventually resulting in ring opening at C-8′ to produce a glutamic acid derivative of the molecule. These metabolites of bromocriptine do not possess the biological actions of the parent molecule as evidenced by the discrepancy between the duration of action on prolactin secretion (usually complete in 12-14 hours after a small oral dose) and the long second-phase elimination half-life found in radiolabelled drug disposition studies (up to 50 hours following a 1 mg dose, Aellig et al., 1977).

Figure 3-16: Serum prolactin and plasma bromocriptine concentrations following acute dosing



Serum prolactin and plasma bromocriptine concentrations in 8 prolactinoma patients given 2.5 mg bromocriptine orally

Pre-treatment basal prolactin levels varied between 2,240 and 196,000 mU/L.

Values shown are mean $\pm$ SEM.

It is important therefore to use an antiserum which recognizes the intact tripeptide moiety in pharmacokinetic studies of bromocriptine. This will be essential when examining plasma bromocriptine levels following slow-release formulations that have been developed (Landolt et al., 1984; Benker et al., 1986; Grossman et al., 1986). The antiserum used in this assay had been raised with these criteria in mind, the conjugate being produced by linkage through the indole nitrogen at position 1 in the ergoline moiety. The 8′-hydroxyl derivative of bromocriptine showed 0.005% crossreactivity in a tritium based bromocriptine radioimmunoassay using the same antiserum (Rosenthaler et al., 1983). The plasma bromocriptine concentration profiles obtained in this study after 2.5 mg bromocriptine orally are broadly similar to those obtained by Thorner et al (1980) using a comparable protocol and a tritium assay employing a similar tripeptide specific antiserum.

The product of DHE iodination was not identified but the binding results would suggest that the iodine had been incorporated into the ergoline portion of the molecule as predicted theoretically. An apparently identical product was formed from DHE, as judged by thin layer radiochromatography, using Iodo-Gen or N-bromosuccinimide iodination techniques. The stability of ^{125}I-DHE on storage, its high specific activity compared with most tritiated ergopeptines (Schran et al., 1979) and the ease with which it could be made were all attractive features of the new radiolabel.

Two sequential incubations, firstly with antiserum and secondly with radiolabel, increased the sensitivity of this assay three-fold, compared with the simultaneous addition of antiserum and radiolabel. The method could be used to detect as little as 0.05 nmol/L of bromocriptine using 100 µL of plasma; this represents 5 fmol/assay tube. It could be

sensitised further by the use of a 200 µL sample volume, if desired, without increasing non-specific binding excessively. Using a tritiated radiolabel a previous bromocriptine radioimmunoassay required a 500 µL sample volume to achieve a detection limit of 0.23 nmol/L (Rosenthaler et al., 1983).

The assay had additional advantages in terms of precision and convenience. A typical tritium assay used to measure patient samples had between and within assay coefficients of variation of 21 and 13% respectively (Thorner et al., 1980). All variabilities in the present assay were <10%. This was probably due mainly to the robust nature of Sac-Cel second antibody separations compared to more temperamental charcoal methods (Moore, 1985). Undoubtedly gamma counting, rather than beta counting using liquid scintillation, would also have contributed to the improved precision. The method had high throughput and the total turnaround time for a batch containing 50 unknowns was approximately 2.5 hours.

The marked tendency of ergot peptide alkaloids to bind to glass/plastic surfaces and plasma proteins was investigated with particular care. The dilution of bromocriptine standards in plasma to avoid losses was found to be of critical importance. The choice of acid buffer greatly reduced tracer binding to plasma proteins. Antibody binding was thereby enhanced and the inclusion of gelatin to protect the antibody at low pH was unnecessary. The situation was analogous to the use of low pH buffer to prevent the binding of cortisol to transcortin (Rolleri et al., 1976) but in this present assay, buffer and plasma standard curves did not become superimposable. The use of the surfactant Triton X-100 overcame a similar problem in the radioimmunoassay of buprenorphine (Hand et al., 1986); for bromocriptine, however, concentrations of Triton X-100 as low as 0.01% simply inhibited tracer binding to antibody. In view of its

structural similarities to the ergoline moiety of bromocriptine the use of pergolide (10 μmol/L) as a blocking agent was attempted but with no success.

Finally standard curves were made in plasma. Tritium methods, although frequently developed using a normal plasma pool to construct standard curves (Schran et al., 1979), in practice, when used to measure patient samples, have to use pre-treatment plasma from a patient to make a standard curve for the measurement of that patient's samples (Thorner et al., 1980). A major advantage of the new assay was that, provided plasma samples had been stored frozen for less than 2 months, interpatient variation in non-specific binding was so low relative to antibody-bound counts that several patients' samples could be measured against the same standard curve made in normal pooled plasma.

In trials of pituitary tumour shrinkage with bromocriptine it is important to be able to demonstrate that a patient whose tumour has not regressed is actually taking the drug, which in high doses not infrequently produces side-effects. The three non-functioning tumours in this study showed no tumour shrinkage after bromocriptine therapy (20-40 mg daily) despite plasma bromocriptine concentrations of 2 to 5 nmol/L (See Chapter 2.C.3).

CHAPTER 4. LABORATORY METHODS II: TUMOUR CELL PERIFUSION

This chapter describes the validation of a tumour cell perifusion system which was used to characterise hormone secretion by pituitary tumour cells and to investigate the effects of a variety of physiological and pharmacological agents on hormone release.

A. INTRODUCTION

1. Purpose of the perifusion studies

The initial purpose was to examine the secretory behaviour of pituitary tumours specifically pre-treated with bromocriptine before surgery, and to correlate the results with in-vivo bromocriptine responsiveness. It was hoped to study any macroprolactinomas that had proved resistant to bromocriptine in-vivo in terms of lack of tumour shrinkage, failure of prolactin to suppress or both. The aim was to examine the dose responsiveness of such tumours to dopamine and bromocriptine and compare the results with tumours fully responsive to bromocriptine in-vivo and also those not given pre-operative bromocriptine. As will be seen these aims were only partly realised, largely because of the unexpected deleterious effects of bromocriptine on subsequent surgery for macroprolactinomas that have been described in Chapter 2. Furthermore most of the prolactinomas studied were responsive to bromocriptine.

A second aim was to characterise secretory products, if any, from non-functioning tumours, particularly those that had been exposed to pre-operative bromocriptine. In tumours secreting gonadotrophins or their sub-units the effects of dopamine, TRH and LHRH on secretion were to be studied. An important aim was to exclude prolactin secretion by large tumours associated with mild hyperprolactinaemia when the latter was presumed to be on the basis of hypothalamo-pituitary disconnection.

Since prolactinomas seldom respond to TRH in-vivo (Chapter 2.A.3) I wished to examine the responsiveness of prolactinoma cells to TRH in-vitro and to investigate whether dopamine could inhibit such a response. If this were the case it would provide further evidence consistent with the presence of increased hypothalamic dopaminergic tone in patients with hyperprolactinaemia.

A critical factor in the choice of method for the secretion studies was the relatively small amount of human tissue obtained at surgery, particularly as I wished to conserve as much as possible for dopamine receptor measurements on the same tumours.

2. Previous studies

Since the early 1970s there have been numerous reports concerning the regulation of pituitary hormone secretion in animal pituitary tissues, particularly from the rat, and, more recently, in human pituitary tumours. A variety of techniques have been used including short-term incubation of cells (Prysor-Jones et al., 1981), hemipituitaries (Shaar and Clemens et al., 1974) or tissue fragments (Peillon et al., 1983), long-term cell culture (Adams et al., 1979) and perifusion of cells (Lowry et al., 1974) or tissue fragments (Lawton et al., 1981). Each system has advantages and disadvantages as will be discussed.

Dispersed cell preparations require enzymatic treatment which may alter the secretory responsiveness of the cells (MacLeod and Cronin, 1983) and destroys the intercellular junctions between pituitary cells which are probably of importance in the local paracrine control of secretion (Denef, 1986). Cronin and Weiner (1979) showed that pronase treatment reduced the dopaminergic binding of [^{3}H]spiperone to bovine pituitary membranes. Furthermore enzyme dispersal is relatively expensive in terms

of the amount of pituitary tissue required to produce an adequate cell yield.

During long-term cell culture hormone secretion frequently decreases and cellular responsiveness to various stimuli may change with time. Furthermore the duration of exposure to various stimuli is frequently non-physiological or non-pharmacological (Surmont et al., 1983; Adams et al., 1979).

With static incubation of cells, hemipituitaries or fragments secretory products accumulate in the medium and may themselves have feedback effects. Hemipituitaries particularly may suffer from poor diffusion of substances into and out of cells which are not in direct contact with the incubation medium and this may lead to cell necrosis as discussed by Lowry et al., 1974. Continuous perifusion of pituitary cells suspended on an inert matrix was first described by Lowry et al (1974) and obviates a number of these problems. The use of small tissue fragments in a similar system avoids the need for enzyme treatment and does not result in internal cell necrosis (see below). Dieguez et al (1984) described a method for culturing anterior pituitary cells within artificial capillaries in a system which although technically complicated exhibited the advantages of both cell culture and perifusion.

Perifusion techniques have been criticised on a number of grounds. Firstly, several investigators have not used control columns to exclude the possibility of declining secretion rates due to poor cell viability. This is particularly important when examining the inhibitory effects of compounds such dopamine or bromocriptine (Yeo et al., 1979; Delitala et al., 1980). Secondly, tumour tissue obtained at surgery is occasionally contaminated with normal anterior pituitary. None of the reports

describing tumour perifusions mention this as a potential problem (Lawton et al., 1981; Bression et al., 1982; Chihara et al., 1984) or describe any methods to identify normal pituitary on column. These two factors have been assessed critically in the validation of the perifusion system to be described.

Although some researchers have correlated in-vivo dynamic tests with in-vitro secretory responses (Chihara et al., 1984) there have been no reports, to the best of my knowledge, comparing dynamic tests and tumour shrinkage of adenomas specifically pre-treated with dopamine agonists with the in-vitro secretory characteristics of the tumour cells. Furthermore although non-functioning tumours have been studied in cell culture (Mashiter et al., 1981; Surmont et al., 1983) there have been no reports of such tumours studied in a dynamic perifusion system. The following studies also describe some unique experiments in which pituitary tumour and normal anterior pituitary from the same patient have been perifused and stimulated in parallel.

B. PERIFUSION METHOD

1. Tissue preparation

Fragments of pituitary adenoma were collected at the time of transsphenoidal surgery and placed immediately into 10 mL of sterile perifusion medium supplemented with 5 μM dopamine at room temperature. The basic perifusion medium was Earle's balanced salt solution (EBS, Gibco Ltd, Paisley) containing human albumin (0.2%, w/v, salt poor fraction, Blood Products Laboratory, Elstree, Herts), penicillin (30 μg/mL, Glaxo Laboratories Ltd, Greenford) and streptomycin (50 μg/mL, Evans Medical Ltd, Beaconsfield) and gassed with 95% oxygen and 5% carbon dioxide. Solutions of dopamine hydrochloride were protected from oxidation by the inclusion of ascorbic acid (100 mg/L) after Delitala et al (1980).

a. Dispersed cells

Early experiments were performed on enzyme-dispersed tumour cells using the general approach of Yeo et al., (1979). In brief, pituitary adenoma tissue was cut into small fragments and placed in a Teflon dispersal apparatus with 5 mL of EBS containing dopamine (5 μM) and trypsin (2.5 mg/mL, Worthington Bioc. Corp., New Jersey, USA). The medium was gassed and maintained at 37°C. The stirring paddle was driven at 200 rpm and four or five 15-minute harvests were pooled and spun at 800 rpm for 40 minutes. The cells were resuspended in 1.0 mL EBS, filtered through 100 micron gauze and counted using a haemocytometer. Pre-swollen Bio-Gel P2 was then added and the cell columns prepared as described below (1-3 x 10^6 cells per column). The initial perifusate contained lima bean trypsin inhibitor (1.0 mg/mL, Worthington).

Trypsin dispersal was abandoned for several reasons. Firstly,

particularly from bromocriptine-treated macroprolactinomas, there was sometimes less than 100 mg tissue available. This would have been insufficient for enzyme dispersal but was sufficient for four fragment columns. Secondly, tumours did not digest uniformly; some were resistant to digestion, and this would certainly have been a problem with the fibrosed prolactinomas, whereas others digested excessively with loss of tumour tissue. Lastly, although >95% dispersed cells excluded trypan blue in most cell preparations there were some tumours whose cells exhibited blebs and other deformities when examined at the light microscopic level and hormone release progressively declined throughout the perifusion. Other workers have dispersed tumour cells using mechanical means, for example, repeated trituration through a Pasteur pipette (Peillon et al., 1983); in my experience this resulted in 40-50% cells being unable to exclude trypan blue. There were other reasons why tumour fragments were finally chosen and these will be described below.

b. Tumour fragments

Adenoma tissue was distributed to weighed 5 mL sterile plastic tubes each containing 1.0 mL gassed EBS with dopamine (5 μM). The tubes were reweighed and the tumour weights calculated. The tissue for each column was diced into small fragments (1 mm^3) using a sterile scalpel and mixed with 0.5 g pre-swollen Bio-Gel P2. Up to four tumour fragment columns for each tumour were then prepared.

The following figures give an indication of the amount of tissue used per column; untreated prolactinomas (32.9 $\pm$ 3.0 mg, mean $\pm$ SEM, n=16), bromocriptine-treated prolactinomas (35.5 $\pm$ 5.4 mg, n=13), non-functioning tumours (110.3 $\pm$ 5.6 mg, n=18) and periadenomatous pituitary (13.2 $\pm$ 5.9 mg, n=4)

2. Perifusion apparatus

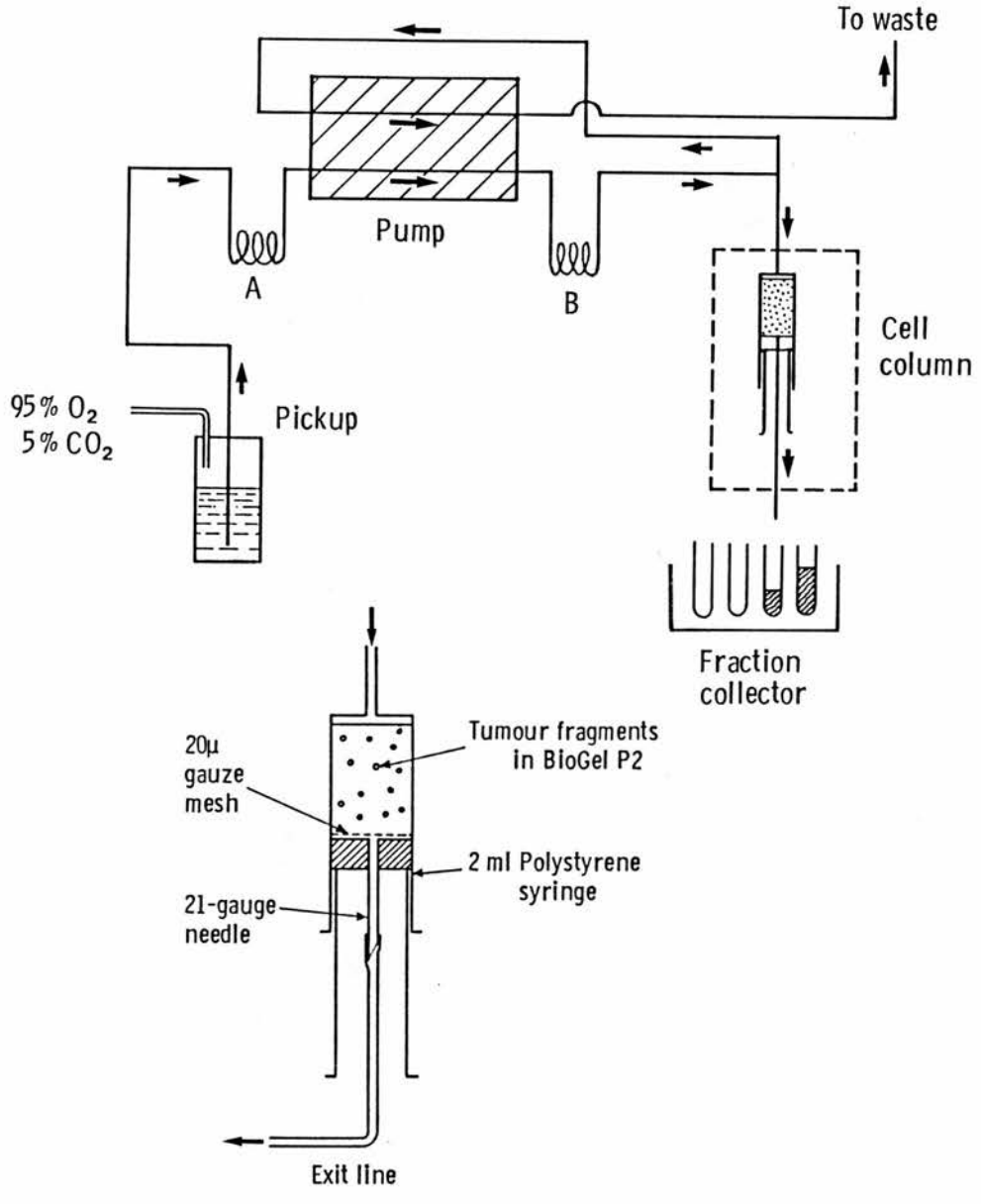
Cell columns were prepared by a modification of the methods of Lowry et al (1974) and Yeo et al (1979). A cell column and the complete perifusion system are shown in Figure 4-1.

The columns were constructed from disposable polystyrene 2 mL syringes (Gillette UK Ltd, Isleworth, Middlesex). A 21 gauge disposable needle was inserted through the septum of the plunger and broken off flush with the surface. The surface of the plunger was covered with 20 micron nylon gauze and the needle connected to a polythene exit line (0.5 mm i.d.).

Cells or tumour fragments were mixed with Bio-Gel P2 (200-400 mesh, Bio-Rad Laboratories, Richmond, CA, USA) which had been swollen overnight in sterile saline and equilibrated with perifusion medium on the day of the experiment; each column contained 0.5 g gel. The tumour-gel mixture was drawn into the perifusion chamber which was connected, nozzle uppermost, to the perifusion apparatus. The cells and Bio-Gel were packed down under the flow of perifusion medium. When this was complete the syringe was disconnected and the plunger raised so that only 0.1 mL of liquid remained between the top of the gel and the syringe nozzle. The syringe was then reconnected and placed in a water bath at 37°C.

Using an eight-channel peristaltic pump up to four columns were perfused with medium which was debubbled just before it reached each column The flow rate through each column was 0.56 mL/min and 5 minute fractions were collected. The deadspace of the system was approximately 2 mL, or just less than one fraction. The cells or fragments were perifused with medium which usually contained dopamine (5 μM) for 2 hours before each experiment was started, to allow the cells to recover from the preparation and to achieve a stable hormone baseline.

Figure 4-1: Perifusion apparatus and cell column



Upper panel shows the perifusion system for one column. The pickup probe could be moved to different test solutions each of which was gassed and pre-warmed to 37°C. A and B denote two heating coils maintained at the same temperature. The peristaltic pump was manufactured by Technicon (Model AA1). Flow rate to the column was 0.56 mL/min and flow rate to waste (debubbler) was 0.18 mL/min. A single fraction collector (Ultrorac II, LKB, Sweden) was used to collect the eluate from all four columns.

Lower panel shows the detail of each cell column (described in the text).

3. Preparation of test solutions

This brief sub-section gives details of the test solutions used in the perifusion studies. All solutions were made up fresh on the day of the experiment. The Earle's balanced salt solution contained the following ionic concentrations; Na^+, 144 mmol/L; K^+, 5.3 mmol/L; Ca^{++}, 1.8 mmol/L; Mg^{++}, 0.81 mmol/L. The salts were supplemented with glucose (5.6 mmol/L) and buffering was achieved using phosphate and bicarbonate systems.

Dopamine A stock solution (2.56 mmol/L) of dopamine hydrochloride (Sigma) was prepared by dissolving 5.0 mg in 10 mL sterile saline (0.9%, w/v, Ivex Pharmaceuticals Ltd, Larne, N.Ireland) which contained ascorbic acid (100 mg/ L) and penicillin/streptomycin. Solutions in the range 1-5000 nmol/L were then prepared by dilution in EBS which contained ascorbic acid (100 mg/L) in addition to the human albumin and antibiotics previously described.

Bromocriptine Bromocriptine methane sulphonate was initially dissolved in ethanol and then diluted in 5 mM tartaric acid to produce a stock solution of 1.0 mmol/L. This was prepared in a silanised glass container to prevent adsorptive losses. Stock bromocriptine was then serially diluted in perifusion medium to produce test solutions covering the range 0.01-10.0 nmol/L.

Thyrotrophin releasing hormone (TRH) TRH (Roche Products Ltd, Welwyn Garden City) was dissolved directly in basic medium to give solutions of 1-100 ng/ml (2.76-276 nmol/L).

Gonadotrophin releasing hormone (LHRH) LHRH (Hoechst UK Ltd, Hounslow, Middlesex) was dissolved directly in basic medium to give solutions of 1-100 ng/mL (0.85-84.6 nmol/L).

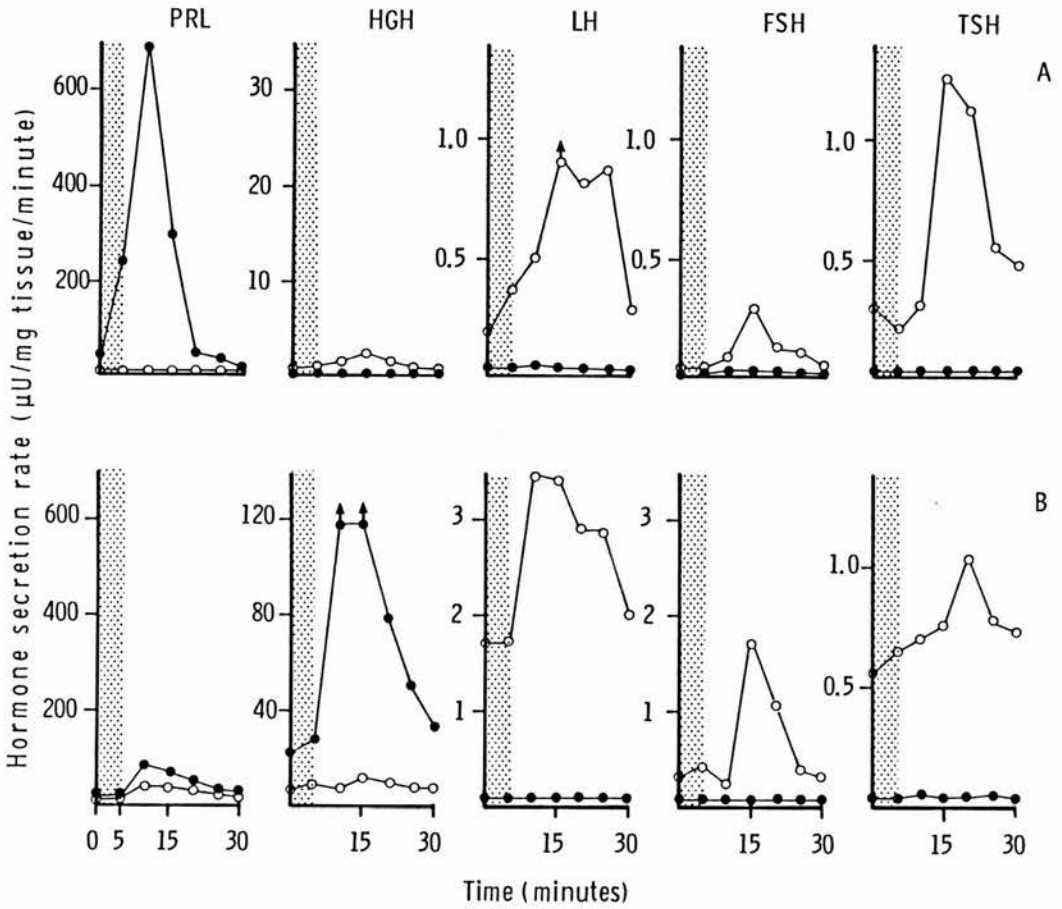
4. Cell viability and identification

After initial equilibration of the tumour fragments the actual perifusion experiment was rarely continued for longer than eight hours during which time careful attention was paid to the oxygenation of the perifusion medium and its maintenance at 37°C. It was important however to make an assessment of cell viability at the start and finish of each study.

One method which was examined to assess both the viability and the identity of the perifused cells was the use of depolarising concentrations of potassium (55 mM) at the end of each experiment. This seems to be a non-specific secretagogue for all anterior pituitary cell types (Eto et al., 1974) and acts by opening voltage-dependent calcium channels leading to calcium influx (Tan et al., 1984). Viable cells with maintained membrane potentials would be expected to show a marked secretory response to depolarising concentrations of potassium in medium containing physiological concentrations of calcium. This stimulus, given for only five minutes, proved to be the most potent secretagogue in all the studies reported in this thesis and indicated satisfactory cell viability at the end of each experiment although, taken alone, this evidence did not prove satisfactory viability of **all** the cells.

High K^+ also proved to be a useful way of detecting anterior pituitary contamination as shown in Figure 4-2. As expected from the hormone contents of normal pituitary (McLean et al., 1981) the measurement of growth hormone in the perifusate after high K^+ was the most sensitive and TSH the least sensitive indicator of anterior pituitary contamination. All tumour perifusions described in this work, apart from the two GH-secreting tumours, had virtually undetectable growth hormone concentrations in the perifusate after cell depolarisation.

Figure 4-2: Potassium depolarisation of normal & tumourous pituitary cells



The release of PRL, HGH, LH, FSH and TSH from two different pituitary adenomas and adjacent normal anterior pituitary tissues following exposure to 55 mM K^+ for 5 minutes (shaded area). Solid circles represent tumour columns and open circles normal anterior pituitary columns. Histology following the perifusions confirmed the purity of tumour and normal anterior pituitary fragments from the different columns.

(A) Prolactin-secreting adenoma (32.6 mg) and adjacent anterior pituitary (30.6 mg). The adenoma immunostained for PRL alone.

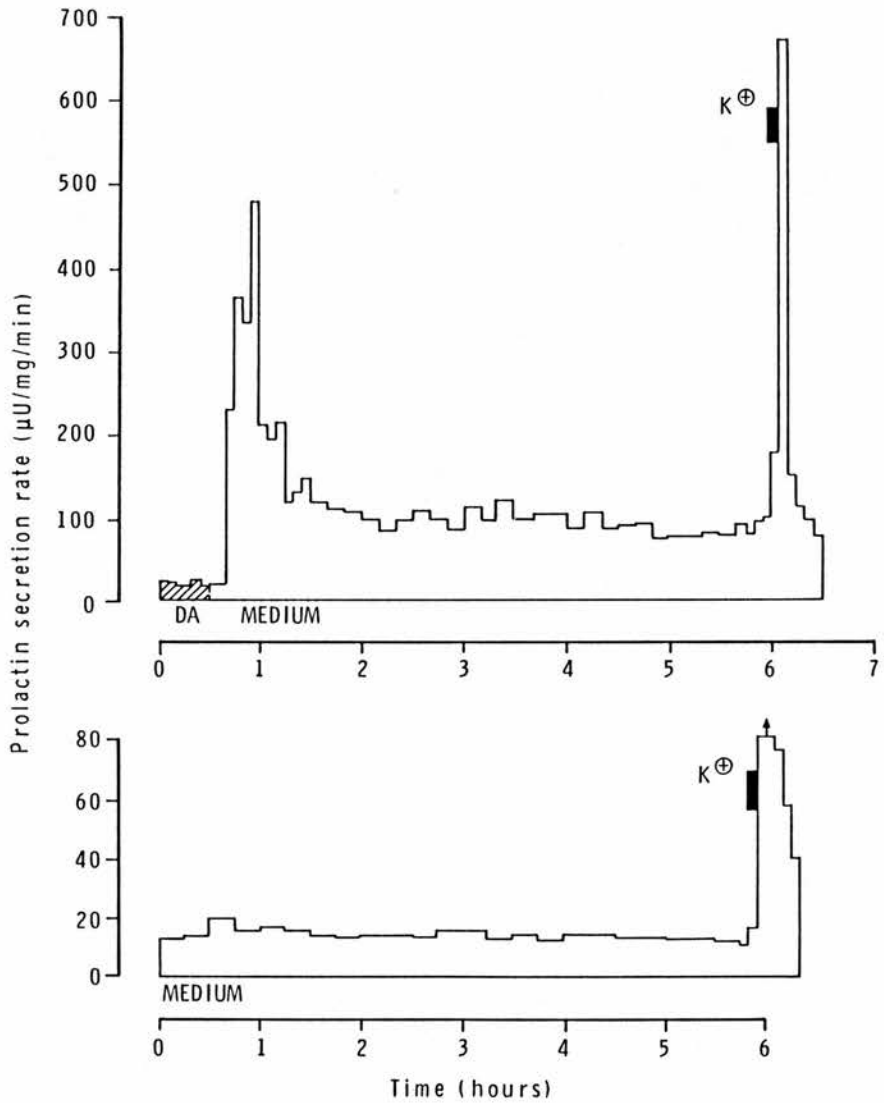
(B) Growth hormone-secreting adenoma (23.7 mg) and adjacent anterior pituitary (5.0 mg). The adenoma immunostained for HGH and, to a lesser extent, PRL.

In both cases secretion of LH, FSH and TSH by adjacent normal anterior pituitary is shown, but secretion of these by the tumour tissue is completely absent.

A further sign of cell viability was the constancy of hormone secretion rates in control columns which were included in most perifusions (Figure 4-3). Preparation stability could also be demonstrated by applying the same physiological stimulus at the start and finish of a perifusion, for example the release of prolactinoma cells from dopamine inhibition, to determine whether the response was similar at both times. The later experiments illustrated in Figures 6-2 and 6-4 illustrate that tumour fragments remained viable after at least 6 hours by this criterion.

A disadvantage of the dispersed cell column was that cells could not be recovered at the end of an experiment for microscopic analysis. At the end of each tumour fragment perifusion tissue from individual columns was recovered into 10% neutral formalin for light microscopy if required. On three occasions tissue was fixed in 2.5% glutaraldehyde and examined ultrastructurally. No tumour showed evidence of cell disruption or gross vacuolation following a six hour perifusion. Furthermore there was no evidence of cell necrosis in the centre of the tumour fragments. Several untreated prolactinomas were immunostained for prolactin following a perifusion and all contained abundant immunoreactive prolactin. Light microscopy was not performed in all cases but was extremely useful in confirming the presence of suspected anterior pituitary on a tumour column or determining the purity of an anterior pituitary column.

