A NEW APPROACH TO HORMONE DEPENDENCE IN HUMAN BREAST CANCER

by

HERSCHEL FLAX M.B. Ch.B. (Cape Town), F.R.C.S (England).

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PREFACE

It is generally agreed that although dramatic regressions may sometimes be achieved by hormonal therapy for disseminated breast cancer, only about one in three patients responds to such measures and no satisfactory system of selection is available, despite many attempts to devise one. The research to be described, which was carried out at the Westminster Hospital, London, between March, 1972 and July, 1973, constitutes a fresh approach to the problem.

The method which has been developed includes a number of innovations and has been designed with the primary aim of clinical applicability in mind. Its basis is a twenty-four hour organ culture system for human breast cancer slices which maintains the tissue close to its original state in the patient and is not intended for proliferation. Among its other advantages are its rapidity, the use of a fully-synthetic, chemically-defined medium and the avoidance of single-cell suspension techniques.

In addition to appropriate controls, the tumours have been cultured with 17-beta-oestradiol and testosterone, which have been known for many years to influence some breast cancers, and also with prolactin. The introductory section of this thesis reviews modern research linking prolactin to animal and human breast cancer. The existence of prolactin as a separate hormone in Man has only recently been established.

Assessment of the results of the culture has been based on histology and on a very sensitive multi-phase histochemical estimation of the total dehydrogenase activity of the pentose shunt pathway, which is an excellent marker of neoplastic activity.

The technique has been standardised and is being routinely performed with a high degree of reliability and reproducibility. It requires no elaborate apparatus and could be established in any laboratory. One hundred and fifty breast cancer patients have now been studied and 53% of the tumours exhibited hormone dependence according to the criteria used. Analysis of the results showed a meaningful relationship to the menstrual status and to the treatment the patient had received prior to testing.

Perhaps the most striking single feature of the project has been the demonstration that 50 of the 150 tumours were dependent on prolactin in vitro. Although a good deal of investigation has been carried out on the role of prolactin in rat mammary tumours, this is to the best of our knowledge the first large-scale study of the effects of prolactin on human breast cancer.

Another important new concept has emerged from the project. A number of tumours were found to be testosterone-dependent in vitro and clinical observations on these patients tended to support this result. This concept may help explain several apparent paradoxes in the hormonal treatment of the disease.

The clinical course of all the 150 patients was closely followed, although it is emphasised that this project was not a controlled clinical trial. However, where it has been possible to compare the results of the test with the response of the patient to hormonal therapy, there has been an extremely encouraging correlation of the order of 90%. It is gratifying that these early results have prompted the establishment of a formal trial at the Westminster Hospital to determine unequivocally whether the test does represent a significant improvement over the present unsatisfactory selection of patients for hormonal treatment.

Apart from its specific applications in the present study, the technique offers a very useful laboratory tool for the study of breast cancer generally and for a number of other tumours and pharmacological agents as well.

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CONTENTS

INTRODUCTION AND REVIEW

In this introductory section I propose to summarise the main evidence, in a historical context, that led to the belief that breast cancer was at least in a proportion of cases not entirely autonomous, and that its behaviour could to some extent be controlled by endogenous and exogenous hormonal manipulations. Charles Huggins, one of the great pioneers in the field of hormones and cancer, has stated that "Cancer is not necessarily autonomous and intrinsically self-perpetuating. Its growth can be sustained and propagated by hormonal function in the host which is not unusual in kind or exaggerated in rate but which is operating at normal or even subnormal levels." (1972). The validity of this remark is well-known to everyone concerned with breast cancer, while the accuracy of his final words in particular will often be manifested as we examine the subject in depth in the following pages. Despite now overwhelming evidence linking sex hormones and prolactin with the growth of breast cancer, attempts to demonstrate a simple quantitative relationship between circulating blood levels of these hormones and the natural history of the disease have consistently failed.