Figure 4-3: Control perifusion columns



Control columns for two different prolactin-secreting adenomas. DA = 5µM dopamine. The solid vertical bar indicates the depolarising pulse of 55 mM K^+.

Two different experimental protocols are shown:

In the upper panel tumour tissue (35.5 mg) was collected and equilibrated in 5 µM dopamine. Removal of dopamine from the medium resulted in a rebound increase in the prolactin secretion rate which stabilised after 1 hour and remained constant for the rest of the experiment.

In the lower panel tumour tissue (19.6 mg) was collected into medium containing 5 µM dopamine but the latter was removed for the 2 hour equilibration period (preceding time 0) and the prolactin secretion rate remained constant throughout the perifusion.

C. ADVANTAGES OF TUMOUR FRAGMENT PERIFUSION

It has been shown that the use of tumour fragments in a perifusion system had a number of advantages which will now be summarised in conclusion to this section on perifusion methods.

Conservation of scarce human tissue.

The production of four perifusion columns from 100 mg prolactinoma tissue permitted unused tissue to be stored for receptor studies. The bromocriptine-treated macroprolactinomas could probably have not been studied by cell dispersal.

Comparison of secretion rates between columns

The accurate weighing of fragments permitted the calculation of hormone secretion rates which allowed comparisons between columns and between experiments. There was generally excellent agreement between the secretion rates for different fragment columns from the same tumour. The hormone secretion rate for each fraction was calculated using the following simple formula, which shows prolactin as an example:

$$\text{PROLACTIN SECRETION RATE } (\mu U/\text{mg tissue/minute}) = \frac{\text{PROLACTIN CONCENTRATION } (\mu U/\text{mL eluate}) \times 0.56}{\text{WEIGHT OF TISSUE ON COLUMN (mg)}}$$

(0.56 = column flow rate, mL/min)

Convenient, avoiding exposure to enzymes

Apart from the possible deleterious effects of enzyme exposure that have been discussed, the avoidance of enzyme dispersal ensured a more convenient and rapid method for establishing the cell columns. The cells seemed to remain viable throughout the perifusion experiment. Some authors using animal pituitary tissue have found responses of fragments to secretory stimuli to be more sluggish than those of dispersed cells,

presumably due to diffusion delays (MacLeod et al., 1983). This was not a particular problem with these experiments on human tumours perhaps because many were more friable than normal pituitary.

Recovery of fragments for microscopy

This, together with potassium depolarisation, was a major advantage for the assessment of cell viability and identity following a perifusion experiment.

CHAPTER 5. LABORATORY METHODS III: DOPAMINE RECEPTOR ASSAY

A. INTRODUCTION

1. Purpose of the receptor studies

The role of dopamine as the main physiological inhibitor of prolactin secretion by the anterior pituitary gland has been reviewed in Chapter 1. Many studies have shown that this inhibition is the result of a direct effect of dopamine on anterior pituitary tissue (Shaar and Clemens, 1974; Weiner and Ganong, 1978; Foord et al., 1983; Bethea, 1985). The first step in this process is thought to be the interaction of dopamine with specific high affinity receptors in the plasma membrane of the lactotroph. Using radioligand-binding techniques, dopamine receptors have been measured directly in anterior pituitary membrane preparations from several species, including man (see below). These receptors fulfil the accepted criteria for receptor binding of saturability, reversibility and specificity. Dopaminergic agonists, in competing for dopamine receptors in particulate preparations of bovine anterior pituitary glands, have a relative potency similar to that shown by the same agents in inhibiting the secretion of prolactin by rat anterior pituitary cells in monolayer culture (Caron et al., 1978). The significance of dopamine receptors in the normal and abnormal pituitary gland has been reviewed by Cronin and Evans (1983).

Using similar radioligand-binding techniques dopamine receptors have been described in human pituitary adenomas, particularly prolactinomas (Cronin et al., 1980a; Bression et al., 1980, 1982), although no correlation has been made with in-vivo dopaminergic responsiveness. Using intact prolactinoma cells in culture Bethea et al (1982) demonstrated a strong correlation between dopaminergic receptor binding and inhibition of prolactin secretion. At the start of my studies these

were the only substantive publications concerning dopamine receptors and human pituitary adenomas.

The ergot derivatives that had been shown to shrink pituitary adenomas were all dopamine agonists and included bromocriptine (MacGregor et al., 1979b; Prescott et al., 1982; Wass et al., 1982), lisuride (Chiodini et al., 1981) and pergolide (Kendall-Taylor et al., 1982). It seemed likely that the presence of dopamine receptors in the tumour membranes was necessary for the tumour-shrinking action of these drugs. Conversely, I hypothesised that the failure of some tumours to regress during dopamine agonist therapy might be due to absent or malfunctioning membrane dopamine receptors. Furthermore I wished to investigate whether there was evidence of tumour dopamine receptor down-regulation during chronic bromocriptine therapy.

The main aim of this work was to correlate dopamine receptor measurements with the tumour shrinkage achieved during dopamine agonist therapy in patients with pituitary macroadenomas. This work has been recently published (Bevan and Burke, 1986) and is the only correlative study of its type in the literature.

2. Previous studies of pituitary dopamine receptors

The first reports of dopamine receptors in membranes derived from rat, sheep and cow anterior pituitaries appeared in the late 1970s (Brown et al., 1976; Caron et al., 1978; Calabro and Macleod, 1978; Cronin et al., 1978; Cronin and Weiner, 1979; Stefanini et al., 1980). Enzyme marker studies suggested that the majority of the binding sites were associated with the plasma membrane fractions (Caron et al., 1978) and subsequent studies demonstrated their presence on intact cells (Bethea et al., 1982; Foord et al., 1983). A variety of tritiated dopamine agonists and

antagonists were used and the relative merits of each will be discussed later. These early studies showed that individual dopamine agonists and antagonists had equivalent potency in the displacement of [^{3}H]ligand binding to anterior pituitary dopamine receptors and the modulation of prolactin secretion in-vitro, suggesting a close relationship between the two events (reviewed by Cronin and Evans, 1983).

The receptor methodology developed using normal animal pituitary was applied to the study of human tumours by Cronin et al (1980a) and Bression et al (1980). Cronin et al (1980a), using the dopamine antagonist [^{3}H]spiperone, demonstrated dopaminergic binding sites in five samples of pooled prolactinoma cell membranes. These receptors were of similar affinity to those present in normal pituitaries removed from patients with metastatic cancer but the number of binding sites was greater in the tumour membranes. Bression et al (1980) used [^{3}H]domperidone to demonstrate dopamine receptors in membranes from 26 human prolactinomas. In competition experiments these were shown to have the pharmacological characteristics of a dopaminergic binding site. In contrast to the findings of Cronin et al (1980a) the number of binding sites in prolactinoma membranes appeared to be similar to those in membranes from normal anterior pituitaries obtained at necropsy. Furthermore, [^{3}H]domperidone defined two dopaminergic binding sites of high and low affinity. Two non-functioning adenomas described in the same paper showed no saturable binding at concentrations of up to 40 nmol/L [^{3}H]domperidone.

A variety of rat lactotroph cell clones have been used as models of the tumourous human lactotroph and have been investigated in an attempt to define possible receptor or post-receptor defects. Early studies emphasised the importance of membrane-bound dopamine receptors in the mediation of the action of bromocriptine. For example, bromocriptine had

no effect on the growth of the prolactin-secreting 235-1 clone in which radioligand binding studies had revealed no dopaminergic binding sites (Cronin et al., 1982). Similarly, dopamine did not reduce the secretion from GH_3 cells, another prolactin-secreting clone without dopamine receptors (Cronin et al., 1980b). In contrast, the clone 7315a was refractory to dopamine despite the presence of apparently normal dopamine receptors (Cronin et al., 1981). In a series of elegant experiments Judd et al (1985) have recently deduced that the post-dopamine receptor defect in this clone is related to the intracellular mobilisation of calcium and the inhibitory action of dopamine was restored in the presence of calcium ionophores.

3. Choice of radioligand

Firstly I shall consider the ideal properties of a radioligand for the identification of dopamine receptors in pituitary tissues. This will then be followed by a discussion of the properties of the various radioligands that have been used in previous pituitary studies. The reasons for my selection of [^{3}H]spiperone for the tumour studies will then be enumerated.

The ideal radioligand would have the following properties:

1. Ligand binding to the pituitary dopamine receptor would be of high affinity.
2. The ligand would bind to dopaminergic receptors alone and not, for example, to adrenergic receptors.
3. The ligand would exhibit a close correlation between dopamine receptor binding and modulation of secretion or a linked intracellular process such as adenylate cyclase activity.
4. Non-specific binding of the ligand would be low. (1) Lipid

solubility would be low to prevent non-specific binding to membrane components and the subsequent evaluation of specific binding over a large "blank" value. (2) It would be advantageous if the ligand did not bind non-specifically to inert materials such as glass or plastic since this might result in losses of non-radioactive ligand during the preparation of solutions which could not be corrected for.

5. The specific activity of the radioligand would be as high as possible to maximise the sensitivity of the assay.
6. The ligand would be chemically stable under incubation conditions and not susceptible to oxidation or to metabolism by the biological material being investigated.
7. The kinetics of ligand dissociation from the dopamine receptor would be slow enough to permit the separation of bound and free radioactivity.

No dopaminergic radioligand meets this specification and each of those used in previous pituitary studies has some disadvantages.

Dopamine itself was used in some early studies (Calabro and Macleod, 1978; Cronin et al., 1978) but [^{3}H]dopamine was unsatisfactory for several reasons. It was an unstable compound and required the presence of an anti-oxidant, usually ascorbate. Furthermore dopamine may be metabolised by pituitary monoamine oxidase and an inhibitor of this enzyme was routinely included in the assay buffer (Calabro and Macleod, 1978). The dissociation constant for dopamine (65 nM, rat and sheep anterior pituitary) was an order of magnitude higher than that obtained for [^{3}H]dihydroergocriptine in the same tissues and specific binding was only 30-35% (Cronin et al., 1978). Lastly, [^{3}H]dopamine rapidly dissociated during membrane washing making separation of bound and free radioactivity difficult.

The ergopeptine [^{3}H]dihydroergocriptine, which is structurally very similar to bromocriptine (see Figure 3-10), has been used by several investigators to examine dopaminergic binding in normal anterior pituitary (Caron et al., 1978; Cronin et al, 1978; Sibley and Creese, 1983; Foord et al., 1983). There is an excellent correlation between receptor binding and biological action (Caron et al., 1978). However the specific binding of 50-60% at K_d concentrations in bovine anterior pituitary (Sibley and Creese, 1983) compared unfavourably to the values of 75-90% obtained in bovine tissue by Cronin and Weiner (1979) using [^{3}H]spiperone. In an early paper Creese et al (1977) argued that only half of the [^{3}H]dihydroergocriptine binding to anterior pituitary homogenates was to dopamine receptors, with the remaining binding accounted for by α-adrenergic interactions. This was not the experience of Caron et al (1978) or Cronin et al (1978) who found little radioligand displacement by noradrenaline or phentolamine. However a recent report by Ramsdell et al (1985) has suggested that [^{3}H]dihydroergocriptine binds with equal potency to both dopaminergic and α-adrenergic receptors in human prolactinoma membranes.

[^{3}H]spiperone is a dopamine antagonist which binds with high affinity to the pituitary dopamine receptor and K_d values of between 0.2 and 0.85 have been obtained in normal animal anterior pituitaries (Creese et al., 1977; Cronin and Weiner, 1979; Sibley et al., 1982). Spiperone is the most potent drug known to antagonise the inhibition of prolactin release by dopamine or dopaminergic agonists (Denef and Follebouckt, 1978). The high specific binding of this ligand has been mentioned above and there is plentiful evidence supporting a good correlation between receptor binding and reversal of dopaminergic inhibition of prolactin secretion (Denef and Follebouckt, 1978; Cronin and Weiner, 1979; Bethea et al.,

1982). Sibley et al (1982) showed that bromocriptine caused 50% displacement of [^{3}H]spiperone binding to bovine anterior pituitary membranes at a concentration of around 6 nmol/L, a value similar to the IC_{50} values for bromocriptine inhibition of prolactin release from normal lactotrophs (Caron et al., 1978; Delitala et al., 1980). There has been some concern that spiperone also binds to serotonin receptors (Baudry et al., 1979). However these receptors are not present in the normal pituitary and high concentrations of serotonin failed to cause significant displacement of [^{3}H]spiperone from anterior pituitary membranes (Cronin et al., 1979, Stefanini et al., 1980). Similar results are described in Chapter 8 for [^{3}H]spiperone binding to human pituitary tumour membranes.

Many of the remarks about spiperone could also be made about the dopamine antagonist domperidone which has been used to investigate dopaminergic binding in the normal and abnormal pituitary (Bression et al., 1980; Sibley and Creese, 1982; Foord et al., 1983). These studies indicate satisfactory performance as a specific dopaminergic radioligand though the compound is marginally less potent than spiperone and shows slightly lower specific binding values (Sibley and Creese, 1982).

A general comment which applies to all of these tritiated radioligands is that the specific activities are low and usually in the range 10-30 Ci/mmol. This is a particularly important factor in studies of human tumours since the amount of available tissue is small and maximal assay sensitivity is desired. The possibility of using ^{125}I-radiolabelled dopaminergic ligands of much higher specific activity is discussed below.

Secondly, many of the compounds exhibit non-specific adsorption to glass and/or plastic; spiperone and dihydroergocriptine are two good examples.

The special precautions required will be mentioned in section B.2.b.

[^{3}H]spiperone was selected as the most suitable radioligand for the tumour studies primarily because of its high affinity for the pituitary dopamine receptor and its low non-specific binding to pituitary membranes. There was sound evidence that the receptor defined by this radioligand was relevant to the inhibition of prolactin secretion and to the action of bromocriptine.

B. DOPAMINE RECEPTOR METHOD

Since an individual pituitary adenoma usually yielded less than one gram of tissue the dopamine receptor assay was established and validated using membranes prepared from bovine anterior pituitary glands. The final method derived from the techniques described by Cronin et al (1979, 1980a) and Bression et al (1980).

1. Materials

a. Pituitary tissues

(i) Bovine anterior pituitary glands

Bovine pituitary glands were obtained from Friesian steers within 15 minutes of death at a local slaughterhouse (FMC, Thame, Oxon). The skull was split and the complete pituitary was removed from above and placed immediately into ice-cold membrane buffer (without ascorbate). The tissue was transported to the laboratory within 30 minutes where each anterior pituitary was bisected and dissected free from connective tissue and neurointermediate lobe. The various pituitary components were verified histologically. Each animal, on average, yielded 1.75 g of anterior pituitary and batches of six pituitaries were routinely collected. The tissue was processed to the end of the low-speed centrifugation step described below and supernatant stored at -70°C until further use.

(ii) Human anterior pituitary glands

Normal human pituitary glands were obtained at necropsies performed within 48 hours of death on previously healthy males (aged 20-50 years) who had died in road traffic accidents. Whole glands were stored at -70°C until membrane preparation at which time posterior pituitary tissue was separated and discarded.

(iii) Pituitary adenomas

Pituitary adenoma tissue was obtained at the time of transsphenoidal adenomectomy. Small tissue fragments were immediately fixed for light and electron microscopy and the remainder was gently washed in Earle's balanced salt solution at room temperature. Tissue for receptor studies was weighed and stored at -70°C until membrane preparation.

b. Chemicals

[^{3}H]spiperone (benzene ring ^{3}H, SA 24.5 Ci/mmol) and [^{3}H]dihydro-ergocriptine (9,10-^{3}H, SA 26.7 Ci/mmol) were purchased from New England Nuclear (Boston, USA). (+)Butaclamol was obtained from Research Biochemicals (Wayland, USA), domperidone from Janssen (Wantage, Oxon) and dopamine, pargyline, serotonin and adrenaline from Sigma. The following compounds were gifts: non-radiolabelled spiperone (Janssen, Wantage, Oxon), (-)butaclamol (Ayerst Laboratories, Rouses Point, NY) and bromocriptine (Sandoz, Basle, Switzerland).

2. Assay development

a. Membrane preparation

(i) Homogenisation

Adenoma or normal tissue was diced using a scalpel to produce approximately 3 mm cubes and then homogenised (10%, w/v) in ice-cold 0.33M sucrose buffer using a Teflon glass homogeniser. The buffer used in these studies comprised 15 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.1% (w/v) ascorbic acid (pH 7.4 at 20°C). The homogeniser was motor driven at 400 rpm and the initial step was 10 strokes with a loose fitting pestle (0.24 mm clearance on the diameter). This was particularly necessary for the initial processing of normal anterior pituitary tissues since these were generally of firmer

consistency than the adenomas. Homogenisation was completed with 20 strokes of a more tightly fitting pestle (0.1 mm clearance). An ice-jacket was used throughout the homogenisation procedure. Dopaminergic binding to bovine anterior pituitary membranes was similar whether 10 or 20 strokes were made with the tighter pestle. Homogenates of greater than 10% (w/v) did not homogenise satisfactorily using the motor driven apparatus. The use of sonication (Bression et al., 1980) for initial tissue disruption was not investigated.

(ii) Centrifugation

The homogenate was centrifuged twice at 850g for 3 minutes at 4°C to remove cell nuclei and debris (Beckman TJ6 refrigerated centrifuge). The supernatant following the second spin was separated (S1) and centrifuged at 100,000g for 60 minutes at 4°C (Beckman L8-70M Ultracentrifuge) to produce the final membrane pellet (P2). The high-speed supernatant (S2) was stored in aliquots at -20°C for subsequent hormone and, where appropriate, bromocriptine analyses. In early experiments using bovine anterior pituitary, high-speed centrifugation at 26,000g for one hour produced a membrane pellet containing marginally less dopaminergic binding than that obtained following centrifugation at 100,000g (data not shown). The latter speed was therefore chosen for routine use. Other investigators have used a wide range of centrifugation forces ranging between 11,000 (Bression et al., 1980) and 145,000g (Cronin et al., 1980a), although most have used >30,000g.

(iii) Membrane pellet

The membrane pellet P2 from bovine anterior pituitary was bilayered with a brown upper layer and grey-white lower layer. This was similar to the findings of Caron et al (1978) who found that the brown layer contained the plasma membranes and dopaminergic binding sites and that the white

layer consisted of secretory granules. A similar pellet was obtained from prolactin-secreting adenomas but non-functioning tumours produced a more uniform pellet. Following high-speed centrifugation of several of the non-functioning tumour homogenates a fat layer was observed floating on the top of the supernatant. This was separated and discarded and it was noted that these tumours usually showed large lipid inclusion bodies on ultrastructural examination. Caron et al (1978) mechanically separated the membrane and secretory granule layers in the high-speed pellet from bovine anterior pituitaries. This would not have been practical for the small pellets obtained from 300 mg or less of pituitary adenoma so in all experiments the complete pellet was resuspended in sucrose-free buffer. This was performed using a 1 mL glass-glass minihomogeniser (10 strokes by hand) and the resulting membrane suspension was that used in the binding assay.

(iv) Tissue storage

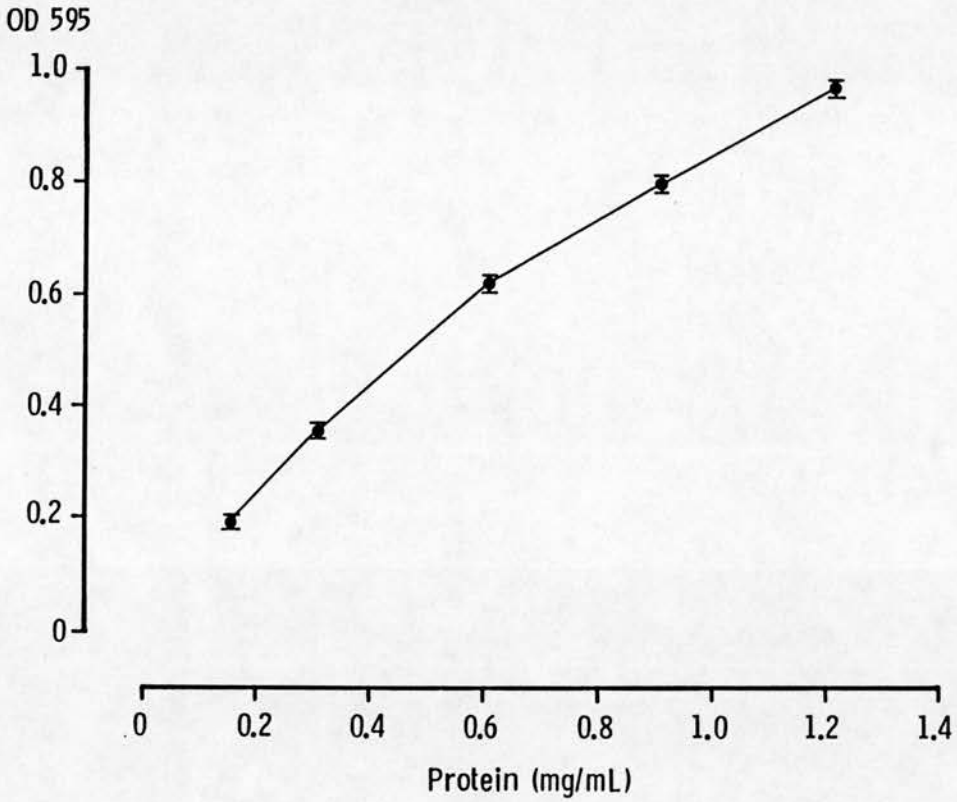
All tissues were stored at -70°C until final processing and radioligand binding. Unprocessed bovine anterior pituitary showed no deterioration in membrane specific binding after such storage for at least 4 months. Similarly bovine S1 and final membrane suspension were stable for at least 2 months. This was in agreement with other workers (Cronin and Weiner, 1979) and validated the use of frozen material for receptor assay. However there was insufficient adenoma tissue to ascertain whether the same receptor stabilities applied to the human tumours during storage.

(v) Protein determination

Membrane protein was determined by the Coomassie Brilliant Blue dye-binding assay (Bradford, 1976) using dye reagant obtained from Bio-Rad Laboratories. The protein standard was bovine serum albumin (Bio-Rad) and covered the range 0.15-1.21 mg/mL. The assay method will not be

described in detail but the standard curve is shown in Figure 5-1. The between assay coefficient of variation was 4.6% at 0.9 mg/mL (n=16). The protein-dye complexes were stable for at least 60 minutes and optical density measurements were routinely made after 20 minutes. In early experiments membrane samples were incubated with 0.1% Triton X-100 for 15 minutes prior to protein determination to solubilise membrane protein. However it was found that serial dilution of pituitary membranes in 0.1% Triton X-100 produced a non-linear effect; the greater the sample dilution the higher the apparent protein concentration. In contrast, dilution of samples in membrane buffer alone was completely linear. Since tumour membrane suspensions required variable dilution for protein estimation the Triton X-100 solubilisation step was omitted.

Figure 5-1: Standard curve for the protein assay



Standard curves from 16 consecutive protein assays. Values are mean ± SEM.
100 µL of samples and standards were mixed with 5 mL diluted Coomassie Blue dye reagant. After 20 minutes the optical densities were measured using a Pye-Unicam SP8-150 spectrophotometer and polystyrene cuvettes.

b. Radioligand binding

(i) Radioligand and early assay problems

The radioligand binding assay proved difficult to establish and a number of pitfalls were encountered. The earliest experiments were performed using [^{3}H]dihydroergocriptine as radioligand and specific binding to bovine anterior pituitary membranes was low compared with previous literature values (approximately 10% of the binding reported by Caron et al., 1978). In retrospect, this was due to a combination of factors. Ascorbic acid was routinely included in the assay buffer as an anti-oxidant and without it specific binding of [^{3}H]dihydroergocriptine was reduced to virtually zero, which was in accord with the observations of Leff et al (1981). However in early experiments the addition of ascorbic acid to the 15 mM Tris buffer reduced its pH from 7.4 to 3.9 and several assays were inadvertently carried out at acid pH. Cronin and Weiner (1979) had demonstrated that specific binding of [^{3}H]spiperone was abolished at pH 2.3. When [^{3}H]dihydroergocriptine incubations were performed at pH 7.4, and not pH 3.9, the specific binding increased but still appeared low compared with literature values. This was found to be largely due to the over-estimation of membrane protein concentrations caused by Triton X-100 solubilisation as discussed above.

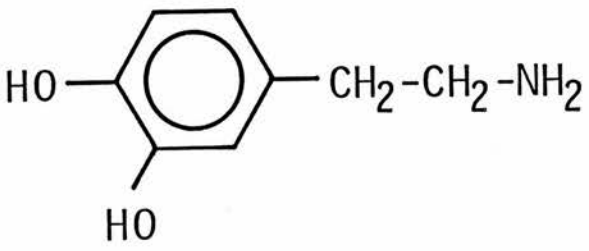
When these two factors were corrected, binding was similar to that described by Caron et al (1978), but specific binding of [^{3}H]dihydroergocriptine to bovine membranes was only 30-65% of total bound counts. [^{3}H]spiperone was therefore finally chosen and specific binding with this radioligand increased to 65-90% of membrane-bound counts.

The structure of spiperone is shown in Figure 5-2, together with dopamine for comparison. Specific dopaminergic binding of [^{3}H]spiperone was determined by displacement of bound radioligand using the potent dopamine antagonist (+)butaclamol. The principle of the dopamine receptor assay is illustrated by the experiment shown in Figure 5-3. Other workers have used a 1000x excess of non-radioactive ligand to define specific binding (Bression et al., 1980) but this may define binding to sites other than dopamine receptors. (+)Butaclamol was dissolved in ethanol acidified with glacial acetic acid (2%, v/v) and then diluted in membrane buffer. The acetic acid was necessary for the complete dissolution of the (+)butaclamol but did not lower the pH of the final incubate.

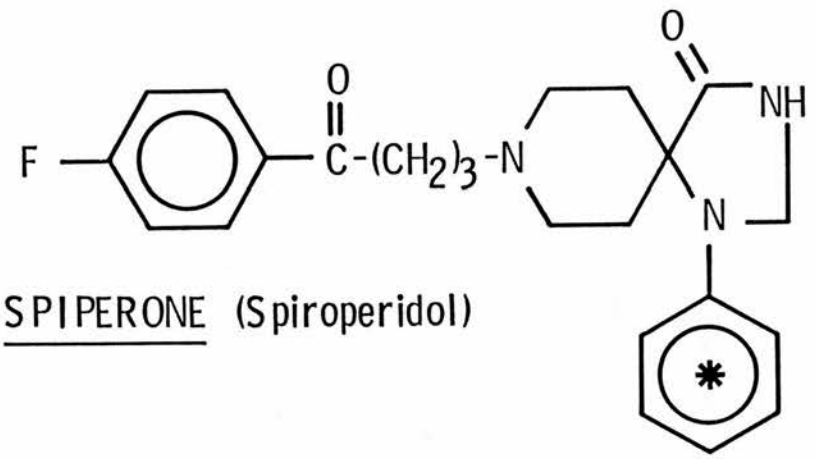
The specific binding of [^{3}H]spiperone was directly proportional to the amount of anterior pituitary membrane protein in the range 0.05-1.22 mg protein per assay tube (Figure 5-4).

[^{3}H]spiperone (and [^{3}H]dihydroergocriptine) showed non-specific adsorption to glass and plastic. This was demonstrated by serial dilution of radioligand using various combinations of glass and plastic pipettes and containers. [^{3}H]spiperone could be diluted in buffer without losses if silanised glass containers and polythene pipette tips were used (data not shown). Similar precautions were taken during the preparation of solutions of non-radioactive spiperone and (+)butaclamol. The radioligand binding assays were performed in silanised glass test-tubes (75 x 12 mm) since the membrane-bound counts were significantly lower when plastic tubes were used, presumably due to non-specific adsorption. Glassware was silanised in the laboratory using dimethyldichlorosilane solution (BDH Chemicals Ltd, Poole, Dorset)

Figure 5-2: Structural formulae of dopamine and spiperone



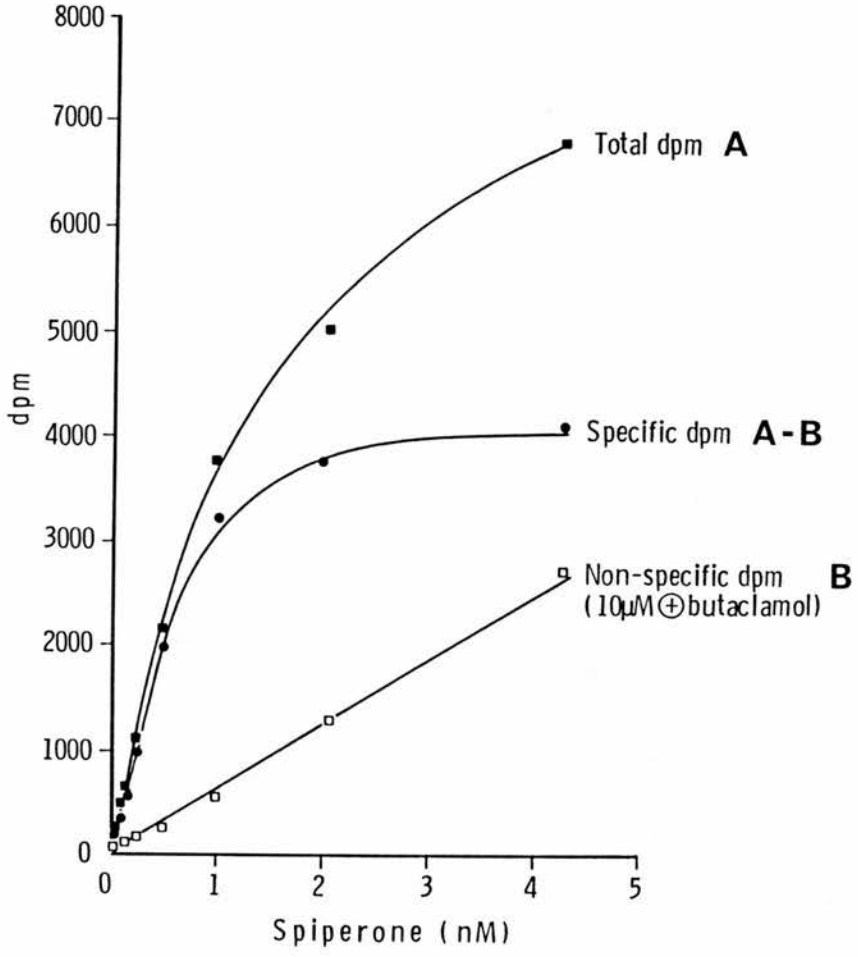
DOPAMINE (3,4 dihydroxyphenylethylamine)



SPIPERONE (Spiroperidol)

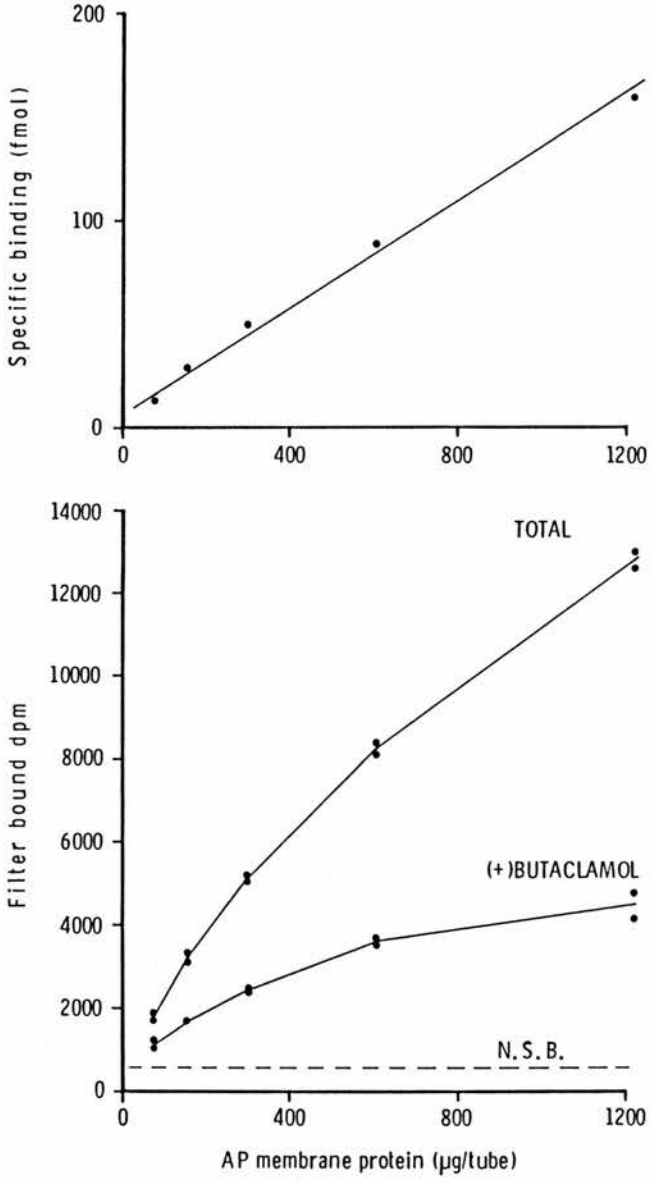
* denotes the site of tritium incorporation in [^{3}H]spiperone

Figure 5-3: Saturation of specific [^{3}H]spiperone binding to bovine anterior pituitary membranes



Bovine anterior pituitary membranes (0.61 mg protein per tube) were incubated for 60 minutes at 30°C with [^{3}H]spiperone in increasing concentrations either alone (total dpm) or in the presence of 10 μM (+)butaclamol (non-specific dpm). Specific dpm (or saturable binding) refers to the difference between the two values. Abscissa: total [^{3}H]spiperone concentration in the incubation (free + total bound). Each point was determined in triplicate.

Figure 5-4: [^{3}H]spiperone binding and membrane protein concentration



Effect of the amount of membrane protein on [^{3}H]spiperone binding to bovine anterior pituitary membranes.

Membranes were equilibrated with 5.1 nM [^{3}H]spiperone for 60 minutes at 30°C. Each point was determined in duplicate and the individual results are shown in the lower panel. Specific binding was defined using 10 µM (+)butaclamol. NSB indicates the non-specific filter binding of 5.1 nM [^{3}H]spiperone.

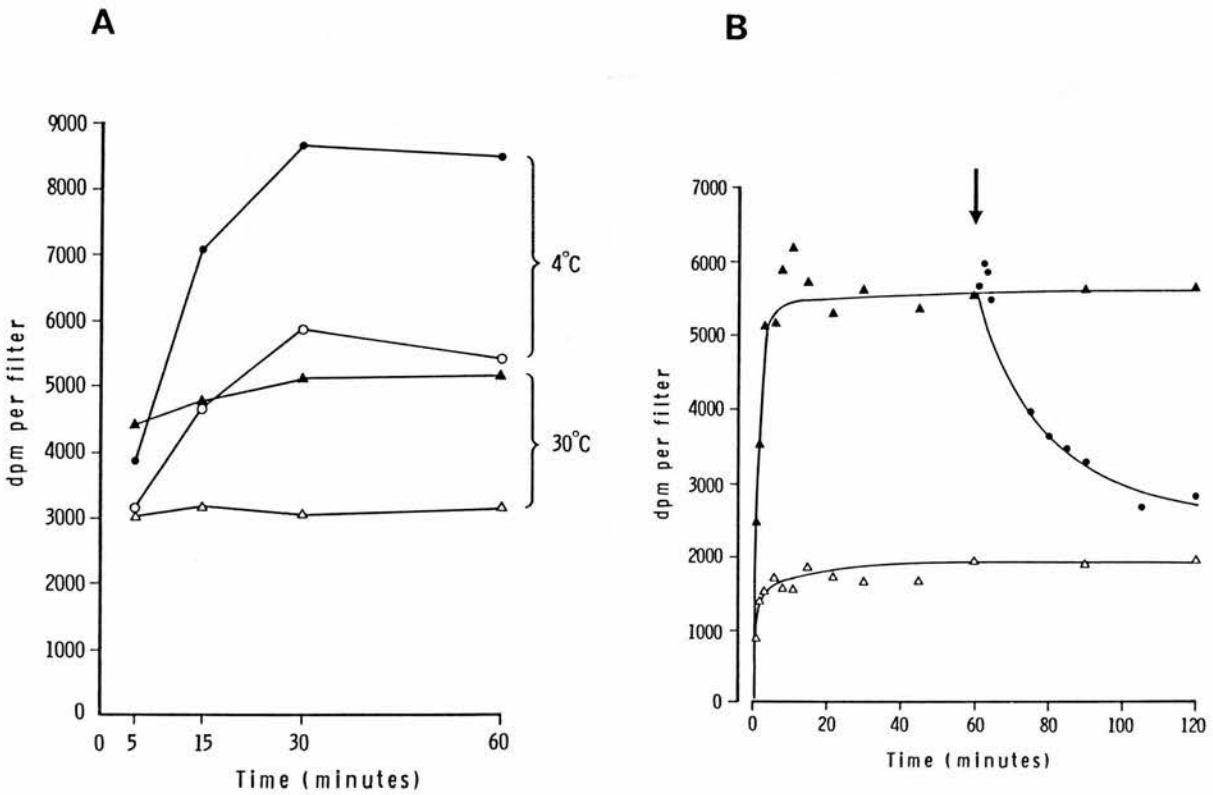
The top panel shows the linear relationship between specific binding (fmol spiperone) and membrane protein (µg/tube)

Dihydroergocriptine was radio-iodinated in an attempt to produce a radioligand of higher specific activity which might improve the sensitivity of the tumour dopamine receptor assay. The iodination technique has been described in Chapter 3. Although ^{125}I-dihydroergocriptine bound to bovine membranes none of the radioactivity was displaced by (+)butaclamol indicating that the binding was not to dopamine receptors. In retrospect this was perhaps not surprising since the subsequent immunoassay data suggested that the ^{125}I had been incorporated into the ergoline part of dihydroergocriptine which confers dopaminergic agonist activity to the molecule. A recent report has described the production of an radio-iodinated dopaminergic radioligand, (N-(p-amino-m-[^{125}I] iodophenylethyl) spiroperidol), with an estimated SA of >2000 Ci/mmol (Amlaiky et al., 1984). This agent defined dopamine receptors in bovine anterior pituitary and also offers considerable promise as an affinity ligand for the purification and characterisation of solubilised dopamine receptors from pituitary membranes.

(ii) Rate of [^{3}H]spiperone binding

Specific binding to bovine anterior pituitary membranes occurred rapidly, reaching equilibrium within 15 minutes of the start of incubation at 30°C (Figure 5-5A). Binding was temperature-dependent and took at least 30 minutes to reach equilibrium at 4°C (Figure 5-5A). Binding at 37°C was complete within 5 minutes. Routine incubations were for 60 minutes at 30°C. Analagous experiments were not performed with tumour membranes because of the small amounts of tissue available but the same kinetic parameters were assumed to apply.

Figure 5-5: Time and temperature dependence of [^{3}H]spiperone binding



(A) Effect of time and temperature on the binding of 4.9 nM [^{3}H]spiperone to bovine anterior pituitary membranes (178 µg protein/tube). ●○=4°C, ▲△=30°C. Membranes were incubated with (open symbols) and without (closed symbols) 10 µM (+)butaclamol.

(B) Rate of association and dissociation between 3.0 nM [^{3}H]spiperone and bovine anterior pituitary membranes (565 µg protein/tube). Duplicate tubes were incubated at 30°C. The triangles indicate incubations with (△) and without (▲) 10 µM (+)butaclamol from the start of the experiment.

Dissociation was examined by the addition of 10 µM (+)butaclamol (arrow) after equilibrium between ligand and membranes had been attained (60 minutes). The [^{3}H]spiperone remaining bound to the membranes was measured by filtrations at various times following this addition and is indicated by the solid circles.

The dissociation for [^{3}H]spiperone was determined by the addition of an excess of (+)butaclamol (10 μM) to membranes at equilibrium (Figure 5-5B). Dissociation was virtually complete within one hour of (+)butaclamol addition.

At the end of a routine incubation 3 mL membrane buffer was added to each tube prior to vacuum filtration to facilitate transfer to the filters. Despite this dilution, radioligand dissociation was shown to be negligible for at least 5 minutes following this addition of buffer.

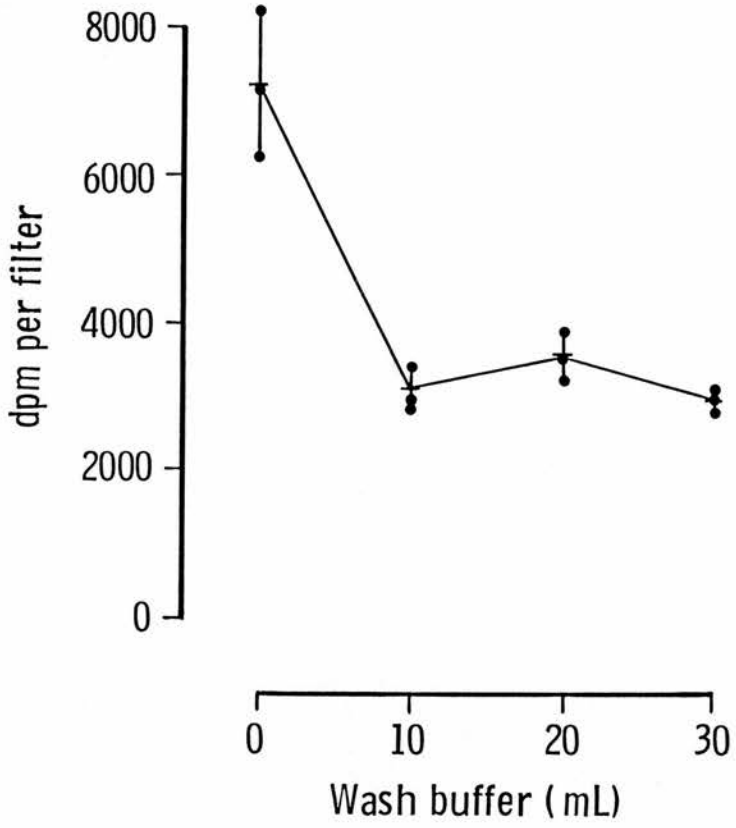
(iii) Separation of bound and free radioligand

Membrane-bound radioligand was separated from free radioactivity by filtration under vacuum through glass microfibre filters (Whatman GF/C, Whatman Ltd, Maidstone, Kent).

Pre-soaked filter discs were mounted on the support screens of a 12-channel sampling manifold (Model 1225-Millipore (UK) Ltd, London) and the vacuum applied (20"Hg, Speedivac, Edwards High Vacuum, Crawley). Prior to filtration 3 mL ice-cold membrane buffer was added to each assay tube. Samples were then poured onto the filters and each filter was washed with 3x10 mL ice-cold membrane buffer with the vacuum still applied. Washing of each filter took less than 5 seconds. Filters were then placed into plastic scintillation mini-vials (Griffiths and Nielsen Plastics Ltd, Billingshurst, England) and dried at 60^{o}C for one hour.

Figure 5-6 shows the effect of volume of wash buffer on filter bound counts. Filter washing was necessary to remove free radioligand from the filter and the 30 mL volume used in all assays provided a very adequate wash. Non-specific binding of [^{3}H]spiperone to the filters was directly proportional to the total [^{3}H]spiperone concentration and constituted

Figure 5-6: Effect of filter washing



Aliquots of bovine anterior pituitary membranes (80 µg protein/tube) were equilibrated with 10.1 nM [^{3}H]spiperone for 60 minutes at 30°C. 3 mL ice-cold membrane buffer was then added to each tube and the contents applied to filters under vacuum. Various volumes of wash buffer were used, as indicated in the diagram, and the bound radioactivity counted. Determinations were made in triplicate; the circles indicate the individual results.

0.002% of total counts in the incubate. Estimations of non-specific binding were not routinely included in each assay since this variable applied equally to total and (+)butaclamol tubes and was cancelled in the subtraction to derive specific binding.

(iv) Liquid scintillation counting

Membrane-bound tritium on the filters was measured by liquid scintillation counting in Phase Combining System, a xylene/surfactant scintillation cocktail (PCS, Amersham Corp, USA). Four mL PCS was added to each minivial and allowed to equilibrate at room temperature for at least 6 hours prior to counting, at which time the filters were translucent. Each minivial was placed in a borosilicate glass vial (Extra low background, Poulten, Selfe and Lee, UK) to act as a carrier. Radioactivity was measured by a RackBeta scintillation counter (Model 1215-001, LKB Ltd, Selsden, Surrey) with a counting efficiency of 25-40%. Quench correction was made using the sample channels ratio method and the machine was standardised with a quench curve generated from a series of acetone-quenched [^{3}H]hexadecane standards (courtesy of Dr G.E.Newman).

The presence of the glass filter did not cause quenching, neither was its orientation critical. Provided the filters were dry and had been equilibrated with scintillant for at least 6 hours, chemiluminescence was generally less than 0.05%. Phase Combining System solubilised approximately 90% of membrane-bound tritium following overnight equilibration.

Two further scintillation cocktails were evaluated; Cocktail-T, a toluene/Triton X-100 based mixture (BDH) and Ready-Solv CP, an emulsifying cocktail for the elution of samples in filter counting (Beckman). Compared with PCS, Ready Solv CP resulted in a much lower

counting efficiency and Cocktail-T showed a lower capacity for aqueous samples. All three cocktails eluted 90% of filter-bound radioactivity and gave equivalent results after quench correction. PCS was chosen for routine use.

(v) Data analysis

As already mentioned specific binding was estimated using (+)butaclamol and was defined as the counts obtained with no competitor present minus the counts in the presence of 10 μM (+)butaclamol. Data analysis was performed using an iterative computer programme which produced best fit Scatchard plots calculated by regression analysis (courtesy of Dr D.R.Matthews). The programme estimated the minimal least squares deviation by assuming a two component linear Scatchard plot (high and low affinity) and subtracted the low affinity curve from the data allowing an independent estimate of high affinity binding. It made no a-priori assumptions about the intercept between the two regression lines.

(vi) Final assay procedure

The final assay procedure is summarised in Table 5-1. The reaction volume of 400 μL was found to be optimal. All solutions were made up fresh on the day of the experiment. [^{3}H]spiperone solutions were made in membrane buffer to produce final concentrations in the incubate of 0.07-4.0 nM. Final spiperone concentrations of 4-54 nM were achieved by the addition of non-radioactive spiperone. The latter was initially dissolved in acidified ethanol, as for (+)butaclamol, and then diluted in membrane buffer. Usual amounts of membrane protein were 200 μg (non-functioning tumours), 100 μg (normal anterior pituitary and TSH-secreting adenomas) and 50-100 μg (prolactinomas) per assay tube. The amount of membrane protein (mg) obtained per gram wet weight tissue was similar for non-functioning tumours (17.7 $\pm$ 1.0, mean $\pm$ SEM, n=20) and

prolactinomas (17.0 ± 1.9, mean ± SEM, n=5). Normal pituitaries yielded 20.8 ± 3.1 mg protein per gram tissue (mean ± SEM, n=6). Aliquots of bovine membrane suspension (stored at -70°C) were used as quality controls in the tumour binding assays; these gave a between-assay coefficient of variation of 12.2% (n=7) at a [^{3}H]spiperone concentration of 4.5 nM.

Table 5-1: Final assay procedure for tumour dopamine receptor analysis

ADDITION (in order)	VOLUME OF ADDITION (µL/tube)			
	TOTAL BINDING	(+)BUTACLAMOL	NSB[a]	TOTAL COUNTS[b]
Membrane buffer	100	-	200	-
(+)butaclamol (10 µM)	-	100	-	-
Non-radioactive spiperone (3.1-50 nM, final concentration) or membrane buffer	100	100	100	-
[^{3}H]spiperone (0.07-4.0 nM, final concentration)	100	100	100	100
Membrane suspension (0.5-2.0 mg protein/mL)	100	100	-	-

Notes:

a Non-specific filter binding tubes were not routinely included.

b [^{3}H]spiperone for total counts was dispensed directly into scintillation vials.

Procedure:

1. The assay was performed in duplicate in silanised glass test-tubes
2. Membrane suspension was equilibrated at 30°C for 30 minutes prior to assay. The assay was started by the addition of membrane suspension.
3. Tubes were vortex-mixed and incubated at 30°C for 60 minutes.
4. At the end of the incubation 3 mL ice-cold membrane buffer was added to each tube, followed by rapid vacuum filtration.
5. Filters were dried at 60°C for 60 minutes.
6. 4 mL scintillation cocktail was added and membrane-bound tritium measured by liquid scintillation counting; each sample was counted for 20 minutes.

CHAPTER 6. PERIFUSION RESULTS I: PROLACTINOMAS

The background to the perifusion studies and the method validation were outlined in Chapter 4. This chapter describes the results of the prolactinoma perifusion experiments and is in two main sections. The first is concerned with dopaminergic control and the second with TRH/dopamine interactions. The results are discussed, together with the findings of other workers, at the end of each section.

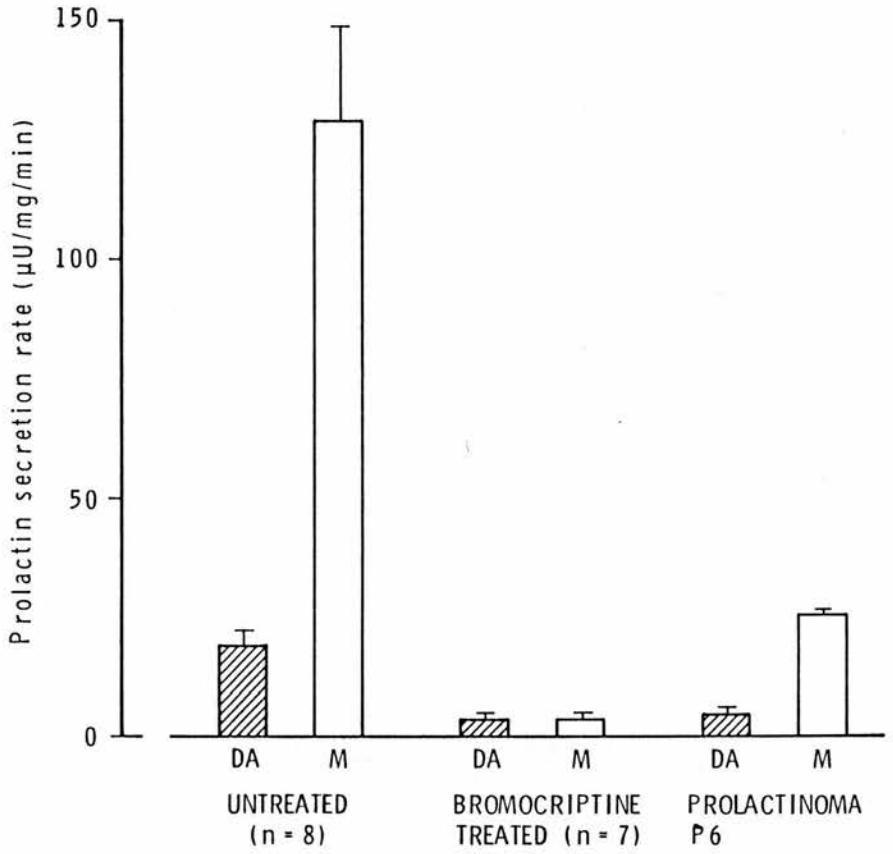
A. DOPAMINERGIC CONTROL OF PROLACTIN SECRETION

1. Prolactin secretion rates

The prolactin secretion rates for bromocriptine-treated and untreated macroprolactinomas are summarised in Figure 6-1. The last dose of bromocriptine had been administered between 40 and 96 hours before surgery to the four patients whose prolactinomas had shrunk during bromocriptine; nevertheless each tumour showed persistent cell size reduction on ultrastructural examination. However tumour regression and fibrosis meant that little tissue was available for in-vitro study from these patients.

During the initial perifusion period, when exposed to 5 μM dopamine, the untreated prolactinomas had a significantly higher mean prolactin secretion rate (19.3 μU/mg tumour/minute) than bromocriptine-treated tumours (3.9 μU/mg/minute) ($P = 0.005$). However in the treated tumours, the presence of excess fibrous tissue might have resulted in some underestimation of lactotroph prolactin secretion since the secretion rate was calculated using total tissue weight.

Figure 6-1: Macroprolactinomas: basal prolactin secretion rates



Prolactin secretion rates in three untreated prolactinomas (8 tumour columns) and four bromocriptine-treated tumours (9 tumour columns). Values shown are mean ± SEM. DA = 5 μM dopamine and M = dopamine-free medium. The secretion rate in DA was the mean of the first six fractions of each perifusion and that in M was the mean of the last 6 fractions of a one hour period of perifusion in dopamine-free medium following the initial 30 minutes perifusion with 5 μM dopamine.

The three BC-treated tumours represented by the third and fourth bars had received BC 10-20 mg daily for 3-36 weeks and BC was withdrawn 40, 72 and 96 hours before surgery.

Prolactinoma P6 received BC 10 mg daily for 20 weeks and BC was withdrawn 96 hours prior to surgery.

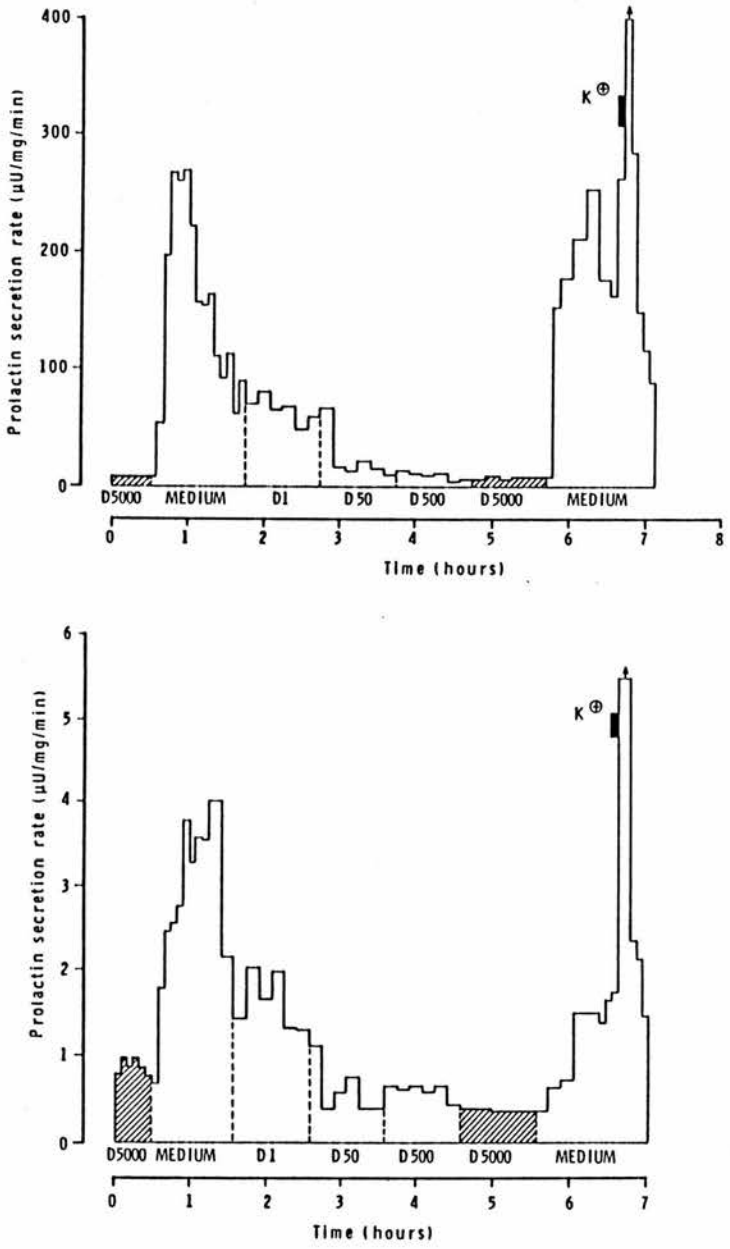
When dopamine was removed from the medium the mean prolactin secretion rate from the untreated tumours rapidly increased to 6.7 times the basal rate (129.7 μU/mg/minute). In contrast, three of the bromocriptine-treated tumours showed no significant increase in the prolactin secretion rate when dopamine was removed. In the fourth however there was a significant increase under these conditions although not to the level observed in untreated tumours. This patient (P6 in Table 2-4, page 50) had shown partial resistance to bromocriptine in-vivo since the serum prolactin level had not fallen below 10,000 mU/L during chronic bromocriptine therapy despite cell and overall tumour shrinkage.

2. Dopamine and bromocriptine dose responses

These studies examined the effects of various concentrations of dopamine and bromocriptine on the prolactin secretion rates from bromocriptine-treated and untreated prolactinomas. The basic experimental design is illustrated by the individual perifusions shown in Figures 6-2 and 6-3. Following perifusion in dopamine-free medium for 60-75 minutes tumour fragments on separate columns were exposed to four different concentrations of inhibitor, each for 60 minutes, followed by a further 60 minutes of medium alone. The perifusion was terminated by a depolarising pulse of K^+.

These perifusions contrast the rapid onset and offset of dopamine inhibition of prolactin secretion with the more prolonged action of bromocriptine. The lower prolactin secretion rate from bromocriptine-treated tumours is clearly seen. The response to K^+ at the end of each experiment indicated satisfactory cell viability and excluded the presence of contaminating anterior pituitary. The dopamine and bromocriptine dose responses were reproducibly obtained in several different tumours.

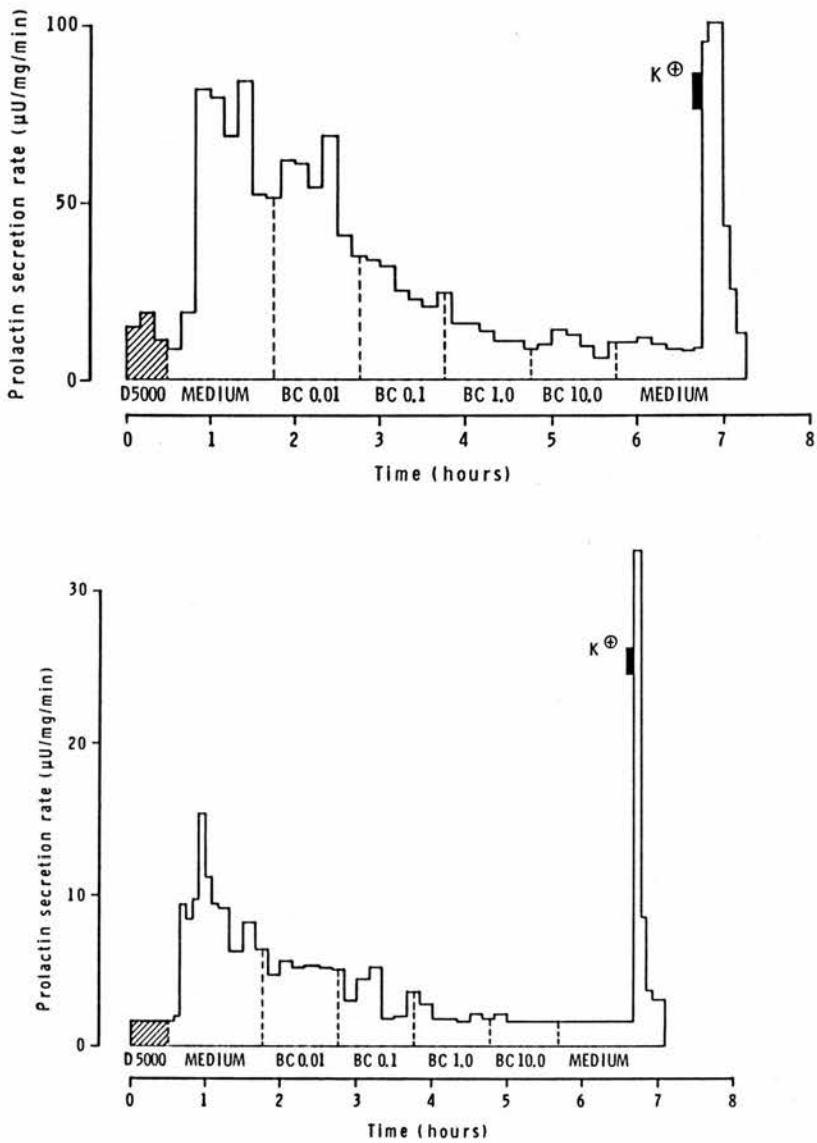
Figure 6-2: Examples of prolactinoma perifusion: effect of dopamine



Upper panel shows a macroprolactinoma (21 mg) not exposed to bromocriptine in-vivo (pre-operative serum prolactin 80,400 mU/L). Note the similar rebound increase in prolactin secretion rate following the removal of 5 μM dopamine from the medium at both the beginning and end of the perifusion.