It is also appropriate to state at the outset that there is a large measure of agreement that hormones themselves do not initiate the transformation of benign into malignant breast cells (Bulbrook, 1972). A mammary tumour virus may well be the initiating agent, with the hormones acting as modifiers of viral action, although in this role they may be so powerful that the effects of the initiating agent may be virtually abolished (Furth, 1965). Recent work has shown that some human milk contains particles identical to mouse mammary tumour virus (Moore et al., 1971) and Schlom et al. (1971) have shown that these particles are RNA virions and that they are found in the majority of human breast tumours. It still remains to be proved that these particles are in fact the initiators of the malignant change, which - once accomplished - is then powerfully influenced and modified by hormones.

After a review of the classical evidence linking the steroid sex hormones with breast cancer, recent work on the physiology of prolactin and its role in breast cancer will follow, while this introductory section will conclude with an account of the main attempts to predict

response to hormonal treatment.

HISTORICAL OBSERVATIONS LINKING HORMONES TO BREAST CANCER

The observation by Sir George Beatson in 1896 that oöphorectomy had a beneficial effect on two women with breast cancer is usually taken as the first documented evidence of the hormonally-controlled nature of the disease. Like many important discoveries, the significance of this one was overlooked for many years and the next development was only in 1932, when Lacassagne succeeded in initiating mammary cancers in specially bred male mice by injection of oestrones. Interestingly enough, the next piece of evidence again came from male breast cancers, namely the observation by Farrow and Adair in 1942 that bilateral orchidectomy may benefit certain cases of human male breast cancer. From this point onwards evidence of hormonal influences began accumulating more rapidly, and may be considered in three main groups. The first illustrated the beneficial effects of certain endocrine-ablative procedures; the second demonstrated varying effects of administering certain exogenous hormones in humans with the disease; and the third developed mammary cancer models in animals, especially rats and mice.

The earliest reports of bilateral oöphorectomy and orchidectomy have already been mentioned. The next endocrine-ablation to be employed in advanced mammary cancer was bilateral adrenalectomy. In 1952 Huggins and Bergenstal reported the use of the operation in a group of patients with advanced prostatic and mammary cancers and also a "miscellaneous" group of tumours. There was some evidence of remission in the prostatic cancers, while of the seven breast cancer patients one died post-operatively, one was unaffected by the procedure, one appeared to be aggravated and four were objectively improved. No specific mechanism to explain the regression was postulated, but Huggins and Bergenstal felt that "it is most reasonable to assume that the regressions are due at least in part to elimination of critical amounts of sex hormones." The success of the operation was later popularly ascribed to removal of residual oestrogen secretion (after ovarian ablation or the menopause) by the adrenal cortex, (Huggins and Dao, 1953; Bulbrook and Greenwood, 1957) but we shall see in due course that it is by no means certain that adrenalectomy always acts by

affecting oestrogen secretion, and that there is indeed considerable doubt that the adrenal cortex is the major site of oestrogen secretion in the postmenopausal woman at all (Grodin et al., 1973). Also in 1952 Luft, Olivecrona and Sjögren achieved remission of advanced breast cancer by hypophysectomy, the mechanism of action being ascribed to removal of pituitary gonadotrophins with a resultant drop in gonadal and adrenal steroid hormone production. These three operations, then, oöphorectomy, adrenalectomy and hypophysectomy, became and have remained the principal forms of indirect surgical relief for disseminated breast cancer. Like hormonal therapy in general, as will be discussed in detail later, they produced remission rates of the order of only 30 - 40%.

At the same time as some successful remissions were being obtained by ablative procedures, successes were being achieved in other cases by additive hormonal treatment. In 1944 Haddow et al. described the use of phenolic oestrogens and in 1947 Adair reported success following administration of the male sex hormone, testosterone, to women with advanced breast cancer.