Lower panel shows a macroprolactinoma (30.8 mg) from a patient given 10 mg BC daily for 3 weeks (last dose 40 hours prior to surgery). Note the lower prolactin secretion rate and the less impressive increase in secretion when dopamine was removed.

Figure 6-3: Examples of prolactinoma perifusion: effect of bromocriptine



Upper panel shows a macroprolactinoma (32.6 mg) not given bromocriptine therapy immediately prior to surgery. In fact this patient had previously shown some dopamine agonist resistance in-vivo but this was not apparent in-vitro. Pre-treatment serum prolactin was 20,300 mU/L but fell no lower than 3500 mU/L during therapy with bromocriptine (30 mg daily) or pergolide (1000 μg daily) and amenorrhoea persisted; there was little tumour shrinkage. No dopamine agonist was administered for six months prior to surgery.

Lower panel shows the bromocriptine-treated macroprolactinoma (27.3 mg) described in the lower panel of Figure 6-2.

Note the longer duration of action of bromocriptine compared with dopamine (Figure 6-2) evidenced by persistent suppression of secretion when the drug was withdrawn at the end of the experiment.

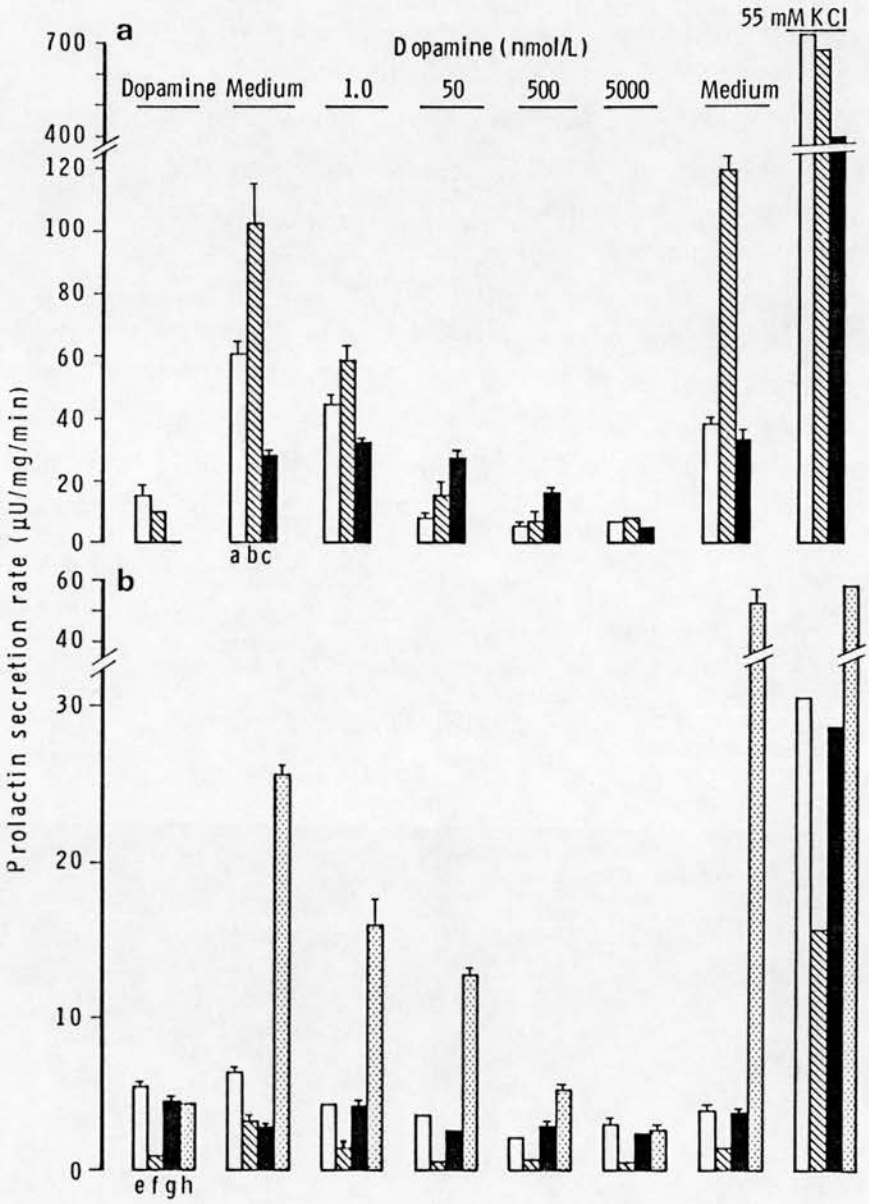
The effects of dopamine on the prolactin secretion rates of seven macroprolactinomas, four of which had regressed during pre-operative bromocriptine therapy, are summarised in Figure 6-4. The untreated prolactinomas showed a rapid increase in prolactin secretion when dopamine was removed from the medium and all responded to dopamine with a dose-dependent inhibition of prolactin release, including tumour "a" which had shown partial bromocriptine resistance in-vivo (see legend to Figure 6-3). In contrast, three of the four bromocriptine-treated tumours appeared to have fully suppressed prolactin secretion rates, even in the absence of dopamine, despite drug withdrawal for at least 40 hours prior to surgery. Dopamine dose responsiveness was therefore impossible to evaluate in these tumours.

Overall, the 50 nM dose of dopamine reduced prolactin secretion rates by approximately 50% in the prolactinomas studied.

The dose responses of eight macroprolactinomas to bromocriptine are summarised in Figure 6-5 for comparison with the dopamine responses in Figure 6-4. A clear dose-response over the concentration range 0.01 to 10 nmol/L, which includes the therapeutic range, was shown by the four untreated tumours. In general, bromocriptine concentrations of 0.1-1.0 nmol/L, which are similar to the plasma concentrations measured in Chapter 3.D, reduced prolactin secretion rates by at least 50%. Tumour "a", which had shown in-vivo dopamine agonist resistance (Figure 6-3) was fully responsive in-vitro to bromocriptine as well as dopamine.

The bromocriptine-treated tumours again showed virtually complete suppression of basal prolactin secretion and in three cases a clear dose-response relationship could not be seen.

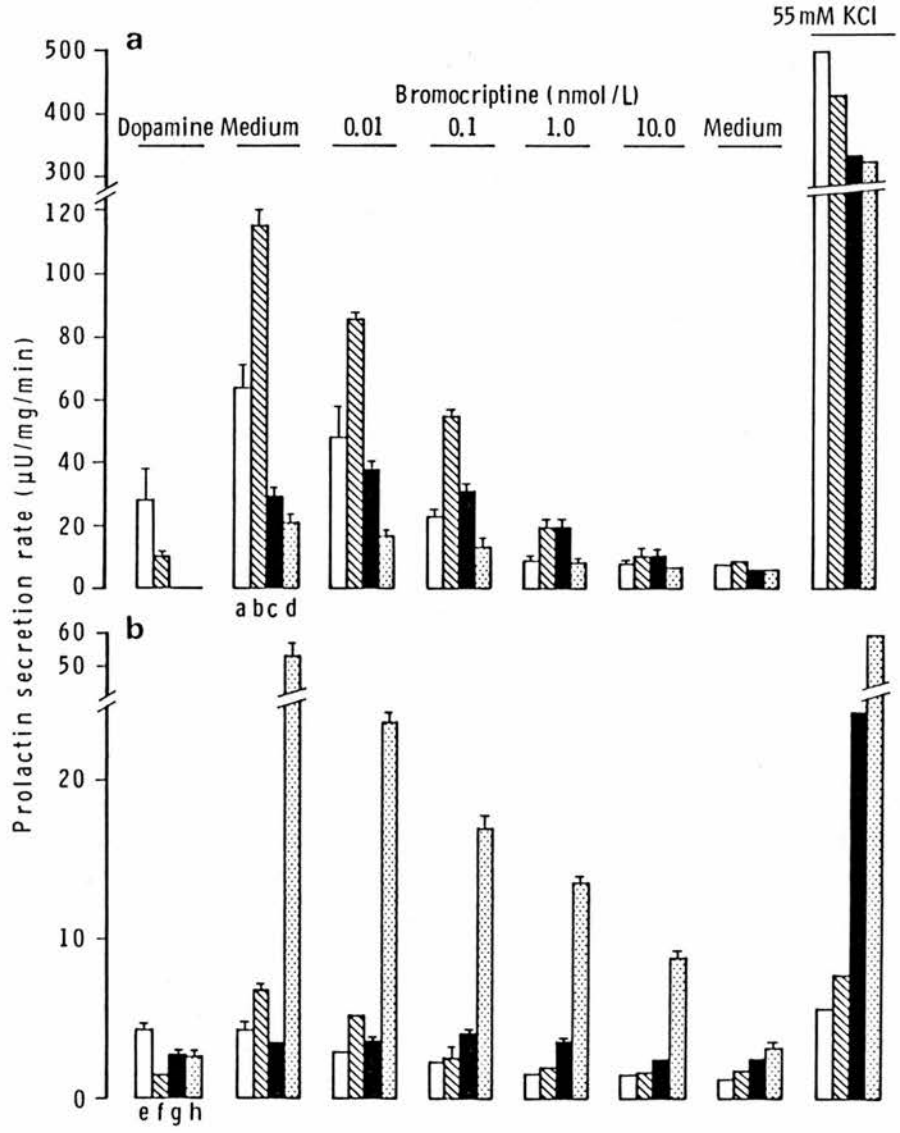
Figure 6-4: Macroprolactinomas: dopamine dose responses



Effects of dopamine on prolactin secretion rates from bromocriptine untreated (a-c, upper) and treated (e-h, lower) macroprolactinomas. In each panel individual tumours are identified by the shading pattern. Tumour "c" was equilibrated in dopamine-free medium hence there is no bar for "c" under "dopamine".

The overall experimental design is illustrated by Figure 6-2. Values shown in this figure are mean $\pm$ SEM for the last six fractions of each treatment period, except for K^+ which shows the secretion rate in the fraction immediately following the high K^+ depolarisation. SEM is not shown if it was too small to be plotted.

Figure 6-5: Macroprolactinomas: bromocriptine dose responses



The format is the same as Figure 6-4 and the same seven tumours are presented with the same code letter and shading. A further untreated tumour was studied (d) which was equilibrated in dopamine-free medium. Note that bromocriptine had a prolonged duration of action in all eight patients but high K^+ was able to overcome this inhibition.

Bromocriptine showed a prolonged duration of action on the inhibition of prolactin secretion which persisted when the drug was removed from the perifusion medium. Depolarising concentrations of potassium were always able to overcome this inhibition however.

3. Bromocriptine "resistance"

One bromocriptine-treated tumour (tumour "h" Figures 6-4 and 6-5, from patient P6 in Table 2-4), which had shown partial bromocriptine "resistance" in-vivo, showed in-vitro responses qualitatively similar to untreated prolactinomas. The explanation for this was unclear. Bromocriptine had been withdrawn for 96 hours prior to surgery so perhaps its effect had started to reverse. However bromocriptine had been given to this patient for 20 weeks and the biopsy specimen still showed cell size reduction. There was no doubt that dopamine, and bromocriptine, reduced prolactin secretion from this tumour in-vitro and possibly a bromocriptine dose of greater than 10 mg daily would have reduced the serum prolactin level to less than 10,000 mU/L. Only 90 mg of tissue was obtained at surgery from this patient so dopamine receptor studies could not be performed. However tumour fibrous tissue content might have made the results difficult to interpret with confidence.

4. Discussion

At the start of this work there was a vast literature on the control of prolactin secretion in the rat but relatively few reports of in-vitro secretion studies on human prolactinomas. Some authors reported significant decreases of prolactin release from human tumours by dopamine or bromocriptine (Peillon et al., 1979; Adams et al., 1979; Lawton et al., 1981), whereas others found little or no effect (Tallo et al., 1978; Prysor-Jones et al., 1981). Since then a number of more comprehensive publications on human prolactin-secreting adenomas have appeared (Bethea et al., 1982; Spada et al., 1983; Peillon et al., 1983; Chihara et al., 1984, Ishibashi and Yamaji, 1984, 1985). Furthermore, our knowledge of the control of prolactin secretion in a number of primate species has increased (Neill et al., 1981; Bethea, 1985; Cronin et al., 1985).

The concentrations of test substances used in in-vitro studies are of critical importance. Neill et al (1981) found that dopamine concentrations in pituitary stalk plasma from Rhesus monkeys (females in the follicular phase) ranged from 0.41-1.23 ng/mL (2.6-7.8 nmol/L). The corresponding dopamine concentrations in man are unknown but Davies et al (1984) found levels between 0.2 and 2.0 ng/mL in blood draining from the tumour beds of resected human prolactin-secreting adenomas and undetectable dopamine in similar samples from pituitary tumour patients without hyperprolactinaemia. Although such measurements are difficult to interpret, the slightly higher dopamine levels in the hyperprolactinaemic patients may have been due to short-loop feedback by prolactin causing increased hypothalamic dopamine release.

Spada et al (1983), using static incubation of tumour fragments, found that human macroprolactinomas showed dose-related inhibition of

prolactin release by dopamine concentrations between 1 and 10,000 nmol/L. In their study the concentration giving half-maximal effect (IC_{50}) was 50 nmol/L for macroprolactinomas (>10 mm diameter), which is very similar to the values I obtained for non-bromocriptine treated macroprolactinomas (Section A.2). In contrast to the large tumours, Spada and colleagues found that the dopamine IC_{50} was in excess of 500 nmol/L for microprolactinomas.

Broadly similar results were obtained by Bethea et al (1982) using human prolactinoma cells cultured on extracellular matrix. Dopamine inhibited prolactin secretion from tumourous and non-tumourous lactotrophs (the latter from patients with metastatic cancer) in dose-dependent fashion, with IC_{50} values of 243 and 76 nmol/L respectively. This study found no difference in the dopaminergic sensitivity of small and large prolactinomas. Bethea has recently reported a dopamine IC_{50} of around 10 nmol/L for inhibition of prolactin release in the Rhesus monkey using either dispersed pituitary cells in culture or pituitary stalk-transected animals treated with dopamine infusion (Bethea, 1985). Ishibashi and Yamaji (1984) found a similar value for normal human pituitary cells in culture. Whether tumourous lactotrophs are less sensitive to the inhibitory actions of dopamine than non-tumourous lactotrophs remains an issue of considerable controversy.

Very few studies on human prolactinomas have correlated in-vivo and in-vitro dopaminergic responsiveness. An exception is the study of Chihara et al (1984). In two patients with prolactin-secreting adenomas dopamine infusion (2.0 μg/kg/min) did not significantly reduce serum prolactin concentrations; the tumours removed from these patients and studied in a fragment perifusion system proved to be resistant to dopamine inhibition in-vitro. Unfortunately only one dopamine concentration was studied (500 nmol/L) so it is unclear whether the resistance was absolute or whether

the dose-response curve was markedly shifted to the right.

The rebound release of prolactin from untreated macroprolactinomas when dopamine was withdrawn from the perifusion medium has been described by other workers using both normal animal pituitaries and human pituitary tumours (Lawton et al., 1981; Denef et al., 1984). It has also been demonstrated in-vivo following the cessation of dopamine infusions (Ho et al., 1984). Interruption of hypothalamic dopamine release may, at least in part, account for the marked suckling-induced increase in prolactin secretion as reviewed by Leong et al (1983). Furthermore suckling undoubtedly stimulates TRH release in the rat and the normal lactotroph may become hyperresponsive to TRH following a brief fall in dopamine (Fagin and Neill, 1981). Whatever the physiological significance of rebound release of prolactin following dopamine withdrawal the phenomenon has obvious implications for the study of inhibitory compounds in a perifusion system since prolactin secretion rates will be unstable for some time following the withdrawal of dopamine. This was evidently not appreciated in some earlier studies (Yeo et al., 1979). In the experiments reported in this thesis inhibitory compounds were not introduced until at least one hour after dopamine withdrawal by which time the rebound phenomenon had passed.

The concentrations of bromocriptine used in earlier in-vitro studies of human prolactinomas (Mashiter et al., 1977; Adams et al., 1979; Prysor-Jones et al., 1981) were 100-1000 times higher than either the IC_{50} values observed with normal rat pituitary cells in culture (Caron et al., 1978) or the low nanomolar concentrations measurable in plasma after therapeutic dosing (Thorner et al., 1980; Bevan et al., 1986).

Prysor-Jones et al (1981) expressed surprise at the apparent resistance of seven out of ten prolactinomas to bromocriptine at the very high

concentration of 3 μmol/L. In fact six of their patients had presented with pressure symptoms from large tumours (three had visual failure) associated with pre-treatment serum prolactin concentrations of less than 1800 mU/L. It seems very likely that these tumours were non-functioning with associated disconnection hyperprolactinaemia. Furthermore four of these six patients had very low prolactin production rates in-vitro and five of the six were apparently resistant to bromocriptine. No immunocytochemistry was reported but the lack of bromocriptine-induced changes in the secretory organelles reinforces the suspicion that most of the tumours were not prolactin-secreting.

Adams et al (1979) also used high bromocriptine concentrations of 1-10 μg/mL (1.3-13 μmol/L) to investigate the inhibition of prolactin release from dispersed human tumour cells. In contrast, Bethea et al (1982) found that the bromocriptine IC_{50} for inhibition of prolactin release from dispersed prolactinoma cells was 0.05 nmol/L. This latter result was more comparable to bromocriptine IC_{50} values obtained from studies using non-tumourous animal lactotrophs; 2.9 nmol/L (Caron et al., 1978, dispersed rat cells in culture), 7 nmol/L (Delitala et al., 1980, perifused dispersed rat cells) and 10 nmol/L (Bethea, 1985, dispersed monkey cells in culture). Bromocriptine IC_{50} values for macroprolactinomas in my studies ranged between 0.1 and 1.0 nmol/L and were therefore in the same range as previously reported values. As already discussed in Chapter 3 bromocriptine showed a marked tendency to adsorb non-specifically to a variety of glass and plastic surfaces; it is quite possible that some of the discrepancies in the literature are due to this factor.

The persistence of bromocriptine inhibition of prolactin release in-vitro for at least 4 hours after removal of the drug from the medium is

well recognised (Delitala et al., 1980; Cronin et al., 1984). A similar effect is seen in-vivo as plasma bromocriptine levels fall following an acute bromocriptine challenge to patients with prolactinomas (Chapter 3, Figure 3-16). It will be demonstrated in Chapter 8 that bromocriptine was bound to dopamine receptors in resected pituitary tumours even when the interval between the last dose of the drug and surgery was as long as 19 hours. Depolarising concentrations of potassium were able to overcome this persistent action of bromocriptine in the prolactinoma perifusions described above. Ishibashi and Yamaji (1985) found that high K^+ or the calcium ionophore A23187 were able to partly overcome the inhibitory effect of dopamine (100 nmol/L) on prolactin secretion from dispersed adenomatous and non-adenomatous lactotrophs in cell culture. The interaction between adenylate cyclase and calcium mobilising systems within the lactotroph is still incompletely understood and will be discussed further in Chapter 10.

In-vitro dopaminergic responses of macroprolactinomas shrunk by bromocriptine prior to surgery have not been studied before. Discussion of these results requires a brief consideration of the current evidence pertaining to the rate of onset and the rate of reversal of bromocriptine effects on the tumourous lactotroph. The clinical evidence implies that suppression of prolactin secretion and tumour cell size reduction occur within a few days of the commencement of bromocriptine therapy (Nissim et al., 1982). Hassoun et al (1985) studied the time course of bromocriptine effects on the morphology of lactotrophs derived from macroprolactinomas not exposed to the drug in-vivo. Dispersed cells in culture from three tumours were studied after 1 and 16 days exposure to bromocriptine at the relatively high concentration of 10 nmol/L. Reduced prolactin secretion rates and accumulation of intracellular prolactin-immunoreactive granules occurred in all three tumours after 24

hours of bromocriptine exposure. Cell size reduction of the type described in Chapter 2 was seen in two of the three tumours after 16 days but, interestingly, not in the third despite persistent suppression of prolactin secretion.

The effects of bromocriptine on prolactin secretion and cell size in prolactinomas are reversible. Thorner et al (1981) documented tumour re-expansion within 2 weeks of drug withdrawal in two macroprolactinoma patients treated for one year with bromocriptine. Nissim et al (1982) found similar early re-expansion in three prolactinomas when therapy was withdrawn after 6 weeks treatment; in one patient this occurred after 5 days bromocriptine withdrawal. After cessation of prolonged therapy (mean duration 3.7 years) Johnston et al (1984) found a return of hyperprolactinaemia in 14 out of 15 prolactinoma patients within 5-14 weeks, though it might have occurred earlier than this. In a morphological study of prolactinomas which had been exposed to pre-operative bromocriptine, cell size reduction was present only in tumours treated with the drug to within 2 days of surgery. Tumours removed more than 2 days after bromocriptine had cell morphology identical to untreated tumours (Landolt et al., 1983). Prysor-Jones et al (1983) studied the effects of bromocriptine on the morphology of the spontaneous prolactin-secreting tumours which occur in aging rats. Tumour and cell size reduction occurred within 2 days of commencing treatment and there was tumour re-expansion within 3 days of stopping bromocriptine.

The prolactin secretion rates in the four perifused bromocriptine-treated macroprolactinomas were low compared with untreated tumours and each tumour showed minimal immunocytochemical staining for prolactin. This was despite a bromocriptine-free interval of 40-96 hours. Furthermore tumour cells showed persistent cell size reduction compared

with control tumours. The shortest treatment period was 3 weeks and the longest 36 weeks. The failure of the prolactin secretion rate to significantly increase following dopamine withdrawal in three of the tumours suggested that little pre-synthesised prolactin was stored ready for release. (However, my results show there was a pool of stored prolactin in these tumours which was releasible following TRH or high K^+ stimulation - see page 171). It is concluded that the bromocriptine actions on prolactin secretion and cell size reduction may persist for four days or longer following cessation of therapy.

Since all the treated macroprolactinomas regressed as a result of bromocriptine treatment it would seem likely that each possessed functional dopamine receptors which mediated the action of the drug. One tumour regressed without full suppression of prolactin; the possible reasons for this have been discussed in section A.3. The tumour which had shown previous partial bromocriptine resistance in-vivo (Figure 6-3, upper panel) was normally responsive to dopamine and bromocriptine in-vitro. The reason for the discrepancy is unclear. I conclude that **partial** in-vivo bromocriptine resistance can be due to factors other than lack of dopamine receptors or post-receptor mechanisms.

Do prolactinomas that completely fail to regress during bromocriptine treatment lack dopamine receptors ? There is no clear answer to this question since tumours which do not show radiological evidence of shrinkage may, paradoxically, show cell shrinkage in the resected tumour (Nissim et al., 1982). Furthermore suppression of prolactin secretion is not always accompanied by cell size reduction (Hassoun et al., 1985) or tumour regression (Chiodini et al., 1981). There have been no detailed in-vitro studies of truly bromocriptine-resistant prolactinomas since it appears to be quite exceptional for a prolactinoma to be resistant to

dopamine agonist therapy in terms of complete failure of prolactin to suppress and tumour mass to regress. The macroprolactinoma described by Breidahl et al (1983), in which bromocriptine resistance occurred during therapy with tumour enlargement and increased serum prolactin levels, was refractory to 1000 nmol/L bromocriptine in-vitro, though detailed studies were not reported. It is possible that this tumour dedifferentiated and lost its dopamine receptors or acquired a post-receptor defect. Bromocriptine resistance developed during therapy in the prolactinoma described by Ahmed and Shalet (1986) but the tumour subsequently responded to pergolide. The mechanism of this differential resistance is obscure.

B. INTERACTIONS OF TRH AND DOPAMINE

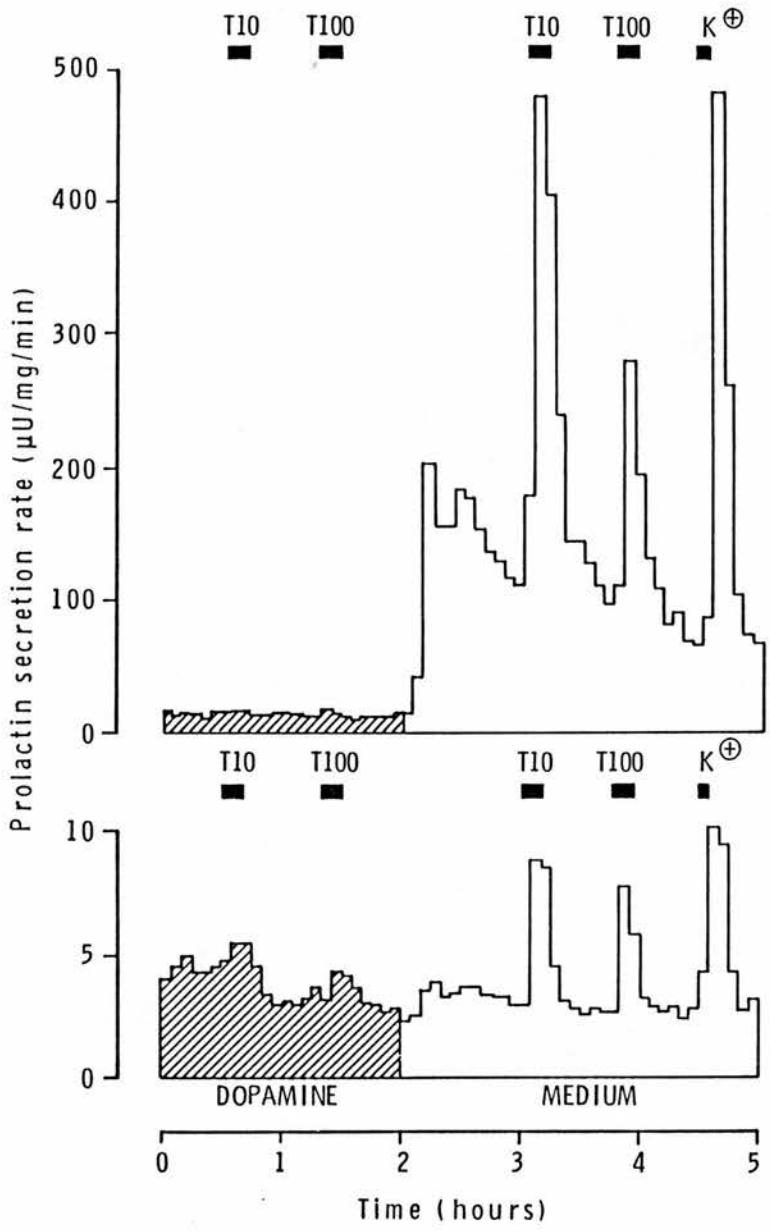
1. Perifusion results

The effect of TRH on prolactin release from eight prolactin-secreting adenomas was examined in the presence and absence of 5 μM dopamine. The usual experimental protocol is illustrated by the two prolactinomas in Figure 6-6. Individual TRH stimulations (10 and 100 ng/mL) were given for 10 minutes to four tumours and 5 minutes to the other four tumours. In most cases the initial stimulations were in the presence of 5 μM dopamine followed by stimulations in medium alone. The results were qualitatively similar if this order were reversed.

Statistical analysis was performed on the basis of fiducial inference (Chihara et al., 1984) and prolactin stimulation was judged significant ($P < 0.05$) when the peak response was beyond 3 standard deviations of the baseline hormone level (mean of 5 fractions preceding the TRH).

The results for six of the tumours are summarised in Figure 6-7. On the above statistical criteria none of these tumours showed significant TRH-induced prolactin release in the presence of 5 μM dopamine. As a group however the bromocriptine-treated prolactinomas showed a small increase in prolactin release to TRH 100 ng/mL in the presence of dopamine which just reached statistical significance using the Mann-Whitney U test. All six tumours showed significant TRH-induced prolactin release in the absence of dopamine. Prolactinomas that had received pre-operative bromocriptine therapy and had low basal prolactin secretion rates showed percentage increases similar to the untreated adenomas.

Figure 6-6: Macroprolactinomas: effect of TRH stimulation



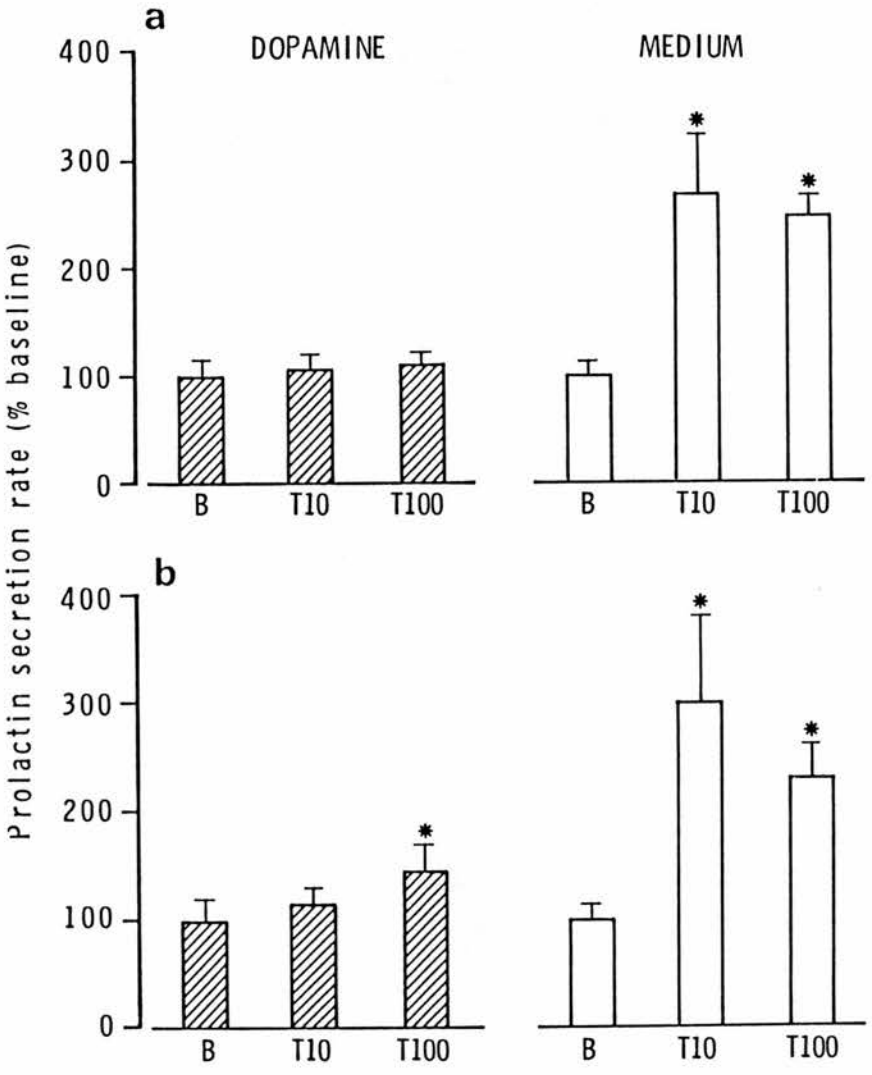
The effects of TRH on prolactin release from two prolactin-secreting adenomas.

T10 and T100 = TRH, 10 and 100 ng/mL. Dopamine = medium containing 5 μM dopamine. TRH stimulations were for 10 minutes.

Upper panel shows a prolactinoma (30 mg) not given bromocriptine and lower panel shows a prolactinoma (28.4 mg) exposed to 20 mg bromocriptine daily for 36 weeks (last dose 96 hours prior to surgery).

Dopamine prevented TRH-induced prolactin release from both tumours.

Figure 6-7: Macroprolactinomas: magnitude of the TRH-induced prolactin responses



The peak prolactin secretion rates following TRH in three untreated (a) and three bromocriptine-treated (b) prolactinomas expressed as a percentage of the baseline secretion rate. The general protocol is illustrated in Figure 6-6. Values shown are mean ± SEM. Dopamine = 5 μM dopamine (shaded bars). Medium = dopamine-free perifusion medium (open bars). B = baseline prolactin secretion rate and T10 and T100 = TRH, 10 and 100 ng/mL.

Statistical analysis was with the Mann-Whitney U test. * denotes $P < 0.05$ and a significant increase in prolactin secretion rate relative to baseline. There was no significant difference in the magnitude of the TRH responses in the absence of dopamine between bromocriptine-treated and untreated patients.

One other tumour showed significant TRH-induced (10 and 100 ng/mL) prolactin release in the **presence** of 5 μM dopamine though the peak secretion rates were considerably lower than in the absence of dopamine; this tumour had apparently normal dopamine receptors on the basis of dopamine and bromocriptine dose-responses and radioreceptor assay measurements (Chapter 8).

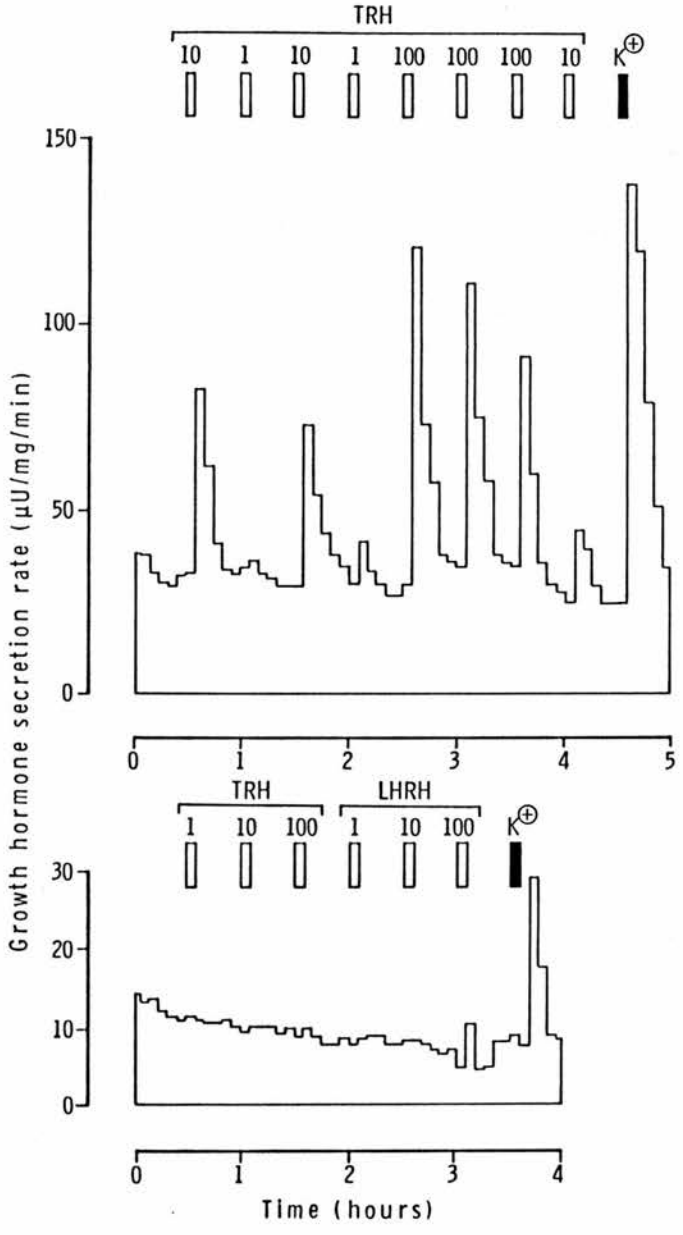
One further tumour did not respond to TRH 10 ng/mL and showed a significant increase in prolactin secretion rate only after stimulation with TRH 100 ng/mL in the absence of dopamine. Whether this was due to abnormal lactotroph TRH receptor function in this tumour remains speculative.

Four of the tumours in Figure 6-7 were tested with TRH 1.0 ng/mL in the absence of dopamine; only two showed a significant stimulation of prolactin release following this lower dose. There was no statistically significant difference between the peak prolactin secretion rates induced by TRH 10 or 100 ng/mL in the absence of dopamine. Furthermore there was no difference in the magnitude of these peaks between bromocriptine-treated and untreated macroprolactinomas. The present studies did not permit a detailed evaluation of the TRH dose-response relationship for macroprolactinomas but concentrated on using relatively large doses of TRH to see whether a prolactin response could be elicited at all. From animal studies one would predict TRH doses of greater than 25 nmol/L to be approaching the top end of the dose-response curve (Tashjian et al., 1971; Ray and Wallis 1984).

Periadenomatous normal pituitary, perifused in parallel with prolactinoma tissue from the same patient, showed TRH-induced prolactin responses identical to those described above (data not shown) suggesting that similar TRH receptors were present on both tumourous and normal

lactotrophs. These data contrasted with the results of a perifusion of periadenomatous pituitary and tumour tissue from a patient with acromegaly who had shown a marked paradoxical growth hormone response to TRH in-vivo. Only the tumour tissue responded to TRH in-vitro suggesting that TRH receptors were present on only the tumourous somatotrophs (Figure 6-8). These experiments were in agreement with the findings of Ishibashi and Yamaji (1984).

Figure 6-8: TRH-induced growth hormone responses by a HGH-secreting adenoma and adjacent anterior pituitary



The growth hormone responses of a HGH-secreting adenoma (23.7 mg, upper panel) and adjacent normal anterior pituitary tissue (5.0 mg, lower panel) to TRH. Prior to surgery the serum growth hormone concentration rose from 29.2 mU/L to >320 mU/L, 20 minutes after the intravenous injection of 200 μg TRH.

Doses of TRH (ng/mL) were administered for 5 minutes, and in random order, to the tumour tissue. In addition, the anterior pituitary tissue was exposed to various doses of LHRH. The identity of the tissue fragments on the two columns was confirmed histologically and the K^+ results are shown in Figure 4-2 (lower).

Note the clear TRH dose-response of the tumour tissue over the range 1-100 ng/mL.

2. Discussion

TRH induces prolactin release by a direct action on the pituitary (Tashjian et al., 1971) and is present in pituitary stalk plasma in higher concentrations than those found in peripheral blood (Fink et al., 1982). The role of TRH in suckling-induced release of prolactin has been recently reviewed by Leong et al (1983) and de Greef and van der Schoot (1985). The mechanism of TRH action on the lactotroph involves phospholipid hydrolysis and the mobilisation of intracellular calcium (reviewed by Gershengorn, 1986).

Tashjian et al (1971), using the GH_3 rat lactotroph cell line, showed that TRH released prolactin in a dose-dependent manner and that the maximum response was elicited by a concentration of 10 ng/mL (28 nmol/L). A similar relationship has been shown for TRH stimulation of prolactin from normal animal anterior pituitary cells (Ray and Wallis, 1984). Ishibashi and Yamaji (1984) found that TRH (10 nmol/L) released prolactin, but not HGH, from dispersed normal human pituitary cells in culture. Some human prolactin-secreting tumours respond to TRH in-vitro in similar fashion and Adams et al (1979) found that a dose of 10 ng/mL gave a maximal response.

In normal human volunteers a marked increase in serum prolactin is observed following the intravenous injection of 200 µg TRH (Cowden et al., 1979; Ghigo et al., 1985; Johnston et al., 1985). The prolactin response to TRH in normal man is abolished by the concurrent administration of dopamine in pharmacological (Besses et al., 1975) and physiological (Connell et al., 1985) concentrations. This effect has also been demonstrated using normal animal pituitary cells in-vitro (Ray and Wallis, 1984).

In contrast to normal controls, patients with prolactinomas or hyperprolactinaemia of any cause (except primary hypothyroidism) show a greatly impaired serum prolactin response to TRH (See Figure 2-2 and Cowden et al., 1979; Scanlon et al., 1986). There is now a major body of evidence that prolactin can influence its own secretion by short-loop feedback on the hypothalamus causing dopamine release (reviewed by Franks, 1983 and de Greef and van der Schoot, 1985). The evidence is based on both animal and human studies. Weber et al (1983) showed that hypophysial stalk dopamine levels were increased in rats inoculated with a prolactin-secreting tumour. Clinical studies with domperidone, a dopamine antagonist which does not cross the blood-brain barrier (Pourmand et al., 1980), have suggested increased dopaminergic activity on the pituitary in patients with prolactin-secreting pituitary adenomas (Scanlon et al., 1981; Rodriguez-Arnao et al., 1983).

If the subnormal response of prolactin to TRH in patients with prolactinomas is due to the presence of increased hypothalamic dopamine release the prediction would be that all of these tumours ought to respond to TRH, in-vitro, in the absence of dopamine. Chihara et al (1984) investigated 8 prolactinomas from patients who showed a negligible pre-operative response of serum prolactin to 500 µg TRH. In 3 of the tumours significant stimulation of prolactin release followed exposure to TRH at the high concentration of 1000 nmol/L, although an interaction with dopamine was not studied. Conversely 5 of the eight prolactinomas were apparently resistant to TRH in-vivo and in-vitro. A possible explanation is that these tumours had absent or deficient TRH receptors. On the basis of ligand binding experiments Le Dafniet et al (1985) showed TRH receptors to be clearly present in 13 out of 18 prolactinomas. However their study did not evaluate any aspects of post-TRH receptor function.

In my studies all eight prolactinomas showed a prolactin response in-vitro to the much lower TRH doses of 10-100 ng/mL in the absence of dopamine. These tumours included 3 whose basal prolactin secretion rates had been reduced to very low levels by pre-operative bromocriptine therapy and in which secretion did not greatly increase following removal of dopamine from the medium. Despite this, the percentage increases in prolactin secretion following TRH were the same as in the untreated patients (Figure 6-7). The significance of this finding is unclear. Ho et al (1985), however, have presented evidence which is consistent with the existence of separate functional pools of prolactin within the lactotrophs, one of which is releasible by TRH and another by dopaminergic modulation. It is conceivable, but entirely speculative, that bromocriptine has a differential effect on different prolactin pools.

The other notable finding in my TRH studies was the marked attenuation of the TRH prolactin response when prolactinoma fragments were exposed to 5 μM dopamine. In many cases the TRH response was completely abolished by this concentration of dopamine. A similar effect was noted in combined PRL/HGH-secreting tumours by Ishibashi and Yamaji (1984) using dopamine concentrations of 100 nmol/L. Bromocriptine-treated macroprolactinomas showed the same responses suggesting that dopamine receptor down-regulation had not occurred during chronic dopamine agonist therapy. None of the patients that I studied showed a significant prolactin response to TRH in-vivo; that they responded in-vitro is consistent with dopamine inhibition of TRH action in-vivo.

CHAPTER 7. PERIFUSION RESULTS II: NON-FUNCTIONING TUMOURS

Ten non-functioning tumours were studied, including six from patients given pre-operative bromocriptine without tumour shrinkage. All were large tumours and four were associated with modestly elevated serum prolactin concentrations between 1000 and 2500 mU/L. None of the patients had clearly elevated serum FSH or LH levels, although three were post-menopausal women with FSH levels up to 28 u/L, and in no case did these hormones respond paradoxically to TRH as has been described in some gonadotrophin-secreting adenomas (Snyder et al., 1980). In the patients given dopamine agonist therapy there was no suppression of serum gonadotrophin concentrations during treatment. No patient whose tumour partly immunostained for FSH/LH showed a significant reduction in the serum concentrations of these hormones following surgery suggesting that the major contribution to serum gonadotrophins in such a patient was not from the tumour but from normal pituitary.

Tumours were studied by fragment perifusion and the results were compared with immunocytochemistry. All the tumours described in this section had dopamine receptor measurements and this provided an opportunity to correlate tumour FSH, LH and alpha subunit contents with the perifusion and immunocytochemistry results. The complete results are summarised in Table 7-1 and various aspects will be considered in the rest of this section.

Table 7-1: Non-functioning tumours; comparison of tumour immunocytochemistry, perifusion and hormone content

No	Immunochemistry	Perifusion							Tumour hormone content		
		Hormone(s) identified				Hormone responses to stimuli					
		PRL	FSH	LH	α	LHRH	TRH	DA	FSH	LH	α
1	FSH and α	-	+	-	nd	$\pm$	-	-	+	-	+
2	FSH, LH and α	-	+	-	+	-	-	nd	-	-	+
3	Nil	-	-	-	-				-	-	-
4	FSH and α	-	+	-	+	-	-	-	+	-	-
5	Nil	-	-	-	-				-	-	-
6	α	-	-	+	+	nd	-	-	-	+	+
7	Nil	-	-	-	-				-	-	-
8	FSH, LH and α	-	$\pm$	$\pm$	+	-	-	-	-	-	-
9	Nil	-	-	-	-				-	-	-
10	Nil	-	-	-	-				-	-	-

(- denotes no hormone detected or no response to perifusion test stimulus, nd = not done)
Tumours 1-3, 5, 7 and 10 had been exposed to pre-operative bromocriptine without tumour shrinkage.
Tumours 1, 6, 8 and 10 were associated with serum prolactin concentrations between 1000 and 2500 mU/L.

Immunocytochemistry: Tumours were immunostained for PRL, FSH, LH and alpha subunit. No tumour showed positive immunostaining in >10% cells and staining was confined to single cells or small clumps of cells.

Perifusion: Hormones were reported negative when no FSH, LH, α or PRL could be detected following K^+ depolarisation. Hormones marked "+" were detected throughout the perifusion period as well as following K^+. Stimulations were with TRH (10 minutes, 1-100 ng/mL), LHRH (10 minutes, 1-100 ng/mL) and dopamine (30-60 minutes, 5 µM).

Hormone contents: The following hormone contents were included as positive; FSH and LH, >20 units per g tumour homogenised and alpha subunit, >10 µg per g tumour homogenised (see section C)

The apparent production of LH by tumour 6 was probably due to crossreaction of alpha subunit in the LH assay.

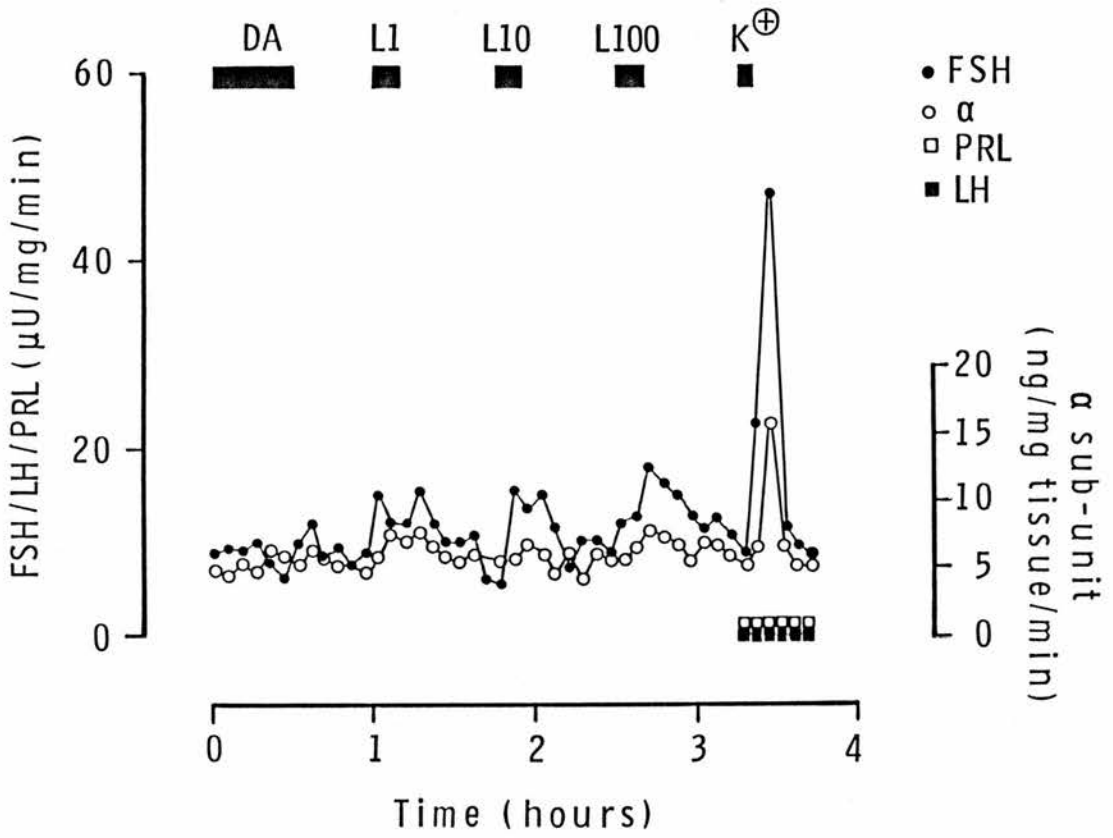
A. GONADOTROPHIN SECRETION

Four tumours secreted FSH and/or LH and alpha subunit in the perifusion experiments. Secretion rates were low and between 50 and 150 mg tissue per column was required to detect this hormone production. This was consistent with the observation that less than 10% of tumour cells immunostained for the various hormones. Immunocytochemistry on thin serial sections of the tumours showed that FSH and/or LH were located in the same cells as alpha subunit. None of these tumours responded to TRH, LHRH or dopamine in-vitro and secretion was stimulated only by depolarising concentrations of K^+. One of the perifusion experiments for tumour 4 is shown in Figure 7-1.

A fifth tumour immunostained for alpha subunit alone and secreted significant amounts in-vitro (Figure 7-2). In apparent disagreement with the immunocytochemistry this tumour also appeared to secrete and contain LH. In fact this was probably due to crossreaction of alpha subunit in the LH radioimmunoassay as described in Chapter 3.B. Unfortunately no serum alpha subunit measurements are available for this patient.

The remaining five tumours showed completely negative immunostaining and no hormones were detected in the perifusates or tumour extracts. Nevertheless each of these tumours showed small secretory granules on ultrastructural examination.

Figure 7-1: Perifusion of non-functioning tumour; release of FSH and alpha subunit

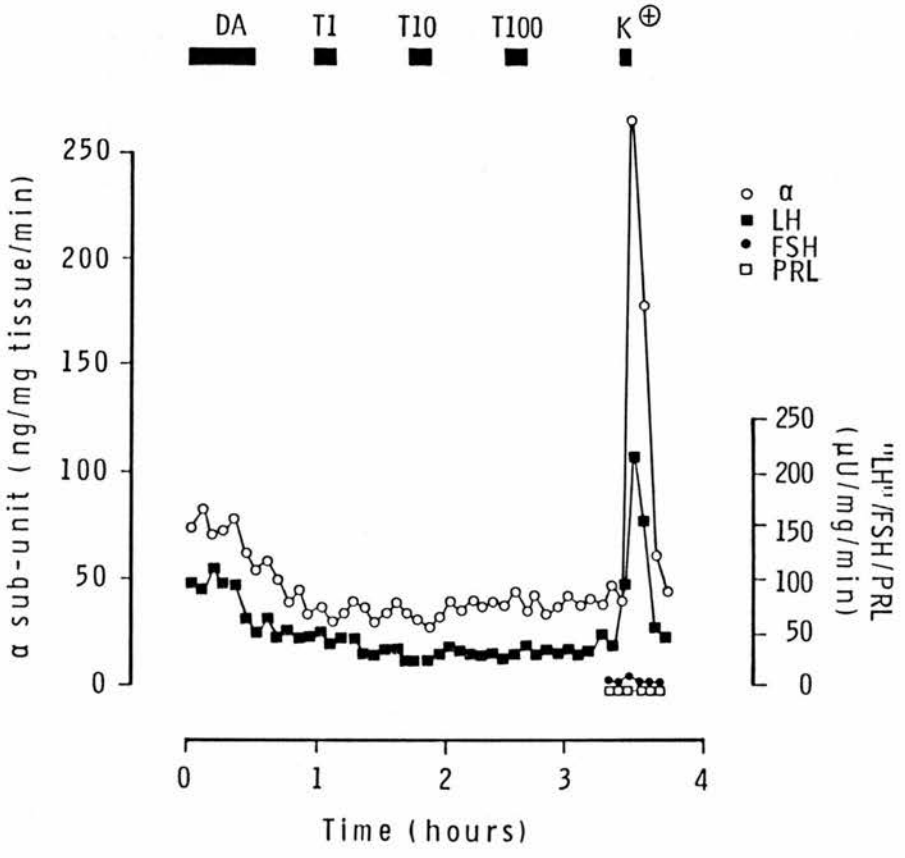


Perifusion of non-functioning tumour fragments (78 mg) from tumour no.4 in Table 7-1.
DA = 5 μM dopamine. L1-L100 = LHRH, 1-100 ng/mL. K^+ = 55 mM.

Immunocytochemistry showed small clumps of cells positive for both FSH and alpha subunit and only these hormones were detected in the perifusate. The tumour contained 41.9 U FSH and 5.3 μg alpha subunit per g tissue homogenised.

In this experiment neither 5 μM dopamine nor LHRH (1-100 ng/mL) significantly altered FSH or alpha subunit secretion rates. Following K^+ depolarisation there was significant release of both hormones but not LH or PRL.

Figure 7-2: Perifusion of non-functioning tumour; release of alpha subunit



Perifusion of non-functioning tumour fragments (108.7 mg) from tumour no. 6 in Table 7-1.
DA = 5 μM dopamine. T1-T100 = TRH, 1-100 ng/mL. K^+ = 55 mM.

Immunocytochemistry showed clumps of cells positive for alpha subunit alone. The tumour contained 38.6 μg alpha subunit per g tissue homogenised.

Dopamine did not inhibit the secretion of alpha subunit neither was the latter stimulated by TRH (1-100 ng/mL). Following K^+ depolarisation there was significant release of alpha subunit but not FSH or PRL.

B. PROLACTIN SECRETION

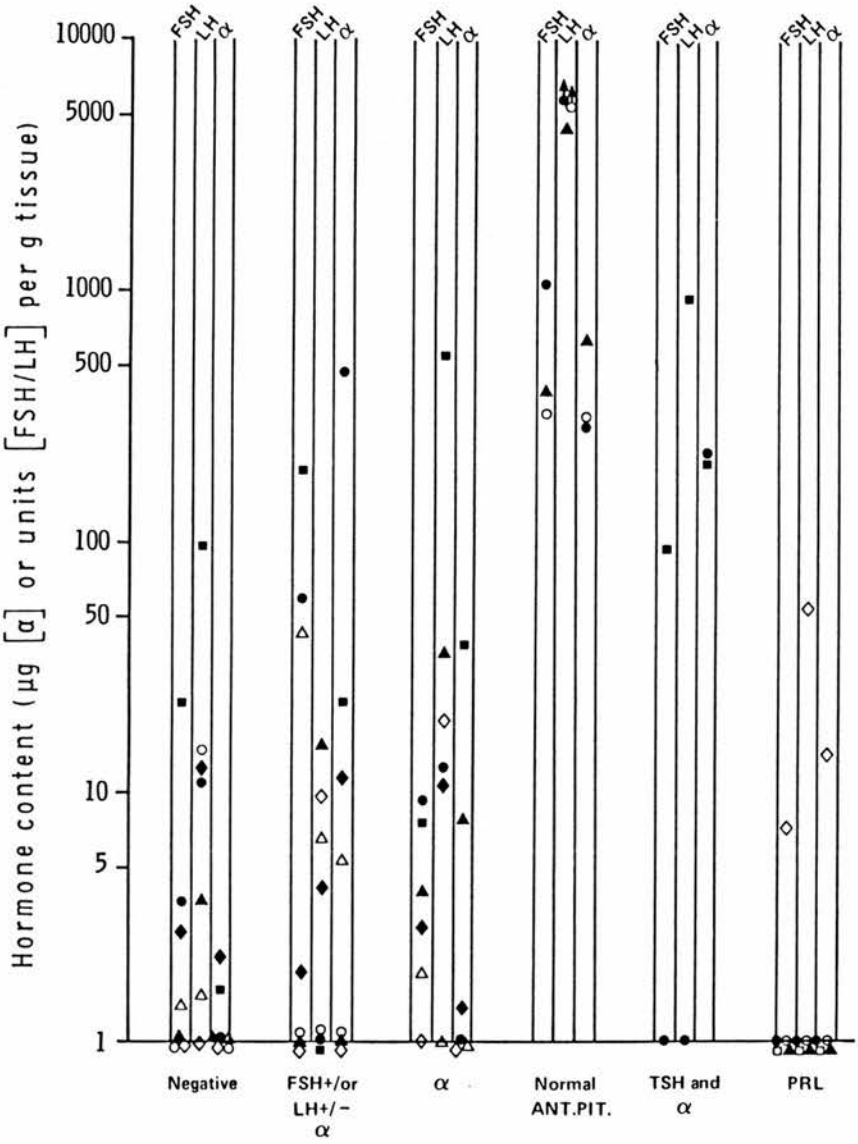
Since four tumours were associated with significant, though modest, hyperprolactinaemia it was important to ensure that the tumours were not in fact prolactin-secreting in-vitro. None of the tumours immunostained for prolactin and prolactin was undetectable in the perifusate from all these tumours following K^+ depolarisation. The tumours were therefore designated as non-prolactinomas with confidence. The hyperprolactinaemia was presumed to be on the basis of hypothalamo-pituitary disconnection and was restored to normal after surgery in all 4 cases.

C. TUMOUR HORMONE CONTENTS

These were calculated by measuring FSH, LH and alpha subunit concentrations in suitable dilutions of the high speed supernatants obtained after homogenisation and differential centrifugation of tumours in the preparation of membranes for dopamine receptor measurement. Figure 7-3 shows the hormone contents of twenty non-functioning tumours, including the ten in Table 7-1, and compares the results with those obtained from TSH and prolactin-secreting adenomas and normal pituitaries. The non-functioning tumours were subdivided into three groups representing negative, gonadotrophin (often with alpha subunit) and alpha subunit immunostaining.

Four of the prolactinomas contained undetectable FSH, LH and alpha subunit as expected. One tumour (◇ PRL column, Figure 7-3) contained detectable amounts of these hormones, presumably due to anterior pituitary contamination. However when these levels were compared with those obtained from normal pituitary it could be estimated that the contamination was, at most, only 1.2%. By a similar estimation the negatively staining tumour marked by the solid square in Figure 7-3

Figure 7-3: Tumour contents of FSH, LH and alpha subunit



The immunohistological category is shown at the foot of the six main columns: Negative (n=7), gonadotrophin/alpha subunit (5), alpha subunit (6, in 4 of these the staining was minimal), normal anterior pituitary (3), TSH/alpha subunit (2) and PRL (5).

Within each immunohistological group the same symbol denotes the FSH, LH and alpha subunit content of an individual tumour/tissue.

was probably also contaminated with some anterior pituitary, but by no more than 3.8%. It was clear therefore that tumour FSH/LH levels of less than 20 U/g tissue and alpha subunit levels of less than 10 μg/g tissue could not distinguish tumour cell content from very small amounts of anterior pituitary contamination.

Only four non-functioning tumours contained more than 10 μg alpha subunit per gram of tumour; all four showed positive immunostaining (but in <10% cells), accompanied by LH in one and FSH in two tumours. The highest alpha subunit content in a non-functioning tumour was 482 μg per gram tissue; the tumour also immunostained for and contained FSH and was associated with a serum alpha subunit concentration of 17 ng/mL (normal range <3). This tumour level was comparable to the alpha subunit contents of two TSH-secreting adenomas in which 25% of cells immunostained for alpha subunit. However one of these tumours gave gonadotrophin results which suggested as much as 20% anterior pituitary contamination (■ TSH column, Figure 7-3).

Due to the problem of possible anterior pituitary contamination the measurement of tumour gonadotrophin contents was a less sensitive method than immunocytochemistry or tumour perifusion (Table 7-1).

D. CORRELATIONS BETWEEN TUMOUR PERIFUSION AND IMMUNOCYTOCHEMISTRY

The excellent agreement between these two methods when applied to the characterisation of non-functioning tumours is summarised in Table 7-1. The only exception was tumour 2 in which immunocytochemistry showed a few foci of FSH, LH and alpha subunit positive cells but perifusion showed low levels of FSH and alpha subunit alone; LH was undetectable even after K^+. The discrepancy was probably a reflection of the very low secretory activity of this tumour.

There was complete agreement between immunocytochemistry and perifusion with regard to prolactin. All tumours positive for prolactin immunocytochemically produced prolactin in-vitro and the converse was also true.

E. DISCUSSION

The enigma of the non-functioning tumour has been introduced in Chapter 1 and will be discussed further in Chapter 10, particularly in the light of the dopamine receptor findings. This section briefly reviews the perifusion results for this group of tumours.

Clearly none of the tumours that I studied in detail were gonadotrophinomas of the type described by Whitaker et al (1985) or Snyder et al (1985), with elevated serum gonadotrophin concentrations and greater than 20% of cells in the resected tumours immunostaining for the relevant hormones. In fact 5 of the 10 tumours in Table 7-1 showed no known anterior pituitary hormones in immunocytochemical, perifusion or hormone content studies, but each contained small secretory granules at the electron microscopic level.