DEVELOPMENT OF EXPERIMENTAL ANIMAL MODELS

Meanwhile, methods of initiating mammary cancers in animals were being discovered and in particular the rat was shown to develop the tumour readily after exposure both to certain aromatic hydrocarbons and to ionising irradiation. Maisin and Coolen (1936) succeeded in producing mammary as well as skin cancers by painting the skin of mice with 3-methylcholanthrene and Shay et al. (1949) produced mammary tumours in rats by the same method even more easily. Finally Huggins, Grand and Brillantes (1969) refined the animal model still in the most widespread use when they succeeded in evoking mammary cancers in all female Sprague-Dawley rats that were given one feed of 7,12-dimethylbenzanthracene (DMBA). Huggins, Morii and Grand (1961) further found that intravenous injection of the lipid emulsion of DMBA was even more effective. Mammary cancers induced in male rats in this way were not influenced by orchidectomy or hypophysectomy, but in female rats the tumours, (whether induced by radiation or by aromatic compounds), were susceptible to various hormonal influences. For example, pregnancy and progestogens accelerated their growth (Huggins, Moon

and Morii, 1962) while ablative procedures, notably hypophysectomy, caused most of them to disappear. At the time it was considered that the effect of hypophysectomy was mediated via the gonadotrophins of the anterior pituitary and the steroid hormones of the target organs, but we shall see later how the DMBA tumour and hypophysectomy relate to the concept of prolactin dependence.

RELATIONSHIP OF TUMOUR TO MENSTRUAL STATUS

By this stage it was clear that addition or removal of both male and female steroid hormones could under certain circumstances produce a remission in breast cancer. Though it quickly became apparent that it was impossible to apply hard-and-fast rules, clinical experience showed that patients could be considered broadly in two groups, separated by the menopause, with the proviso that there was a period of a few years immediately preceding and after the menopause, when the response of the tumour was even less predictable than usual. It appeared that in the premenopausal patient the tumour was likely to be stimulated by oestrogens, (Haddow et al., 1944; Nathanson, 1947) although there had never been any conclusive evidence, (and this remains true today), that high oestrogen levels might play a part in its induction, as it seems to do in rodents. The issue was further complicated by the influence of absorbed oestrogens and the fact that breast cancer tissue itself could aromatise testosterone to oestriol, (Adams and Wong, 1968). However, in general it seemed that ovarian ablation by surgery or irradiation was the likeliest hormonal manoeuvre to succeed in the premenopausal patient. There was also some measure of agreement that although prophylactic ovarian ablation as an adjunct to primary surgical treatment might possibly delay the appearance of metastases slightly, it did not materially alter the long-term life-expectancy or natural history of the disease.

The situation was even more complicated in the postmenopausal woman. It appeared as if oestrogen secretion by the adrenal cortex began to rise, (Huggins and Dao, 1953; Bulbrook and Greenwood, 1957) hence the postulated mechanism for successful adrenalectomy. However, this is a paradox when one considers that oestrogen administration can also induce a remission in a certain number of postmenopausal women, particularly the older ones, (as it does in a

small number of premenopausal women, for that matter). The prevailing theories became less credible as it became evident that no consistent relationship was observed between either the pre- or post-operative excretion of oestrogens and the clinical response to oöphorectomy, adrenalectomy or hypophysectomy. Forrest (1971) concluded that "the view that the remission of advanced breast cancer induced by endocrine surgery resulted from changes in total circulating oestrogens was no longer tenable."

Atkins et al. (1960) and Hayward et al. (1970) showed that transfrontal hypophysectomy gave a significantly better remission rate than bilateral adrenalectomy plus oöphorectomy, and although the rationale was thought to be based on a gonadotrophic and adrenocorticotrophic effect (i.e. the steroid hormones), I think that in the light of recent data, including those from the experimental work to be described, it is reasonable to infer that the success of hypophysectomy has been due to specific removal of an additional pituitary factor as well as the gonadotrophin effect, and this factor is almost certainly prolactin.