The four tumours with low levels of gonadotrophin and alpha subunit correspond to the type of tumour studied by Mashiter et al (1981) and Surmont et al (1983). It is difficult to assign these tumours as definite gonadotrophinomas since more than 90% of cells showed completely negative immunostaining and tumour hormone contents were only marginally greater than completely non-functioning tumours and very much lower than normal pituitary (Figure 7-3). Following short-term exposure to TRH and LHRH in the perifusion studies none of the tumours showed a significant response. Surmont et al (1983) examined four such tumours exposed to TRH and LHRH (both at 100 ng/mL) for prolonged periods (12-41

days) in cell culture. FSH and LH secretion was stimulated by LHRH from one tumour and LHRH and TRH from two tumours. It is difficult to see how the difference in exposure time could explain this discrepancy but the number of tumours studied in each case was small.

The dopaminergic responsiveness of gonadotroph-cell tumours is largely unknown. Berezin et al (1984) described a patient with a macroadenoma which was clearly FSH-secreting, despite a lack of immunocytochemical data, and in whom bromocriptine reduced serum FSH concentrations, but without tumour shrinkage. None of the tumours in my studies showed a reduction of serum gonadotrophin levels during bromocriptine therapy or an inhibition of gonadotrophin release when exposed to 5 μM dopamine in-vitro.

One of the adenomas studied in detail (No.6, Table 7-1) appeared to contain and secrete alpha subunit alone, though the number of cells showing positive immunostaining was less than 10%. Such tumours have been described previously (Ridgway et al., 1981; Snyder et al., 1985). The problem of crossreactivity of alpha subunit in the LH assay was also encountered by Snyder et al (1985) who showed that the LH immunoreactivity eluted in the alpha subunit position on gel filtration of culture medium from an alpha subunit secreting adenoma.

In conclusion, the primary cell of origin in many so called non-functioning tumours remains unclear and I shall return to this again in the final chapter.

CHAPTER 8. DOPAMINE RECEPTOR MEASUREMENTS

Previous studies of pituitary dopamine receptor studies and the method validation were described in Chapter 5. This chapter contains the results of the pituitary tumour dopamine receptor measurements.

A. TISSUES STUDIED

Dopaminergic binding was investigated in tumours removed from patients with non-functioning tumours (20) and prolactinomas (5). The median wet weight of tissue available for membrane studies was 572 mg (range 107-1184) for non-functioning and 375 mg (range 222-820) for prolactin-secreting adenomas. The tumour results were compared with those obtained from fresh bovine and necropsy human anterior pituitaries.

The clinical details, including pre-operative bromocriptine therapy, and tumour pathology for the non-functioning tumour and prolactinoma patients are shown in Tables 8-1 and 8-2. All the non-functioning tumours were large tumours with mean fossa volume 2780 mm^3 and mean suprasellar extension height 14 mm. Nine of these tumours had been exposed to pre-operative bromocriptine without tumour shrinkage (six have been described in Chapter 2). The prolactinomas had a mean fossa volume of 2670 mm^3, though none had a significant suprasellar extension, and none had been exposed to bromocriptine. Unfortunately there was insufficient tissue from bromocriptine-treated macroprolactinomas for receptor studies.

Immunostaining for prolactin was negative in all 20 non-functioning tumours but strongly positive in all of the prolactin-secreting tumours. Furthermore, tumour fragments from 4 non-functioning tumours associated with initial serum prolactin concentrations over 1000 mU/L did not release prolactin in-vitro following exposure to depolarising

Table 8-1: Clinical details and tumour pathology for 20 patients with non-functioning tumours

Code	Age	Sex	Symptoms	Serum PRL (mU/l) (pre-treatment)	Immuno-staining	Bromocriptine Dose (mg/day)	[Duration-mths]
UNTREATED							
1	70	M	Hypopit (ACTH/GH) Visual failure	450	Neg		
2	33	F	Galactorrhoea	4350	FSH+ α+		
3	60	M	Hypopit (ACTH/GH LH) Visual failure	1010	FSH+ LH+ α+		
4	51	M	Hypopit (ACTH/GH LH) Visual failure	370	α±		
5[a]	40	M	Hypopit (ACTH/GH TSH/LH)	390	Neg		
6	51	M	Hypopit (ACTH/GH TSH/LH)	540	α+		
7	30	M	Visual failure	740	Neg		
8	62	F	Visual failure	1370	Neg Onco+++		
9	68	F	Visual failure	1020	α+		
10	47	F	Visual failure	1200	FSH+ α+ Onco+		
11	78	M	Hypopit (ACTH/TSH) Visual failure	<90	Neg Onco++		
BROMOCRIPTINE TREATED							
B1[b]	59	M	Hypopit (ACTH/GH TSH/LH)	220	Neg	20 40	[6] [4]
B2[ab]	53	F	Visual failure	520	FSH+ Onco+++	30	[10]
B3[b]	63	M	Hypopit (ACTH/GH LH)	400	α±	20	[7]
B4	54	F	Visual failure	1070	FSH+ α+	10	[1.5]
B5	73	M	Hypopit (ACTH/GH TSH/LH)	1250	Neg Onco+++	7.5 2.5	[12] [36]
B6	36	M	Visual failure	300	FSH+ Onco++	20	[3]
B7	59	F	Visual failure	600	α± Onco+	5.0	[3]
B8	81	F	Visual failure	770	FSH+ LH+ α+ Onco+	7.5	[1]
B9[c]	39	F	Visual failure	150	α±	20	[6]

a = previous transfrontal surgery and radiotherapy
b = last dose of bromocriptine within 24 hours of surgery
c = previous transfrontal surgery
ACTH, GH, TSH, LH denote hormone deficiencies in patients presenting with hypopituitarism.
Onco = Oncocytic change

Table 8-2: Clinical details and tumour pathology for five prolactin-secreting pituitary adenomas

Code	Age	Sex	Symptoms	Serum PRL (mU/1)	Immuno-staining	Perifusion PRL release
P1	40	M	Headache Impotence	80,350	PRL+++	+ve
P2	39	M	Incidental finding on xray	20,130	PRL+++	+ve
P3	21	M	Galactorrhoea Headache	>68,000	PRL+++	ND
P4	22	F	2^{o} amenorrhoea	12,720	PRL+++	+ve
P5	30	F	2^{o} amenorrhoea	6,570	PRL+++	+ve

ND = not done

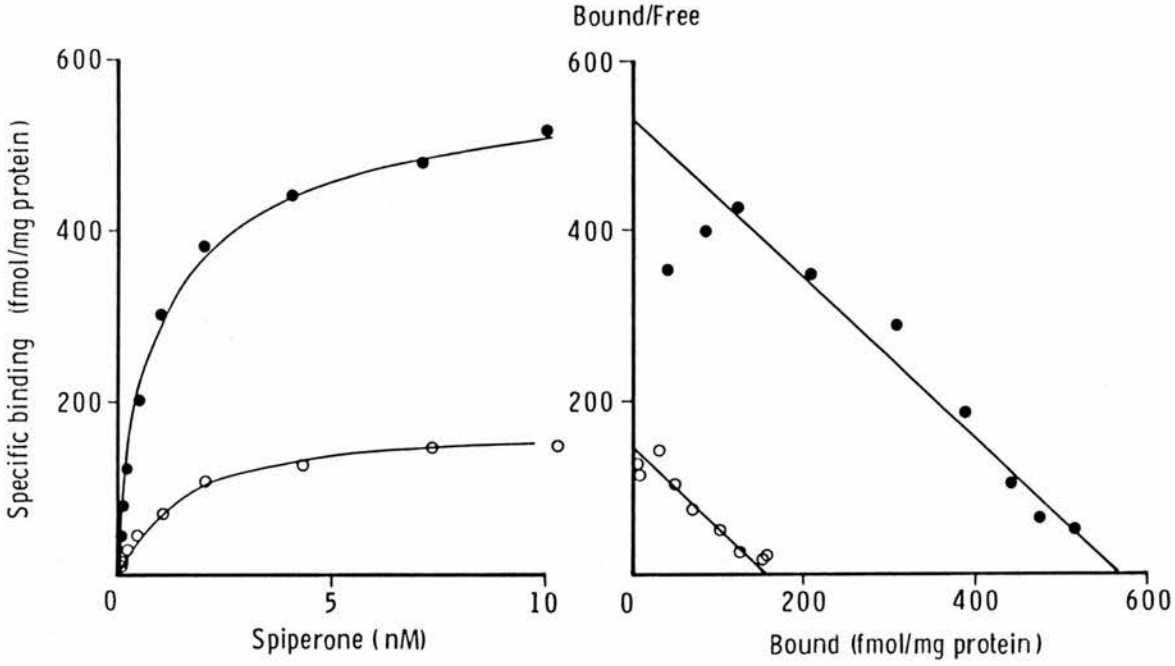
concentrations of potassium. None of the bromocriptine-treated non-functioning tumours showed ultrastructural changes typical of bromocriptine-treated macroprolactinomas (see Chapter 2.C.5). The modest elevation of serum prolactin to between 1000 and 5000 mU/l in 7 patients with non-functioning tumours was considered to be due to hypothalamo-pituitary disconnection.

No tumour in the receptor studies showed any histological or perifusion evidence of contamination with normal anterior pituitary but two showed evidence of minor contamination (<4%) on the basis of hormone content studies (see Figure 7-3 and related text). Major oncocytic change (>10% cells) was found in 5 non-functioning tumours, 3 of which had been exposed to bromocriptine. A variable pattern of gonadotrophin immunostaining, confined to <10% cells in each case, was present in 7 non-functioning tumours.

B. HIGH AND LOW AFFINITY DOPAMINE RECEPTORS

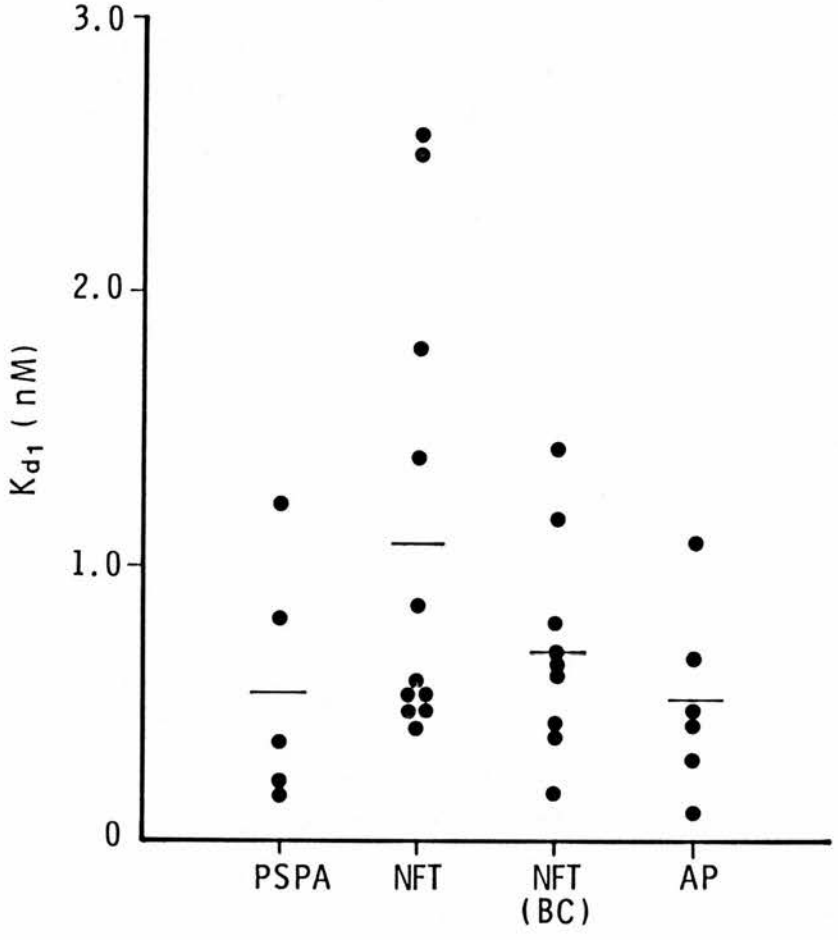
All tissues studied showed high affinity dopaminergic binding sites defined by [^{3}H]spiperone. A typical binding curve and corresponding Scatchard plot for a prolactinoma and a non-functioning tumour are shown in Figure 8-1. The mean dissociation constant (K_{d1}) for the high affinity dopaminergic binding site was not statistically different between the three tissues studied (prolactinoma, 0.55 nM; non-functioning tumour, 0.92 nM; normal pituitary, 0.51 nM) (Figure 8-2). The mean number of high affinity sites (B_{max1}) was significantly greater ($P < 0.01$) in prolactinomas (698 fmol/mg protein) than in non-functioning tumours (131 fmol/mg protein) or anterior pituitary (136 fmol/mg protein) (Figure 8-3).

Figure 8-1: Direct and Scatchard plots of [^{3}H]spiperone binding to prolactinoma and non-functioning tumour membranes



Direct (left) and Scatchard (right) plots of specific [^{3}H]spiperone binding (0.07 - 10 nM) to membranes from a prolactinoma (closed circles) and a non-functioning tumour (open circles). Specific binding was 94 and 76% of total tissue binding respectively at K_d concentrations. Each point represents the difference between two sets of duplicate tubes, one of which contained 10 µM (+)butaclamol.

Figure 8-2: High affinity dissociation constants

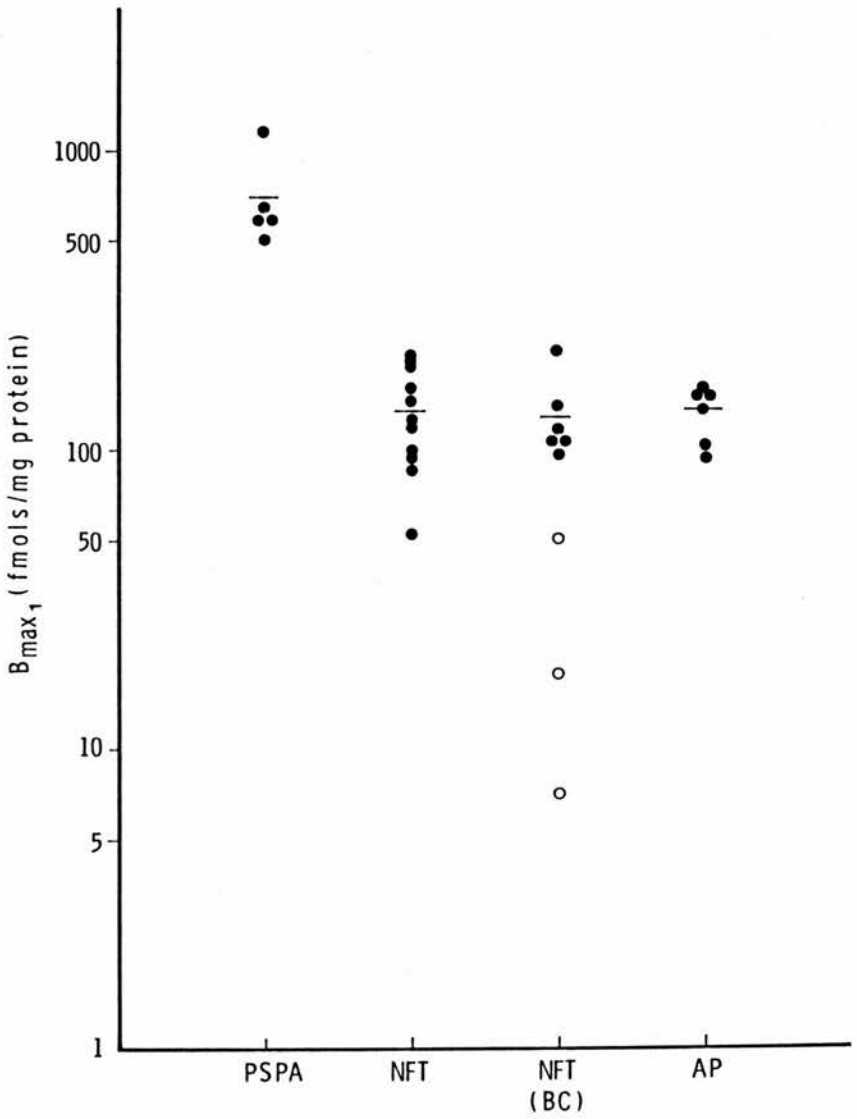


High affinity dissociation constants (K_{d1}) for the tissues studied. Bars indicate the mean values for each group.

PSPA = prolactin-secreting pituitary adenomas
NFT = non-functioning tumours (no bromocriptine)
NFT (BC) = nine NFTs given pre-operative bromocriptine
AP = normal anterior pituitaries (bovine 3, human 3)

There was no significant difference between the groups using the Mann-Whitney U test

Figure 8-3: High affinity binding site numbers



High affinity binding site numbers (B_{max1}) for the tissues studied. Note the logarithmic scale. Abbreviations as for Figure 8-2.

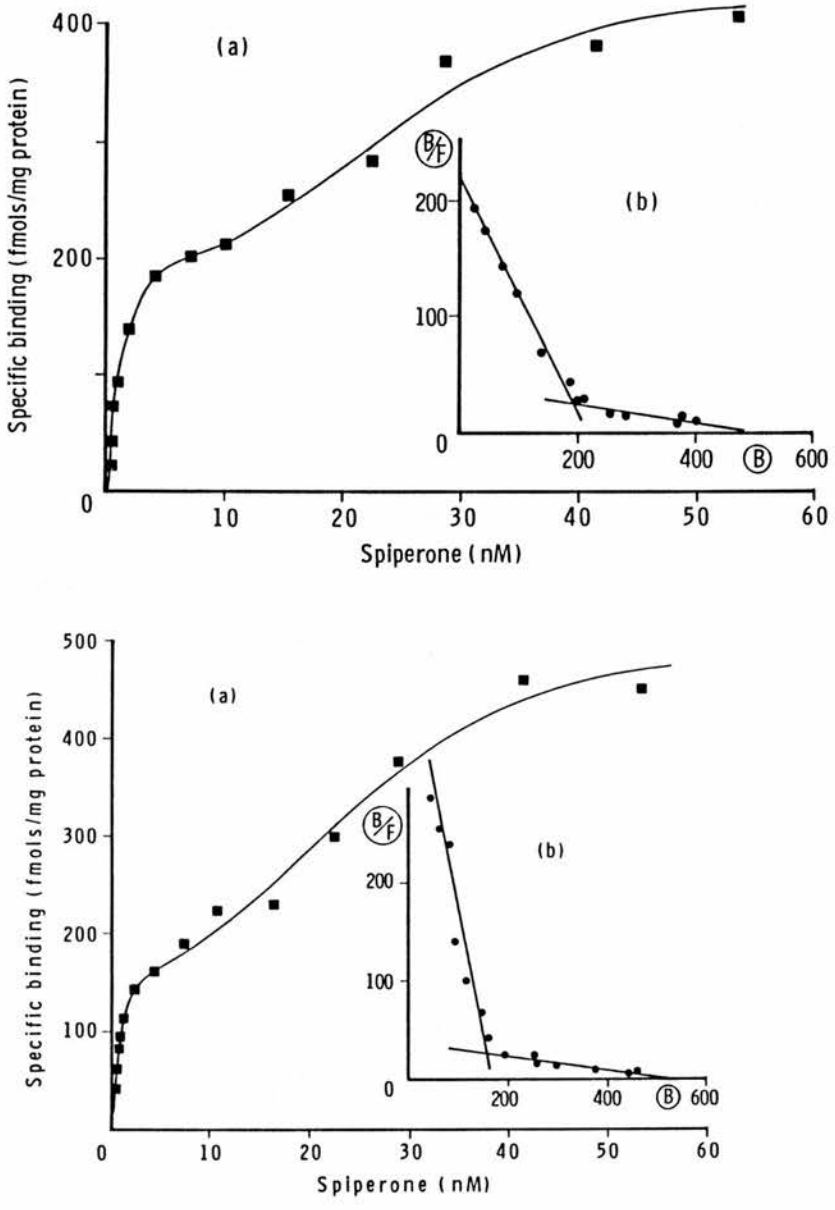
Bars indicate the mean values for each group except for NFT (BC) where the mean does not include the open circles which denote three NFTs exposed to bromocriptine within 24 hours of surgery.

Using the Mann-Whitney U test prolactinomas had significantly higher numbers of high affinity dopaminergic binding sites than the other three tissue groups ($P < 0.01$).

There was no difference in binding characteristics between tumours showing oncocytic change or gonadotrophin immunostaining and the rest of the non-functioning tumours. The binding characteristics of fresh bovine and autopsy human pituitary were virtually identical.

All tissues (except 2 prolactinomas) showed an apparent second dopaminergic binding site of much lower affinity when spiperone concentrations up to 50 nM were used (Figure 8-4). However in a number of cases the low affinity parameters K_{d2} and B_{max2} could not be calculated with confidence and these data will not be further enumerated.

Figure 8-4: High and low affinity binding sites



Upper

(a) Specific [^{3}H]spiperone binding (0.07 - 54 nM) to membranes from non-functioning tumour B9 (Table 8-1)

(b) Scatchard analysis of specific binding showing two sites: K_{d1} 1.2 nM, K_{d2} 22 nM.

Lower

(a) Specific [^{3}H]spiperone binding (0.07 - 54 nM) to bovine anterior pituitary membranes

(b) Scatchard analysis of specific binding showing two sites: K_{d1} 0.46 nM, K_{d2} 20 nM.

B = bound radioactivity (fmol/mg protein);
F = unbound radioactivity (nM).

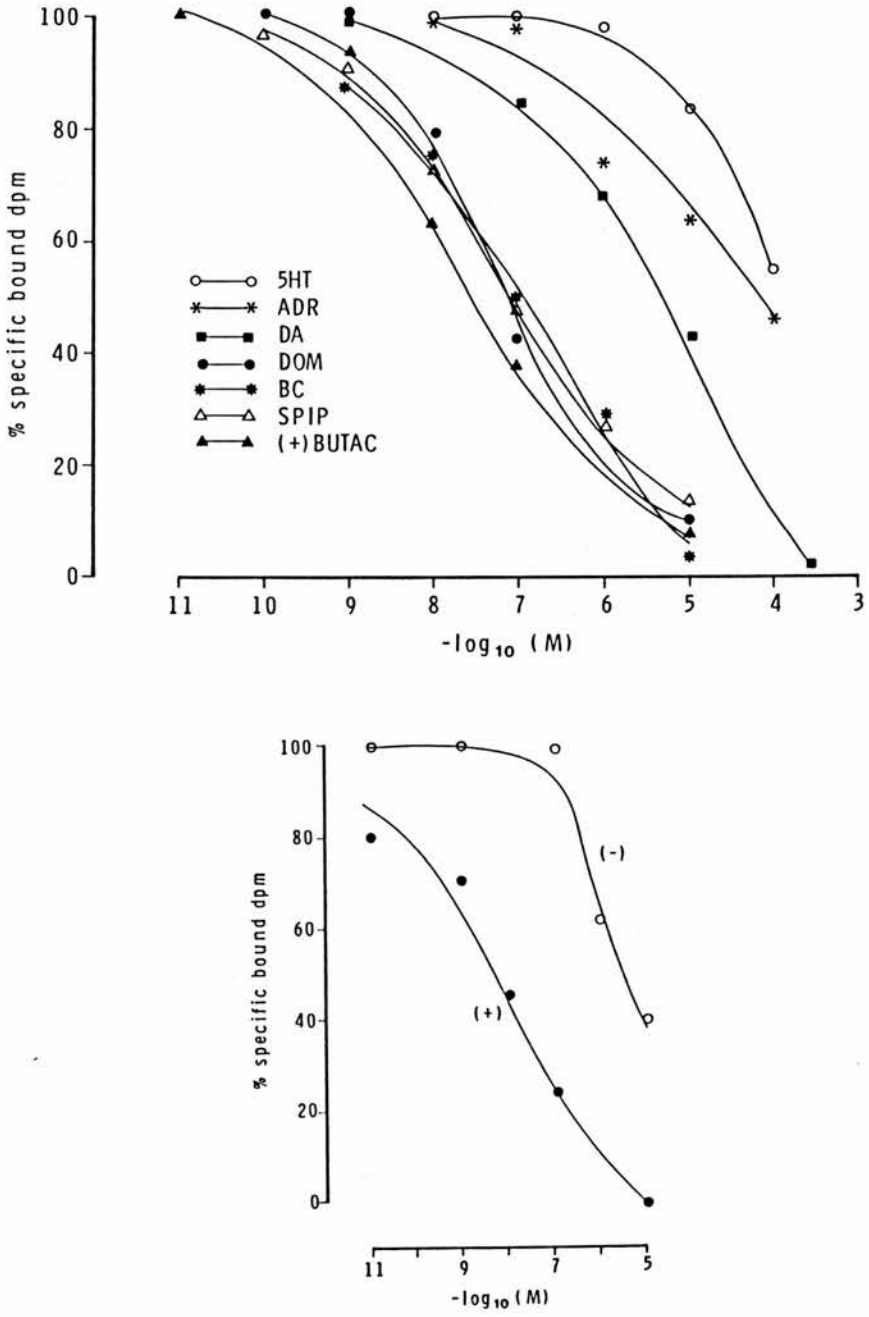
C. SPECIFICITY STUDIES

To further characterise the dopaminergic binding site in non-functioning tumour membranes specificity experiments were carried out on the high affinity site using a pool of membranes prepared from 5 tumours that had not been exposed to bromocriptine. Pargyline (10 μM, Sigma) was included in the membrane buffer to prevent metabolism or binding of dopamine by monoamine oxidase. The competitors displayed a rank order of potency which is typically dopaminergic (Kebabian and Calne, 1979) (Figure 8-5). Virtually identical results were obtained with bovine anterior pituitary membranes (data not shown). Stereospecificity of binding was shown using the isomers of butaclamol; the (-) isomer being 1000x less active than the (+) isomer (Figure 8-5)

D. BROMOCRIPTINE INTERACTION WITH NON-FUNCTIONING TUMOUR DOPAMINE RECEPTORS

B_{max1} for 3 of the bromocriptine-treated non-functioning tumours was less than 50 fmol/mg protein (tumours B1-B3, open circles Figure 8-3). These tumours were from the 3 patients who received the last dose of the drug within 24 hours of surgery. Furthermore, bromocriptine was detectable in the high speed supernatant in these 3 cases only (Figure 8-6). Membranes from cases B1 and B3 were resuspended in buffer and centrifuged three times at 100,000 g; 0.6 and 0.2 pmol bromocriptine per mg membrane protein were recovered in the washings (Figure 8-6). Following this, [^{3}H]spiperone binding significantly increased and B_{max1} values became comparable to those obtained with the other NFTs (Figure 8-6). This suggested that the original B_{max1} values were artifactually low due to bromocriptine occupation of the dopaminergic binding sites.

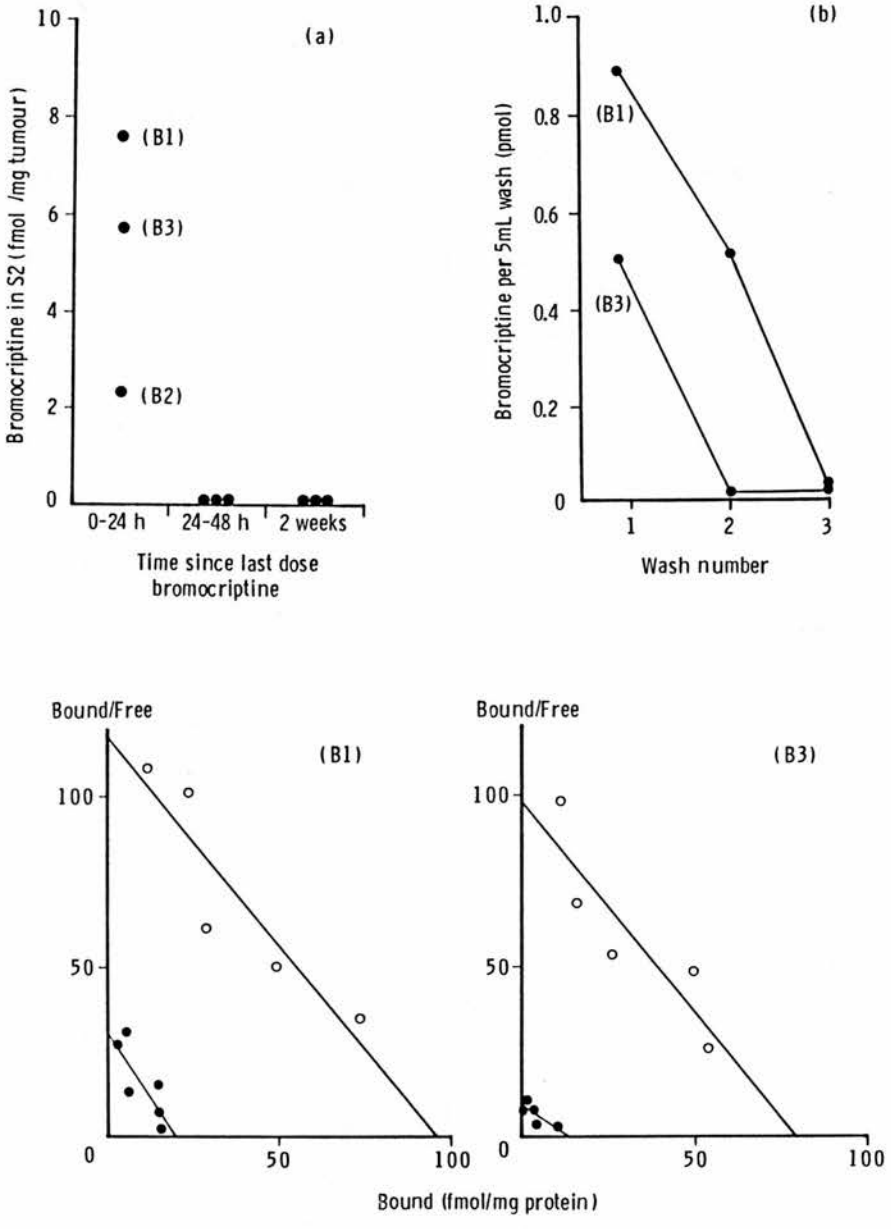
Figure 8-5: Non-functioning tumour competition experiments



Upper
The competition of various agents for specific [^{3}H]spiperone binding (1.8 nM) to a pool of non-functioning tumour membranes (224 µg protein per tube). Each point represents the mean of duplicate tubes. M denotes molar concentration of competitor.
5HT, serotonin; ADR, adrenaline; DA, dopamine; DOM, domperidone; BC, bromocriptine; SPIP, spiperone; (+)BUTAC, (+)butaclamol;

Lower
Stereospecificity of [^{3}H]spiperone binding to non-functioning tumour membranes. Same format as above. (+) = (+)butaclamol;
(-) = (-)butaclamol.

Figure 8-6: Bromocriptine interactions with non-functioning tumour dopamine receptors



Upper

(a) Bromocriptine (BC) in high speed supernatant (S2) of nine non-functioning tumours given pre-operative BC and its relation to the timing of last BC dose (fmol per mg wet weight tumour homogenised)

(b) Bromocriptine removal by washing of membranes from non-functioning tumours B1 and B3 - described in the text.

Lower

Scatchard plots for non-functioning tumour B1 (left panel) and B3 (right panel) before (solid circles) and after (open circles) membrane washing. Open circles represent singlicate determinations due to limited membranes available.

E. DISCUSSION

At the outset of this work there was only one report of dopaminergic binding studies in non-functioning tumours (Bression et al., 1980). These workers were unable to demonstrate saturable dopaminergic binding in two non-functioning tumours using [^{3}H]domperidone at concentrations up to 40 nM, a ligand shown to have a high affinity for the dopamine receptors found in the above studies (Figure 8-5).

During my studies two further reports were published. Serri et al (1984), using enzyme-dispersed tumour cells and [^{3}H]spiperone as radioligand, showed dopamine receptors in two out of four non-functioning tumours. The tumours in this study were not extensively characterised and immunostaining was performed for only prolactin and growth hormone. Furthermore it is unclear whether the two non-functioning tumours apparently without dopamine receptors received pre-operative dopamine agonist treatment which may mask dopamine receptors, as demonstrated above. The mean K_d for the two tumours with dopaminergic binding was 0.67 nM, similar to the values obtained in the above twenty well-characterised non-functioning tumours.

The second report was published in preliminary form by Koga et al (1984) but has not been followed by a substantive publication. Using [^{3}H]spiperone these workers found dopamine receptors in membranes from 13 out of 19 non-functioning adenomas; mean K_d and B_{max} values for these tumours were 2.2 nM and 191 fmol/mg protein, remarkably similar to the values described above. In their abstract there is no indication of how well characterised the tumours were or whether any were exposed to pre-operative dopamine agonists.

The binding parameters obtained for normal bovine anterior pituitary were in excellent agreement with those reported by Cronin and Weiner

(1979). The K_{d1} values for human necropsy pituitary reported here were indistinguishable from bovine tissue although slightly lower than those described for fresh anterior pituitary removed from patients with metastatic cancer (Cronin et al., 1980a). The mean K_{d1} for non-functioning tumours, prolactinomas and normal pituitary were not significantly different in the above studies and competition experiments using bovine anterior pituitary gave identical results to those in Figure 8-5 (data not shown). Thus my studies revealed no binding differences in the high affinity dopamine receptors in any of the four tissues studied.

Using [^{3}H]spiperone concentrations up to 50 nM it was possible to define, in all tissue types, a second dopaminergic binding site of lower affinity, similar to that found in prolactinomas by Bression et al (1980) using [^{3}H]domperidone. Cronin et al (1980a) found only a single class of high affinity sites in prolactinomas and normal pituitary using [^{3}H]spiperone, presumably because the highest concentration used was 6 nM. In separate experiments, not reported above, a similar second site in bovine anterior pituitary membranes was revealed by [^{3}H]dihydroergocriptine at concentrations of up to 50 nM. The significance of this site is unclear. Foord et al (1983) have suggested that it may represent a dopamine uptake pathway; the K_{d2} in the micromolar range and lack of stereospecificity in their intact pituitary cell preparation made it an unlikely site for control of hormone release. The discovery of dopamine associated with the subcellular fraction containing prolactin secretory granules (Nansel et al., 1979) allied with the recent characterisation of high affinity dopamine receptors in the cytosol of bovine anterior pituitary glands (though constituting only 3% of total dopaminergic binding, Kerdelhue et al., 1981) provides evidence suggesting an uptake of dopamine into anterior

pituitary cells.

The situation has been further complicated by the demonstration that the dopamine receptor responsible for inhibition of prolactin secretion in the normal pituitary exists in high and low affinity states (Sibley et al., 1982; De Lean et al., 1982; Sibley and Creese, 1983). Dopamine agonists bind to the receptor in its high affinity form and such binding can be completely abolished by guanine nucleotides (Sibley et al., 1982). It is clear that the functional state of the receptor is in the high affinity form (George et al., 1985). Dopamine antagonists, and paradoxically the ergopeptines, can interact with the receptor in either affinity state (Sibley and Creese, 1982). There remains considerable controversy regarding the precise molecular explanation of these observations but it seems probable that a GTP-sensitive regulatory protein exists which coordinates receptor binding and post-receptor mechanisms, particularly adenylate cyclase activity.

Site numbers for the high affinity site in the five prolactinomas studied were also in close agreement with previously reported values (Cronin et al., 1980a) and significantly higher than in the non-functioning tumour or normal pituitary groups. The difference in site numbers between prolactinomas and normal pituitary might be explained by the fact that immunocytochemically most, but not all, dopamine receptors in the anterior pituitary are related to the lactotroph population (Goldsmith et al., 1979). This constitutes only 10-30% of cells in normal anterior pituitary (Asa and Kovacs, 1982) but greater than 90% in prolactinomas. However the presence of dopamine receptors has been demonstrated or inferred on other normal and neoplastic pituitary cell types (Bression et al., 1982; Marcovitz et al., 1982; Foord et al., 1983; Chanson et al., 1984) and the difference in receptor number may

reflect a greater receptor density on the tumourous lactotrophs.

It is still uncertain which cell type is represented in non-functioning tumours; it may be an undifferentiated progenitor cell or alternatively a dedifferentiated cell derived from a mature cell type (Kovacs et al., 1980). The receptor data do not resolve this question. There was a striking similarity in high affinity site numbers between non-functioning tumours and normal anterior pituitary which suggests that the dopamine receptor might be a feature common to several pituitary cell types.

None of the non-functioning tumours regressed during bromocriptine therapy. The patients treated to within twenty-four hours of surgery provided evidence that the drug bound to non-functioning tumour dopamine receptors in-vivo but this binding did not result in shrinkage. B_{max1} values after membrane washing increased to within the range of the untreated group of non-functioning tumours which showed that chronic bromocriptine therapy had not altered the number of dopamine receptors. Furthermore, non-functioning tumours which had last been exposed to bromocriptine between one and fourteen days before surgery had similar B_{max1} values to the untreated non-functioning tumours.

Similar studies with normal pituitary and prolactinoma tissue exposed to bromocriptine in-vivo would be necessary to confirm the significance of these results. The work reported by Di Paulo and Falardeau (1984) is of considerable interest. These workers induced prolactin-secreting tumours in rats using oestradiol valerate and treated the rats with sub-cutaneous bromocriptine for one month. On a weight basis the rats received a daily dose of bromocriptine 50 times greater than a typical dose used to shrink human prolactinomas (7.5 mg daily). Tumours from rats sacrificed 60 hours after the last dose of bromocriptine showed a

70% apparent reduction in dopaminergic binding sites (using [^{3}H]spiperone) and the authors concluded that chronic bromocriptine had reduced dopamine receptor number. An alternative explanation could be that the massive sub-cutaneous doses of bromocriptine acted as a drug reservoir and that the apparent fall in dopamine receptor number was due to continuing bromocriptine receptor action and occupancy; plasma prolactin concentrations were still fully suppressed at the time of death.

What prevents dopamine agonist induced tumour regression in patients with non-functioning tumours ? Perhaps the dopamine receptors are functionally defective in a analogous way to the rat lactotroph clone 7315a which has apparently normal dopamine receptors but is refractory to dopamine inhibition of prolactin release in-vitro (Cronin et al., 1981; Judd et al., 1985). Another possibility is that non-functioning tumour dopamine receptor density is not great enough to facilitate shrinkage. Perhaps the most likely explanation is that since non-functioning tumours appear to be largely quiescent from the secretion point of view there is no hormone product to be inhibited by receptor activation, with subsequent feedback on nucleic acid and protein synthesis, as is thought to occur in prolactinomas (Brocas et al., 1982; Bassetti et al., 1984). These possible explanations are discussed in the final chapter.

CHAPTER 9. SOME STUDIES OF TWO TSH-SECRETING MACROADENOMAS

A. INTRODUCTION

Only 52 TSH-secreting tumours have been described to date and few have been subjected to detailed in-vitro investigation. This short chapter describes in-vitro studies of two further tumours and correlates the results with the in-vivo TSH responses to TRH and dopaminergic manipulation. These results are included in this thesis since they provide an interesting contrast to the previous sections on TRH/dopamine interactions in the tumourous lactotroph (Chapter 6.B) and dopamine receptors in non-functioning pituitary tumours (Chapter 8).

The TRH responsiveness of the normal thyrotroph is inhibited by dopamine in-vitro (Foord et al., 1981) and in-vivo (Connell et al., 1985). In contrast, most patients with TSH-secreting adenomas do not respond to TRH, and of the small number investigated nearly all have proved resistant to dopaminergic suppression of serum TSH concentrations (Faglia, 1984). Dopamine receptors were present in membranes from the only tumour so investigated, but this patient showed a paradoxical increase in serum TSH following L-Dopa (Chanson et al., 1984).

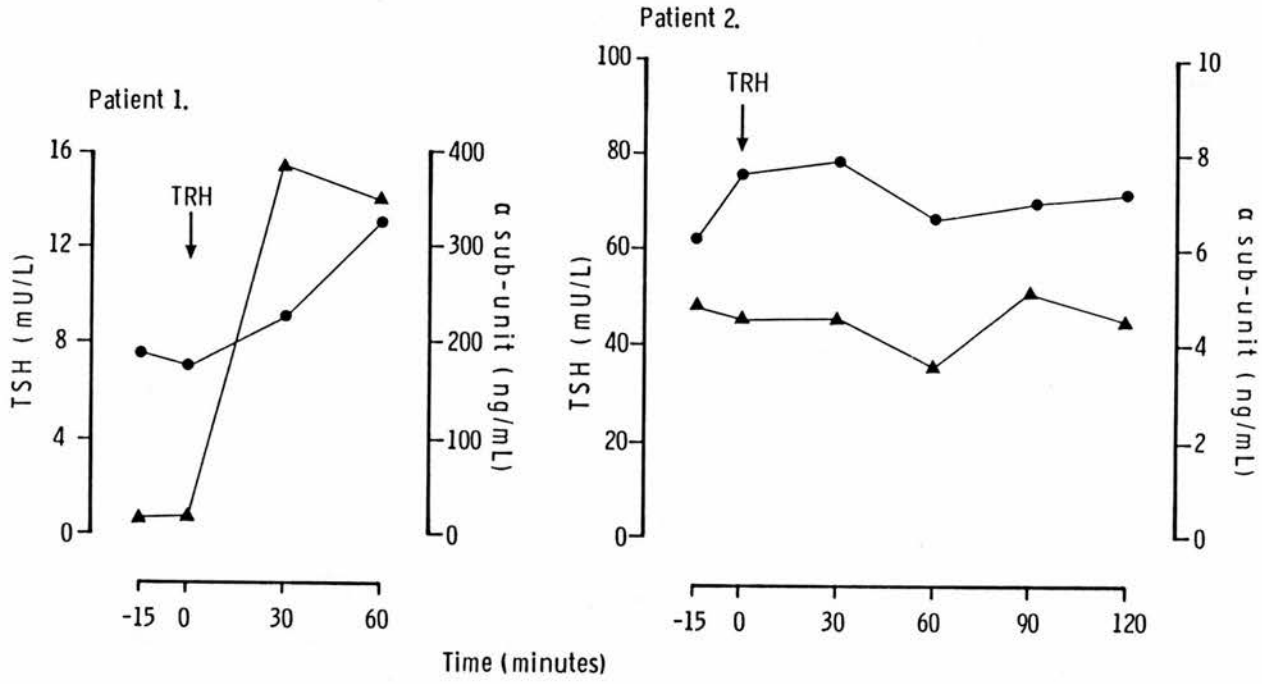
B. IN-VIVO STUDIES

Both patients underwent transsphenoidal removal of macroadenomas in which 25-50% of cells immunostained for TSH and alpha subunit.

Patient 1 was thyrotoxic on presentation and had slightly elevated serum TSH concentrations of 7-8 mU/L (normal <5). Serum alpha subunit was substantially raised (10.4 ng/mL), as was the alpha subunit/TSH molar ratio (4.8). There was a small but significant increase in TSH and a marked rise in alpha subunit following TRH in-vivo (Figure 9-1). Neither domperidone - a D2 dopamine antagonist - nor bromocriptine affected serum TSH concentrations (Figures 9-2 and 9-3)

Patient 2 had received radioiodine in the past for presumed Graves disease and off thyroxine treatment had greatly elevated serum TSH concentrations of 41-77 mU/L in the presence of low-normal thyroid hormone concentrations. Serum alpha subunit was only slightly raised (4.5 ng/mL) and the molar ratio was normal (0.27). There was no increase in serum TSH or alpha subunit following TRH (Figure 9-1), neither did dopaminergic manipulation affect serum TSH concentrations (Figures 9-2 and 9-3).

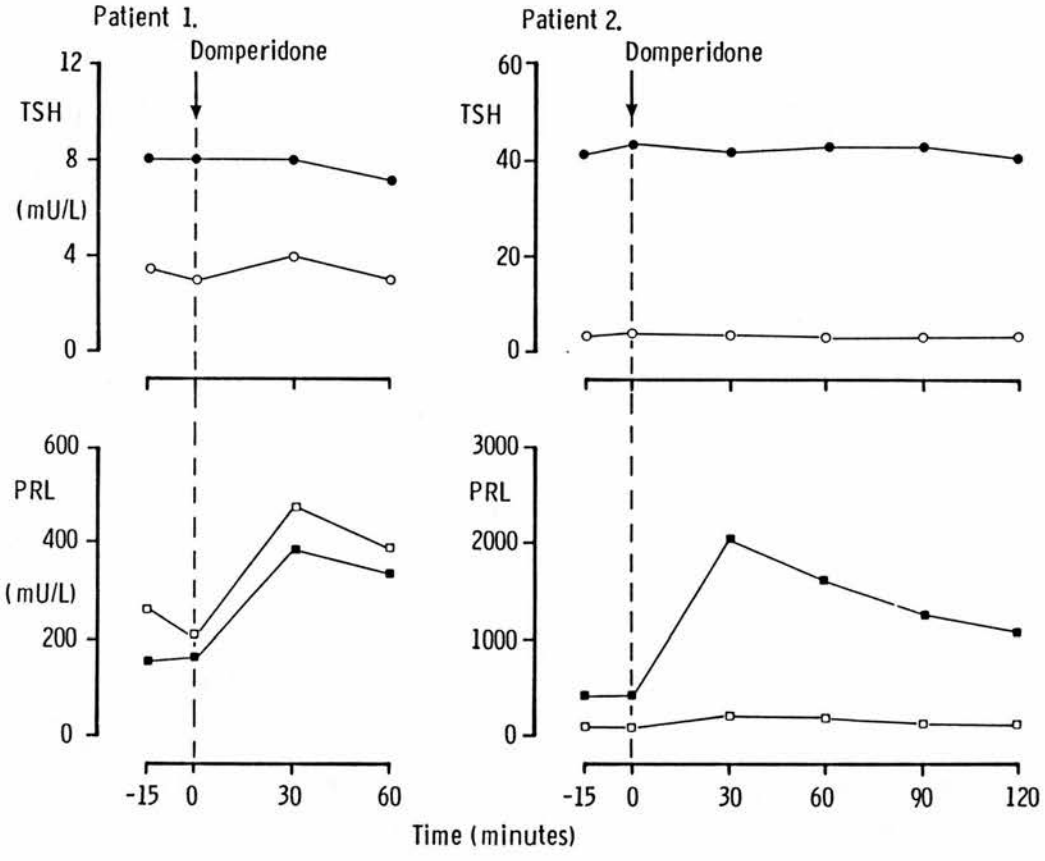
Figure 9-1: TSH-secreting adenomas: serum TSH and alpha subunit responses to TRH



In-vivo responses of serum TSH (●) and alpha subunit (▲) concentrations to 200 µg TRH (iv) in the two patients.

Only patient 1 showed significant hormone release.

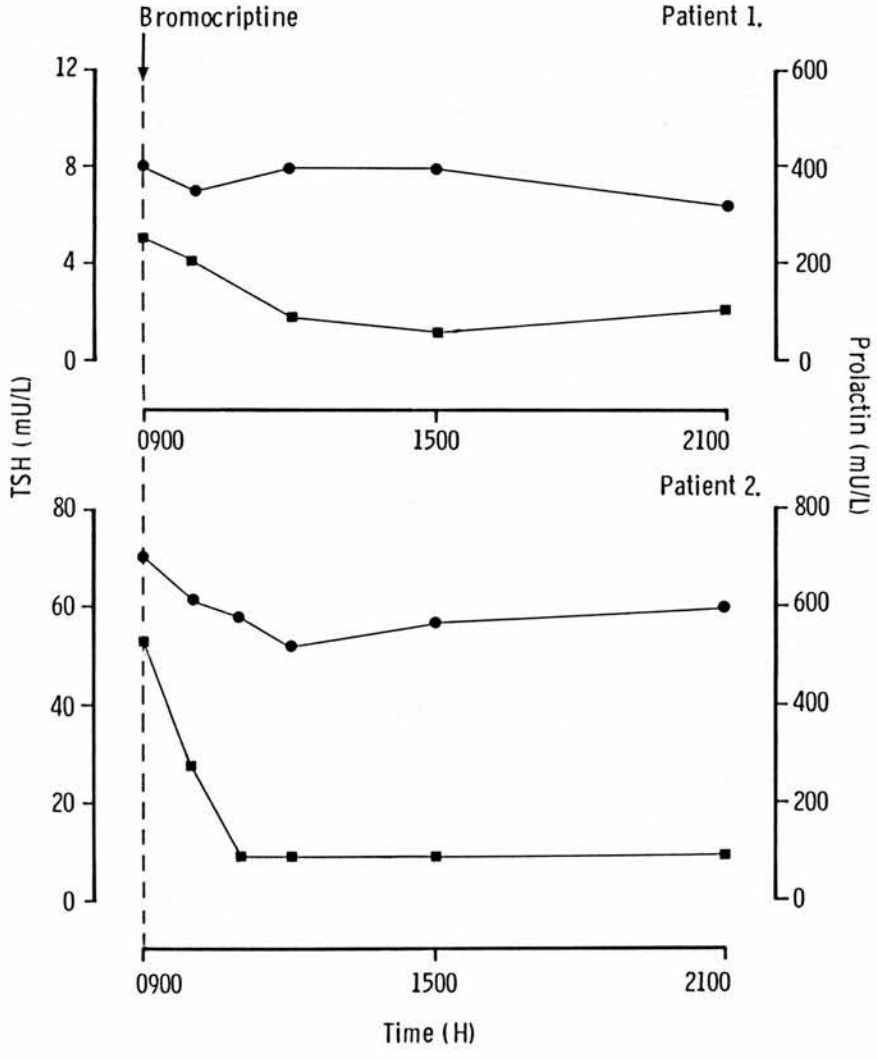
Figure 9-2: TSH-secreting adenomas: in-vivo responses to dopamine antagonism



In-vivo responses of serum TSH (circles) and prolactin (squares) concentrations to 10 mg domperidone (iv) before (solid symbols) and after (open symbols) surgery, in the two patients.

Before surgery, neither patient showed a significant increase in TSH but both showed some increase in prolactin.

Figure 9-3: TSH-secreting adenomas: in-vivo responses to bromocriptine



In-vivo responses of serum TSH (●) and prolactin (■) concentrations to 2.5 mg bromocriptine in the two patients.

Each showed significant suppression of prolactin but not TSH.

C. IN-VITRO STUDIES

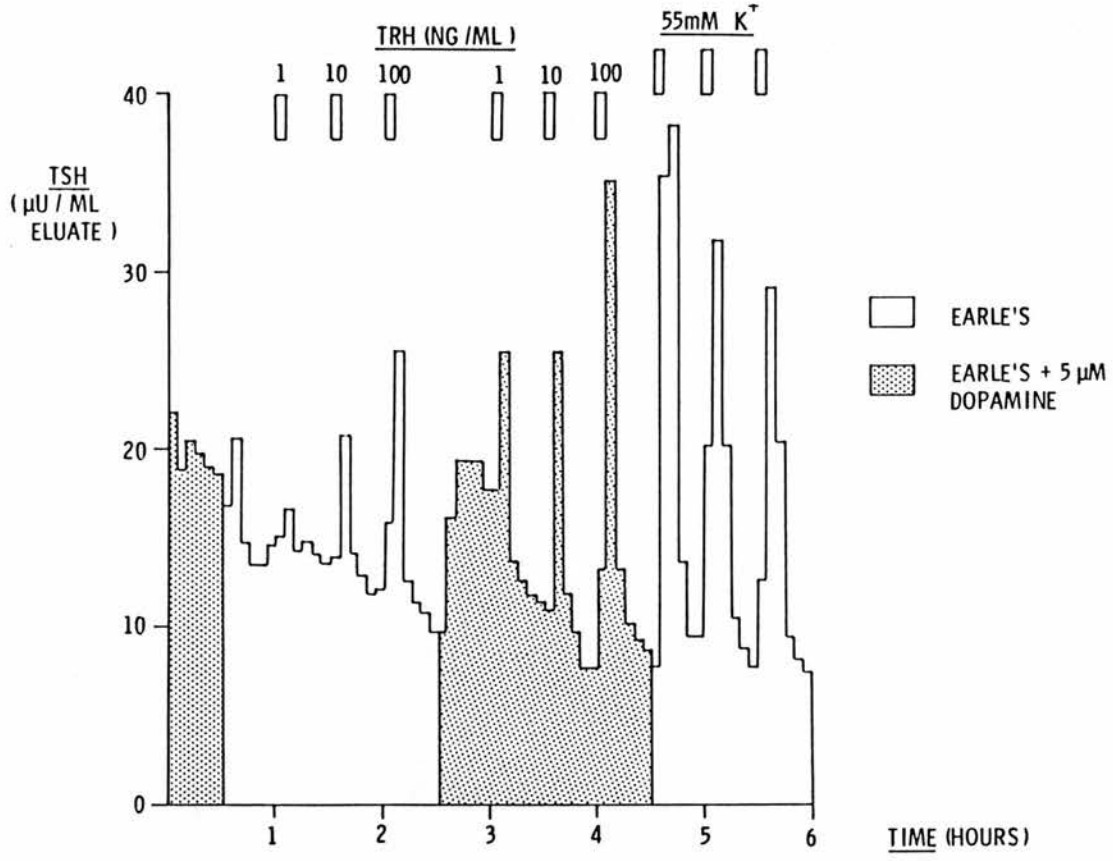
The in-vitro TSH responses were studied using the tumour cell perifusion system and there was complete correspondence between the in-vivo and in-vitro results.

The tumour from patient 1 showed dose-dependent TSH release following TRH stimulation but this could not be inhibited by dopamine concentrations as high as 5 μM (Figure 9-4), quite unlike the responses expected from the normal thyrotroph (Foord et al., 1981). The second tumour showed no TRH-induced TSH release in-vitro or in-vivo.

Both tumours were completely resistant to the actions of dopamine and bromocriptine, even at the high concentrations of 5 μM and 10 nmol/L respectively (Figures 9-5). In contrast, the lactotrophs in the non-adenomatous pituitary showed normal dopaminergic responsiveness as indicated by the in-vivo tests (Figures 9-2 and 9-3).

Membranes were prepared from each tumour for dopamine receptor measurement. Neither tumour showed significant numbers of dopaminergic binding sites as defined by [^{3}H]spiperone. The first tumour had no discernible binding and the other, which had shown possible anterior pituitary contamination of up to 20% from hormone content studies (See Figure 7-3), had ill-defined binding of, at most, 20 fmol/mg protein. A prolactinoma control in the same assay gave a B_{max} value of 318 fmol/mg protein.

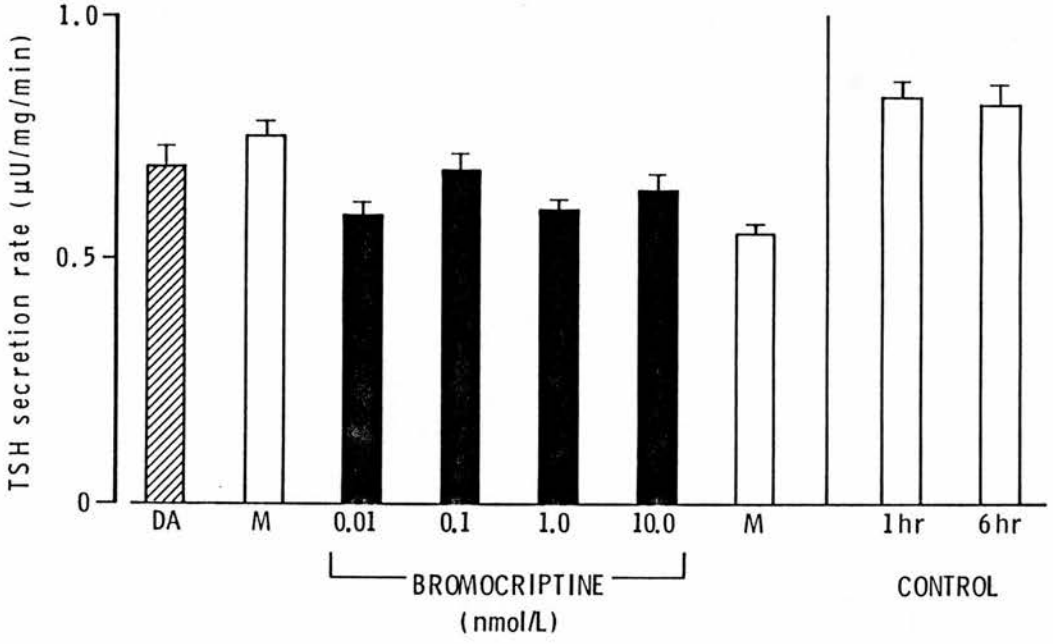
Figure 9-4: TSH-secreting adenoma: in-vitro responses to TRH and dopamine



Perifusion of tumour cells (3 x 10^6 cells) from patient 1.
The TSH responses to TRH (1-100 ng/mL) were not reduced in the presence of 5 µM dopamine.

(Tumour cells from patient 2 were unresponsive to TRH in-vitro - data not shown).

Figure 9-5: TSH-secreting adenoma: in-vitro responses to bromocriptine



Tumour fragment perifusion from patient 2 (50 mg per column). DA = 5 μM dopamine, M = dopamine-free medium, bromocriptine concentrations as shown.

The experimental design was the same as that used for the prolactinomas (for example, see Figure 6-3). Each bar represents the mean of the last 6 fractions of each treatment period (+ SEM). A separate control column is shown on the right of the figure.

There was no significant inhibition of TSH secretion by bromocriptine.

D. DISCUSSION

The TSH-secreting adenomas provided an interesting comparison with the other tumours in this thesis. Most of the prolactinomas showed TRH-induced prolactin release which was abolished by 5 μM dopamine (Chapter 6.B). The first TSH-secreting adenoma showed TRH-induced TSH release which could not be blocked by dopamine suggesting that a dopamine receptor or post-receptor defect prevented the inhibition of apparently normal TRH action. The second showed apparent defects in both TRH and dopaminergic mechanisms. Most of the previously described tumours have been resistant to dopamine suppression of TSH (Faglia, 1984), but in-vitro TRH/dopamine interactions have not been studied before.

The radioreceptor studies suggested that the dopaminergic resistance of both tumours was due to deficiency of membrane-bound dopamine receptors. Since normal thyrotrophs possess such receptors (Foord et al., 1983), perhaps these had been lost during neoplastic transformation. Only one previous TSH-secreting adenoma has had dopamine receptor measurements (Chanson et al., 1984). Although this tumour contained dopaminergic binding sites, it also showed paradoxical stimulation of TSH by L-Dopa, and may not be typical of most TSH-secreting adenomas. Of the 21 previous patients with TSH-secreting tumours challenged with L-Dopa or bromocriptine, 18 (86%) showed no change in serum TSH concentrations and may have lacked dopamine receptors. No TSH-secreting tumour has been shown to shrink during bromocriptine treatment.

Dopaminergic resistance in the TSH-secreting adenomas was therefore due to a lack of dopamine receptors, in contrast to the non-functioning tumours where it occurred despite the presence of such receptors in tumour membranes (Chapter 8).

CHAPTER 10. CONCLUSIONS

The conclusions to this thesis are in three main sections. Firstly I shall seek to summarise the guidelines for the management of patients with large pituitary tumours which resulted from the clinical studies, and set these in the context of recent publications from other centres. Secondly I shall discuss the significance of dopamine receptors in pituitary tumours and possible mechanisms of bromocriptine-induced tumour regression. Lastly, the finding of dopamine receptors in non-functioning pituitary tumours suggested a number of areas for future research, both to define intracellular mechanisms in these tumours and to further characterise the cell type represented.

A. GUIDELINES FOR THE MANAGEMENT OF PITUITARY MACROADENOMAS

It is now clear that only large prolactin-secreting pituitary tumours regress during dopamine agonist therapy. It is therefore important to be confident of that diagnosis before embarking on a course of dopamine agonist therapy in a patient with a large pituitary tumour and visual failure. The results in Chapter 2 show that a large pituitary tumour associated with a serum prolactin concentration of less than 3000 mU/L is unlikely to be prolactin-secreting or to shrink during dopamine agonist therapy. Over the past 3 years the results of dopamine agonist therapy in a total of 60 non-functioning tumours have been reported (Barrow et al., 1984; Pullan et al., 1985; Verde et al., 1985; Grossman et al., 1985b; Zarate et al., 1985; Bevan et al., 1987b). Of these 60 tumours, only 3 showed evidence of tumour regression and the diagnosis of non-functioning tumour has been confirmed in surgical biopsies, usually supplemented with immunocytochemistry. These tumours have been studied mostly by third and fourth generation computerised tomography, in contrast to some of the earlier studies which suggested

that some non-functioning tumours might regress during dopamine agonist therapy (Wolleson et al., 1982). Of the 60 recently reported cases, 41 received bromocriptine in a dose of at least 7.5 mg daily for 3 months or longer; treatment in 10 cases was continued for over 1 year. At least one of the 3 patients with tumours that shrank had a history suggestive of pituitary apoplexy (Grossman et al., 1985b), and in view of the frequency of subclinical pituitary tumour haemorrhage (Wakai et al., 1981), it is conceivable that this was also the explanation in the other two. Importantly 4 of the tumours described by Verde et al (1985) continued to enlarge during bromocriptine treatment.

Most patients with non-functioning tumours can be treated successfully with transsphenoidal tumour decompression, which is the initial treatment of choice (Chapter 2). Furthermore, this approach will reveal non-adenomatous pathology masquerading as a pituitary adenoma.

A large pituitary lesion associated with a serum prolactin of more than 8000 mU/L is virtually certain to be a macroprolactinoma and even above 5000 mU/L there is little risk of misdiagnosis. Most macroprolactinomas can be successfully reduced in size using dopamine agonist therapy which is appropriate initial management. Very few patients seem to develop resistance to the bromocriptine, but perhaps this will increase with longer treatment periods. Furthermore, once the initial response has been achieved, control of tumour size and serum prolactin concentration can be maintained in many patients with a greatly reduced dose of dopamine agonist; Liuzzi et al (1985) found that the bromocriptine dose could be reduced as low as 0.625 mg daily.

However a number of dilemmas remain. The most important is the continuing uncertainty regarding the efficacy of longterm medical therapy alone. Withdrawal of dopamine agonist therapy, even after 7-8

years of treatment, invariably results in the prompt return of hyperprolactinaemia (Johnston et al., 1984; Liuzzi et al., 1985). This must signify the continuing presence of tumourous lactotrophs and a failure of dopamine agonist therapy to cure the prolactinoma, at least when given for this length of time. Immediate tumour re-expansion on drug withdrawal may occur even after one year of therapy (Molitch et al., 1985), although after more prolonged treatment such early re-expansion seems uncommon (Johnston et al., 1984). In view of the bromocriptine-induced fibrosis demonstrated in Chapter 2 this latter finding is not unexpected, and the return of hyperprolactinaemia suggests that tumour enlargement would occur in the longterm. Longterm withdrawal studies are needed to resolve this issue.

A second problem is that a proportion of macroprolactinomas do not reduce in size during dopamine agonist therapy. Benker et al (1986) reviewed 10 major series comprising a total of 274 patients and found that 30% of macroprolactinomas did not regress during therapy. A proportion of these non-responders were probably misclassified non-functioning tumours with disconnection hyperprolactinaemia, nevertheless some which are definitely macroprolactinomas do not shrink. The mechanism of this is obscure, since a large proportion of non-shrinkers show marked suppression of serum prolactin concentrations (Liuzzi et al., 1985). In some cases cystic change or tumour haemorrhage may obscure the response of the tumourous lactotrophs. In others the pre-treatment tumour size is probably important, radiological size change being less easily detected in intermediate sized tumours (10-20 mm) than in the very large tumours of greater than 20 mm diameter. Fahlbusch and Buchfelder (1986) reported that 96% of 24 large prolactinomas (>20 mm diameter) shrank during bromocriptine therapy, which was also my experience with a smaller series of 12 large macroprolactinomas, all of

which shrank by at least 25%.

The timing and nature of subsequent definitive therapy remains controversial. Some recommendations can be made from the clinical studies in Chapter 2. Firstly there is no doubt that bromocriptine treatment of macroprolactinomas for longer than 8-10 weeks causes tumour fibrosis which makes subsequent surgery unproductive and even dangerous. Should surgery be used at all in patients with bromocriptine-treated macroprolactinomas? If compact shrinkage has occurred after 4 weeks of medical treatment then it would seem reasonable to attempt surgical cure of the tumour. If the shrinkage has been asymmetrical or if dopamine agonist therapy has been given for longer than 3 months then surgery is contraindicated and external radiotherapy is more appropriate as definitive treatment. Of course, bromocriptine alone can be given to younger patients with fertility needs provided the tumour shrinks well away from the optic chiasm; but prolonged dopamine agonist therapy precludes the option of future surgery. If a macroprolactinoma does not shrink or if resistance develops during prolonged bromocriptine therapy does this mean that surgery is contraindicated? The answer to this question is unknown. Prolactinoma re-expansion during bromocriptine treatment is rare but there seemed to be little operative difficulty in the tumour described by Breidahl et al (1983). Tumours without radiological evidence of shrinkage may nevertheless show lactotroph cell size reduction (Nissim et al., 1982), and presumably may develop fibrosis, so it is probably safer to avoid surgery in all patients with large prolactinomas who have received dopamine agonist therapy for longer than 3 months.

Large pituitary lesions associated with serum prolactin concentrations between 3000 and 5000 (perhaps 8000) mU/L may or may not be pituitary adenomas and may or may not be prolactin-secreting (Bevan et al.,

1987a). The best approach is to treat such cases surgically as this provides both effective decompression and a histological diagnosis. An alternative approach is to use the radiological response of the lesion to bromocriptine as a diagnostic test. However, if the lesion does not shrink it may be impossible to distinguish the various diagnostic possibilities since the serum prolactin will probably suppress whatever the aetiology.

Despite the development of several alternative dopamine agonists, bromocriptine remains the mostly widely used and studied dopamine agonist. Recently a depot preparation of bromocriptine in polylactic microspheres has become available. A single intramuscular injection of 50 mg suppresses prolactin secretion in normal volunteers and patients with prolactinomas for 4 to 6 weeks (Benker et al., 1986; Grossman et al., 1986). It has been suggested that this formulation of the drug might be a useful way of initiating treatment in patients with macroprolactinomas (Grossman et al., 1986), but its duration of action seems to be somewhat variable, lasting for only 2 weeks in some patients (Benker et al., 1986). It would be of interest to use the sensitive bromocriptine assay described in this thesis to measure plasma levels of active non-metabolised bromocriptine following such an injection, and to correlate these levels with the slow rise in serum prolactin which occurs after 2 to 4 weeks.

B. DOPAMINE RECEPTORS AND PITUITARY TUMOUR REGRESSION

The evidence presented in this thesis is consistent with the view that the effects of bromocriptine on lactotroph prolactin secretion and cell size reduction are mediated via membrane-bound dopamine receptors. However the work described in Chapter 8 demonstrates that the presence of dopamine receptors in pituitary tumour membranes does not necessarily imply that a tumour will regress during dopamine agonist therapy.

In 1982 when this work started the mechanism of bromocriptine-induced tumour shrinkage was unknown. Considerable progress has been made over the past 4 years towards understanding it, although the precise intracellular mechanisms remain unclear. In order to speculate on possible reasons for the varying responses of different pituitary tumour types to bromocriptine I shall review the recent evidence pertaining to the actions of the drug on the tumourous lactotroph.

The inhibitory actions of the drug on DNA synthesis and mitosis in the normal pituitary were well known (Lloyd et al., 1975) but seemed hardly relevant to the rapid shrinkage of slow growing pituitary adenomas. It seemed likely that most progress was going to be made from morphological studies of tumour tissue removed from patients given pre-operative bromocriptine, and from experiments on human prolactinoma cells in-vitro.

The morphological studies have shown that early reduction in tumour size is due to diminution in lactotroph cell size, particularly the cell cytoplasm (Tindall et al., 1982; Nissim et al., 1982; Bassetti et al., 1984; Barrow et al., 1984; Esiri et al., 1986). Ultrastructural studies have demonstrated a rapid involution of rough endoplasmic reticulum and Golgi apparatus (Landolt et al., 1985), which is reversible (Landolt et al., 1983), and to my knowledge, without parallel in any other drug-cell

interaction. What is the mechanism of this dismantling of the protein synthetic machinery in the tumourous lactotroph?

In bromocriptine-responsive macroprolactinoma patients serum prolactin levels fall to 25% of the pre-treatment values 6 hours after the first tablet (Chapters 2 and 3). This rapid effect on prolactin secretion has been similarly demonstrated in-vitro, and after 24 hours exposure to bromocriptine tumourous lactotrophs show a reduction in exocytosis and an intracellular accumulation of prolactin-immunoreactive granules, but no reduction in cell size (Hassoun et al., 1985). It seems reasonable to propose that the initial event in bromocriptine action is the inhibition of prolactin release.

There is still controversy regarding the precise interaction of the intracellular messengers responsible for this inhibition. The dopamine receptor in normal and tumourous lactotrophs is negatively coupled with adenylate cyclase and reduction in intracellular cAMP levels is an important mechanism whereby dopamine and bromocriptine inhibit hormone release (Spada et al., 1983; Enjalbert and Bockaert, 1983; McDonald et al., 1984). Conversely, compounds which stimulate or simulate adenylate cyclase activity promote lactotroph prolactin secretion (Spada et al., 1983; Ishibashi and Yamaji, 1985; Cronin et al., 1985). The GTP sensitivity of dopamine agonist binding to anterior pituitary membranes is analogous to the guanine nucleotide dependency of several other adenylate cyclase-linked receptor systems (Rodbell, 1980).

Other regulators are also involved. Calcium has an important role in prolactin secretion (Ishibashi and Yamaji, 1985) and, in normal animal pituitary, dopamine inhibits phosphatidylinositol turnover which in turn regulates intracellular calcium mobilisation and protein kinase C activity (Canonico et al., 1983). Exactly how these intracellular

messengers **interact** with cAMP in the control of prolactin secretion in the tumourous lactotroph is still not well understood.

Accepting that a reduction in intracellular cAMP concentration is an important component of bromocriptine inhibition of prolactin secretion, how does this relate to subsequent changes in lactotroph cell size? In 1981 Brocas et al showed that bromocriptine administration to rats produced rapid suppression of serum prolactin concentrations (undetectable at 24 hours) followed by a delayed effect on prolactin and prolactin mRNA synthesis (50% reduction after 3 days). Furthermore ergot-induced inhibition of prolactin mRNA synthesis could be reversed using long-acting analogues of cAMP (Maurer, 1981). It is likely that bromocriptine-lowered cAMP levels have the immediate effect of preventing prolactin release and the later effect of reducing gene transcription and prolactin synthesis. Whether prolactin itself is able to exert negative feedback control on the transcription of its mRNA remains unknown. Using biopsy material from prolactinoma patients given short-term pre-operative bromocriptine, Landolt et al (1985) have recently estimated a half-life of 3 days for the removal of lactotroph rough endoplasmic reticulum and Golgi apparatus. Presumably it involves a reduction in ribosomal protein and RNA synthesis but the cause of this phenomenon and whether it is **directly** related to the inhibition of prolactin secretion remains obscure. As discussed above serum prolactin levels may be suppressed in some patients without tumour shrinkage, though the converse is rarely true.

Bromocriptine not only reduces the synthesis and release of prolactin but accelerates its lysosomal degradation within the lactotroph. De Marco et al (1984a) have shown that the drug greatly increases lysosomal acid prolactin proteolytic activity within 24 hours of administration.

This activity is probably due to the concerted actions of the cysteine proteases cathepsins B and D. The overall increase in cellular lysosomal activity with increased autophagic vacuole formation may be one of the mechanisms responsible for the removal of rough endoplasmic reticulum and Golgi. Again the intracellular signal for these changes is unknown.

The occasional "complete disappearance" of giant prolactinomas during prolonged bromocriptine suggests that the drug may be cytocidal to some tumours (Gen et al., 1984; Clayton et al., 1985). However hyperprolactinaemia returned after drug withdrawal even in these cases (Gen et al., 1984) suggesting that at least some lactotrophs had escaped. The inhibitory effects of bromocriptine on lactotroph DNA synthesis are presumably responsible for the cytostatic effects of the drug demonstrated by Arafah et al (1983) using human prolactinoma cells growing in soft agar. Significantly these workers were unable to demonstrate a similar effect on cells from GH, ACTH, FSH or non-functioning tumours.

The studies in Chapter 8 clearly demonstrated that non-functioning pituitary tumours do not shrink during bromocriptine therapy despite the possession of dopamine receptors which bind the drug. What is the explanation for this apparent discrepancy?

The relatively low secretory activity of these cells may be responsible; if no secretory product accumulates within the tumour cells following bromocriptine treatment then perhaps feedback inhibition on nucleic acid and protein synthesis cannot occur. Against this is the observation that GH-secreting tumours rarely shrink and do not show cell size reduction during dopamine agonist therapy, despite the frequent suppression of serum GH concentrations (Oppizzi et al., 1984). Little is known about the actions of dopamine agonists on true gonadotrophinomas; in the

single case reported by Berezin et al (1984) serum FSH concentrations were reduced by bromocriptine but tumour shrinkage did not occur.

Another possible explanation for the failure of pituitary tumours other than prolactinomas to shrink is that the density of membrane-bound dopamine receptors is too low. In the studies in Chapter 8 the number of dopaminergic binding sites in prolactinoma membranes was approximately five times that in non-functioning tumour membranes. The two non-functioning tumours reported by Serri et al (1984) had similar numbers of dopaminergic binding sites to four GH-secreting tumours. Cronin et al (1980a) reported that site numbers for GH-secreting tumours and normal anterior pituitary were similar but that not all GH-secreting adenomas contained dopamine receptors. The two TSH-secreting tumours in Chapter 9 were unresponsive to dopaminergic manipulation in-vivo and in-vitro. These two tumours lacked the dopamine receptors present in non-tumourous thyrotroph membranes (Foord et al., 1983), and presumably would not have regressed during bromocriptine therapy had this been tested.

The localisation of dopamine receptors within non-functioning tumours would be of interest. For example, similar methodology to that described by Goldsmith et al (1979) employing a histochemical dopaminergic probe could be used to determine whether the receptors are uniformly distributed throughout the tumour or whether they are confined to the small number of cells immunostaining for gonadotrophins. The former result seems most likely since 11 of the 20 non-functioning tumours in Chapter 8 had negative, or virtually negative, immunostaining. Although some non-tumourous gonadotrophs appear to possess dopaminergic receptors (Goldsmith et al., 1979), dopamine does not have a direct effect on gonadotrophin release from such cells in-vitro (Weiner and Ganong, 1978). In-vivo inhibitory effects of dopamine on LH release are most likely to be due to an indirect action on the hypothalamic secretion of

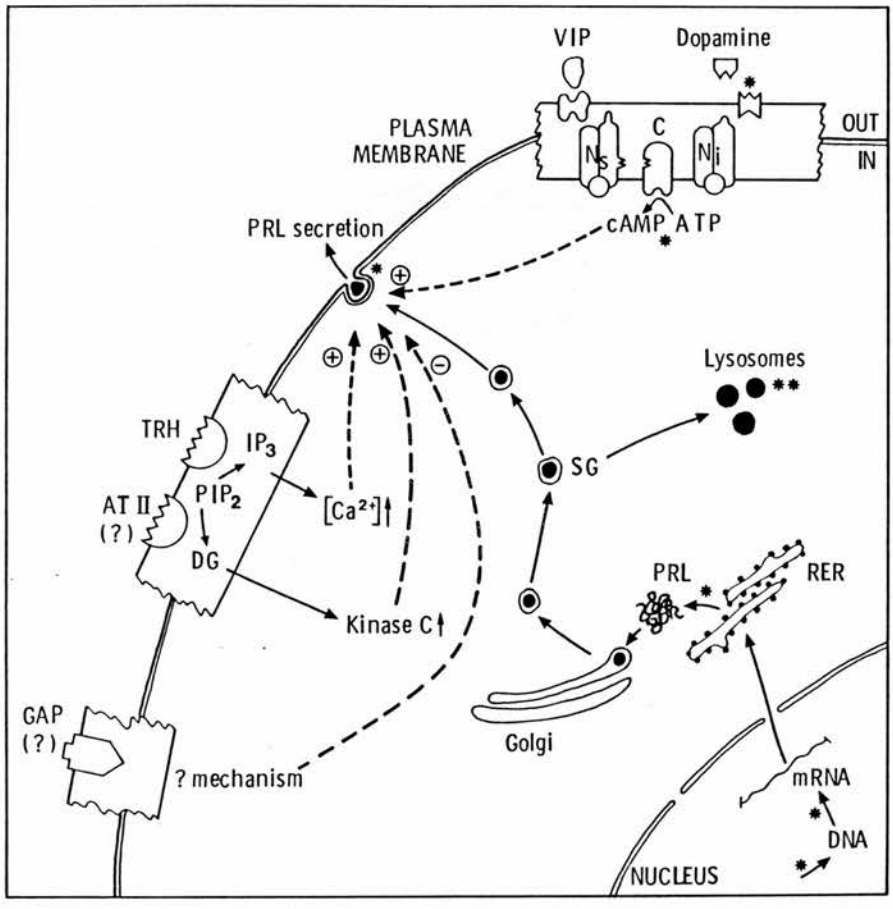
LHRH (Judd et al., 1978), though bromocriptine does not have a similar effect (Martin et al., 1981).

A third explanation for the failure of non-functioning tumours to shrink is that they possess a post-dopamine receptor defect which renders the cells unable either to secrete hormone or to respond to dopamine receptor activation, in a manner analogous to the rat pituitary tumour 7315a (Judd et al., 1985). Nothing is known of post-receptor mechanisms in non-functioning tumours and this suggests a promising area for future research which is discussed below.

A final possibility is that lactotroph size reduction is in some way specifically related to reduced levels of prolactin itself. This is entirely speculative but Gout et al (1980) showed that prolactin is an obligatory growth promoting factor for the malignant Nb2 lymphoma in the rat.

A schematic diagram of the lactotroph is shown in Figure 10-1 to illustrate the various sites of action of bromocriptine and to act as a guide to the last section of this chapter.

Figure 10-1: Intracellular mechanisms in the lactotroph



A diagrammatic representation of the lactotroph showing the pathway of prolactin synthesis and release, membrane receptor complexes and the sites of action of bromocriptine.

Sites of bromocriptine inhibition (*) and stimulation (**)
SG = secretory granules; PRL = prolactin; RER = rough endoplasmic reticulum; mRNA = prolactin messenger RNA.

Adenylate cyclase complex
C = catalytic unit of adenylate cyclase; N_i and N_s = GTP-sensitive regulatory proteins, inhibitory and stimulatory.

Inositol phosphate pathway
PIP_2 = phosphatidylinositol 4,5-biphosphate; IP_3 = inositol 1,4,5-triphosphate; DG = diacylglycerol; Kinase C = protein kinase C.

AT II = Angiotensin II; GAP = GnRH associated peptide; (?) = unknown

C. FUTURE RESEARCH

The detailed characterisation of cells from non-functioning pituitary tumours remains a largely unexplored area of clinical neuroendocrinology. The demonstration of "lactotroph-like" dopamine receptors in these cells has suggested two broad directions for future research. The first would involve a search for other specific cell markers which might identify the pituitary cell type of origin and, in particular, reveal any other similarities to the lactotroph. The second relates to the elucidation of post-dopamine receptor control mechanisms with the aim of explaining the failure of non-functioning tumours to shrink during dopamine agonist therapy.

Cell marker studies would require careful interpretation for a variety of reasons. Firstly, many pituitary adenomas exhibit atypical responses to secretory stimuli, for example the paradoxical responses of some gonadotrophinomas to TRH (Snyder et al., 1980), or some GH-secreting adenomas to TRH or corticotrophin-releasing hormone (Pieters et al., 1984). Such tumours may therefore exhibit membrane-bound receptors not representative of their normal cells of origin. Secondly, several regulatory factors have actions, and possibly receptors, on more than one pituitary cell type and the demonstration of a wide variety of possible paracrine control mechanisms has greatly increased the complexity of anterior pituitary regulation (reviewed by Denef, 1986).

Of central importance would be the further characterisation of the dopamine receptor complex. Although the dopamine receptors in non-functioning tumours and prolactinomas were kinetically similar it is possible that structural differences exist. New dopaminergic ligands have been developed for the affinity purification of solubilised receptors and this may reveal differences between dopamine receptors in

the two tissue types (Amlaiky et al., 1984; Ramwani and Mishra, 1986; Shorr, 1985, personal communication). Furthermore these ligands should also facilitate photoaffinity probe studies of the active site of the dopamine receptor (Amlaiky et al., 1984). Studies of adenylate cyclase activity in non-functioning tumours have not been reported and it would be of great interest to determine whether dopamine receptor and adenylate cyclase are negatively coupled, as they are in the lactotroph (Figure 10-1). Furthermore the presence of VIP receptors and VIP stimulation of adenylate cyclase activity would provide a further similarity to both the normal and abnormal lactotroph (Spada et al., 1983, Enjalbert and Bockaert, 1983). If adenylate cyclase were present in non-functioning tumour cells then the effects of forskolin (a stimulator of adenylate cyclase), dibutyryl cAMP (a long acting cAMP analogue) and phosphodiesterase inhibitors, on secretory granule release, and where appropriate hormone secretion, could be studied.

By further analogy to the lactotroph, TRH receptors could be sought in non-functioning tumour membranes although, as mentioned above, their presence might signify abnormal expression rather than necessarily indicating similarity to the lactotroph. If TRH receptors were present then it would be pertinent to examine the inositol phosphate system as this is considered to mediate the actions of TRH on the lactotroph (Gershengorn, 1986). This is briefly summarised in Figure 10-1. The effects on granule release of compounds which simulate activation of this pathway could be examined; the calcium ionophore A23187 to raise intracellular free calcium levels and phorbol myristate acetate to mimic stimulation of protein kinase C by diacylglycerol.

Recent reports suggest further ways of distinguishing lactotroph-like and gonadotroph-like cells in non-functioning tumours. The putative

precursor molecule for gonadotrophin-releasing hormone contains not only GnRH which stimulates gonadotrophs, but an associated peptide (GAP) which, in nanomolar concentrations, inhibits prolactin release from lactotrophs (Nikolics et al., 1985). Secondly, the renin-angiotensin system in the normal pituitary seems to be involved in paracrine interaction between gonadotrophs and lactotrophs. Angiotensin II (AT II) is present within the LH containing granules of the gonadotroph, converting enzyme-like activity is present in the gonadotroph and AT II stimulates prolactin release from the lactotroph although the intracellular mechanisms remain controversial (Steele et al., 1982; Aguilera et al., 1982; Schramme and Denef, 1984; Marie et al., 1985). From these reports lactotroph-like cells would be predicted to possess specific binding sites for GAP and AT II (Figure 10-1).

As already noted most non-functioning tumours contain secretory granules, yet many show completely negative immunostaining for recognised anterior pituitary hormones. A proportion may be secreting hormones as yet unidentified but in others cellular hormone levels may be too low to be detected by conventional immunocytochemistry. In these cases it is probable that the more sensitive techniques of recombinant DNA technology will enable the detection of low tissue levels of specific mRNA. This technology has not yet been extensively applied to the study of human pituitary tumours but as the cDNA probes become available this will undoubtedly increase. Such probes have been prepared for human alpha subunit, LH$\beta$and prolactin and their use has been recently reported by Jameson et al (1986). These workers described a pituitary tumour which was clearly hypersecreting alpha subunit since serum levels were elevated and many clumps of cells showed positive immunostaining for the hormone. The tumour contained not only alpha subunit mRNA (as shown by blot hybridisation of extracted RNA to the

specific cDNA probe) but also beta subunit mRNA more characteristic of chorionic gonadotrophin than LH, suggesting defective promoter site activation in the tumourous cells. The mRNA measurements were made on **total** extracted RNA so it was impossible to comment on gene expression in tumour cells showing no hormone immunostaining; the application of in situ hybridisation techniques may reveal such information. Furthermore it would be of great interest to see whether there is detectable mRNA for prolactin or gonadotrophin subunits in non-functioning tumours completely negative with conventional immunostaining.

In final conclusion, the studies described in this thesis have resulted in useful guidelines for the clinical management of patients with large pituitary tumours. In particular, the responsiveness of different pituitary tumour types to dopamine agonist therapy has been clarified. The unexpected finding of dopamine receptors in non-functioning tumours has raised several new questions which promise many years of further research. If intracellular post-receptor defects can be defined, then this should, in the longterm, permit definition of genetic defects in these tumours whose origin and pathogenesis remain an enigma.

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APPENDIX: PATHOLOGY METHODS

These techniques were carried out by Dr M.M.Esiri and the laboratory staff of the Department of Neuropathology, Radcliffe Infirmary, Oxford. I did not personally perform this experimental work, hence its inclusion as an appendix.

1.Routine pituitary tumour histology

Tumour fragments were fixed in 10% neutral formalin for 15-30 hours and embedded in paraffin wax. Routine staining was performed with haematoxylin and eosin, and orange G/PAS.

2. Immunocytochemistry

Immunohistology was performed using the immunoperoxidase (PAP) technique with rabbit antisera to human PRL, GH, ACTH, βTSH, βFSH, βLH and αFSH, used at dilutions ranging from 1:50 to 1:400 depending on the results of test dilutions carried out on surgical specimens of tumours and normal pituitary glands obtained at necropsy. Antisera were of high specificity and supplied by Dr S.Raiti (NIAMDD). Additional antisera to GH (Dako), PRL (LKB) and ACTH (Wellcome) were also used. Control sections were treated with normal rabbit serum or specific antiserum preabsorbed with the appropriate hormone.

3. Reticulin staining

Sections of neutral formalin fixed tumour (6-8 μm) were stained by Gordon and Sweet's method for reticulin. Using this stain, reticulin appeared black and collagen brown; both were recorded as fibrous tissue in the tumour fibrosis study (Chapter 2.C). In this study reticulin-stained sections from all tumours were coded and examined by MME without knowledge of tumour type or treatment. Sections were projected on a Nikon profile projector (model V16) at a final magnification of x100 and a point counting grid measuring 20 x 20 cm with points at 1 cm intervals

was placed over the tumour image. This covered an area of tumour equivalent to 2 x 2 mm. The proportion of randomly selected tumour area occupied by tumour cells and fibrous tissue was measured by counting the number of grid points overlying each. Multiple interrupted serial sections at 50 μm intervals were counted in tumours whose area in one section was insufficient to cover at least 80% of the grid.

4. Electron microscopy

Tumour specimens were diced into 1 mm pieces, fixed immediately in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and embedded in Spurr medium. Ultrathin sections were stained with 2% lead citrate and uranyl acetate and examined in a Philips 301 transmission electron microscope.

Ultrastructural examination revealed oncocytic change, defined as an abundance of mitochondria in >10% tumour cells, in several non-functioning tumours. It was graded + (<10% cells), ++ (10-50% cells) or +++ (>50% cells).

5. Cell morphometry

Cells from bromocriptine-treated and untreated prolactinomas were examined at the electron microscopic level (x3000-6000 magnification). Nuclear and total cell areas were measured from photographs using a semi-automatic image analyser (MOP, Reichert).

ADDENDUM

Points for clarification arising from the viva examination of this thesis (16.12.87)

1. Clinical management of macroprolactinomas; the role of bromocriptine

This paragraph clarifies the discussion on pages 65 and 215-217 regarding my recommendations for bromocriptine therapy in patients with large prolactin-secreting pituitary adenomas (serum prolactin >5000 mU/L). Bromocriptine, followed at some stage by external radiotherapy, is the preferred treatment for nearly all such lesions, and surgery now has a minor role in their management. There are three situations where surgery might be used occasionally. Firstly, if short-term (<2 months) bromocriptine produces compact shrinkage resulting in an intrasellar tumour (uncommon with large adenomas) then some of these patients may be cured by subsequent surgery; this possibility remains unproven. Secondly, some macroprolactinomas have considerable residual suprasellar tissue even after prolonged (>1 year) bromocriptine therapy and it may be considered desirable to debulk such tumours surgically prior to radiotherapy; tumour fibrosis may make this difficult however. Lastly, macroprolactinomas which do not shrink at all during bromocriptine therapy will require surgery if there is significant suprasellar tumour, especially if visual failure is present. However these cases comprise the minority and most patients with macroprolactinomas now avoid surgery.

2. Interpretation of serum prolactin levels in the intermediate range; implications for therapy

This section clarifies some aspects of Chapter 2 (section A) and the discussion on pages 217-218. Further analysis of the data in Chapter 2

and of similar data recently reported by Ross et al (1985) gives some indication of the percentage of large pituitary lesions (>1500 mm^3 fossa volume, usually with extrasellar extension) associated with intermediate serum prolactins of 2500-6000 mU/L, that are **not** macroprolactinomas. In my series the figure is 38% (5/13 patients) rising to 50% (5/10) if patients with serum prolactins not fully diluted are excluded. Of the patients described by Ross et al (1985) 41% (7/17) did not have macroprolactinomas - 54% (7/13) if those with undiluted prolactins are excluded. In summary, about one half of such lesions are not prolactinomas and will not therefore respond to bromocriptine.

There is debate as to whether these patients should receive surgery or bromocriptine in the first instance. The decision will depend on a variety of factors including local surgical expertise, the severity of any visual failure, patient preference and clinical judgement. With a 50% chance of the lesion not being a prolactinoma, particularly if vision is severely impaired, I consider that early surgery is desirable. However a closely supervised trial of bromocriptine is perfectly reasonable provided that surgery is performed if there is any deterioration or if there has been no shrinkage of the lesion after treatment for no longer than 3 months. Using bromocriptine, visual failure will persist for longer if the lesion is not a prolactinoma but up to 50% of patients will avoid surgery with its small but definite morbidity.

3. Duration of the bromocriptine trial and the fibrosis conclusions

This point clarifies aspects of Chapter 2 (section C). It is accepted that had the trial of bromocriptine continued for longer than 36 weeks (longest duration in a prolactinoma patient) then more prolactinoma

shrinkage might have been achieved. However, my results and those of others (Weiss et al., 1983; Barrow et al., 1984) indicate that most of the early shrinkage occurs by about 6 weeks. The further regression sometimes seen during more prolonged therapy (up to 2 years) occurs at a much slower rate (Gen et al., 1984; Clayton et al., 1985). Since my objective was to examine the effect of surgery on bromocriptine-treated tumours this was performed when the early shrinkage was maximal, the adverse experience with tumour fibrosis precluding surgery after more prolonged bromocriptine.

Two points have been made about the fibrous tissue study. The first is that the number of tumours was relatively small with only 4 prolactinomas shown to contain excess fibrous tissue after bromocriptine (Fig 2-12) and the second that untreated adenomas occasionally contain excess fibrous tissue. The criticism of small numbers is accepted but the surgical difficulty provided by the tough tumour texture in these 4 cases was greater than that encountered by Mr CBT Adams in a series of more than 300 consecutive operations on pituitary adenomas of all types. Despite the small numbers I believe the preoperative bromocriptine given for at least 3 months to be highly relevant to the problems encountered (Esiri et al., 1986; Bevan et al., 1987b). Lastly, none of the tumours in the control group contained excess fibrous tissue.

J.S.Bevan 7.1.88

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