In addition to the already-mentioned shortcomings of the simple division of breast cancer patients into the pre- and postmenopausal groups, with the therapeutically-difficult group straddling the menopause, a few more complicating factors need to be mentioned. Androgen therapy has been found to be useful in a certain number of patients in both age-groups and - like all hormones - it may act either directly on the tumour cells, or via an oestrogen-blocking effect on the tumour cell, or via a feedback mechanism on any or all of the ovaries, adrenals and especially the pituitary. The observation of a remission in the same tumour to oestrogen and androgen used successively and the rebound response after cessation of androgen administration (Delarue et al., 1955) further cloud the picture. Progestogens are known to cause a worth-while remission in certain cases of breast cancer, often after oestrogen therapy has failed, but the physiology of the progestogens' effects on breast cancer remains relatively obscure even to date, (Hayward, 1970) and again it may operate both directly and via pituitary or other feedbacks.

THE EFFECTS OF CORTICOSTEROIDS

The response of some breast tumours to the corticosteroids completes this review of the role of the main steroid hormones in this disease. Apart from the well-known non-specific effects of the corticosteroids, including induction of euphoria, relief of hypercalcaemia, relief of cerebral oedema in the presence of cerebral metastases and the important local anti-inflammatory action, the corticosteroids probably work on a negative feedback system, lowering the secretion of ACTH and possibly gonadotrophins and thereby the production of both androgens and oestrogens from the ovaries and adrenal cortex. They may in fact have considerably wider feedback effects on the pituitary, resulting in suppression of prolactin secretion as well. Administration of corticosteroids has sometimes been referred to as a "medical adrenalectomy" but Ker (1970) has pointed out that it is clearly not as effective as a surgical adrenalectomy, and furthermore several non-responders to corticosteroids achieved an excellent remission after surgical adrenalectomy. The incidence of side-effects of the drugs also has to be taken into account.

It will already be clear at this stage, before we examine attempts at prediction of response to hormonal therapy, that the need for such prediction is very great, and that furthermore an ideal method should indicate specifically which hormonal manipulation is likeliest to succeed. Despite all the important studies towards this end, and the increased understanding of the pathophysiology of the disease that they have given us, it is agreed that a satisfactory system has not yet been found. This sets the background for the experimental work which I will describe after discussing the advent of prolactin on the breast cancer scene.

EVIDENCE FOR THE ROLE OF PROLACTIN IN BREAST CANCER

The period 1937-1950 saw a great deal of interest in the stimulatory effects of hypophyseal isografts on the breasts of mice, leading in certain strains, (at the time only those strains carrying the Mouse Tumour Virus), to breast tumours. The investigators, chief among them Loeb, (Loeb et al. 1937; Loeb and Kurtz, 1939) assumed without further question that the effects were due to

stimulation of ovarian oestrogen production. In 1951 these experiments were re-performed on a large scale at the Netherlands Cancer Institute in Amsterdam to determine whether the same results occurred in Mouse Tumour Virus (MTV)-free animals and to analyse the hormonal situation more clearly. In their first publication on this work Mühlbock and Boot (1959) showed that the hypophyseal isografts did in fact produce a high tumour incidence in MTV-free mice and raised the possibility that prolactin was involved in the process, as the isografts produced prolactin continuously throughout the life-span of the recipients. Boot has discussed these findings fully in a more recent review (1970). In 1957 Hadfield described a "mammotropic agent," which he thought was probably prolactin, in the urine of women with breast cancer, and showed that it disappeared when there was a favourable response to hypophysectomy. Also in 1957 Pearson noticed that administering ovine prolactin to breast cancer patients after hypophysectomy could induce exacerbation of their disease. In 1959 Pearson and Ray went on to show that oestrogen administration after hypophysectomy produced no observable effect on breast cancer, even in patients with previously hormone-sensitive tumours, (as judged by previous response to hormonal treatment), and they suggested that oestrogens required an intact pituitary gland to act.

On the experimental front Furth and Clifton (1958) described a mammotrophic pituitary tumour in rats and also claimed that the secretion of prolactin by the anterior pituitary is normally stimulated by physiological levels of oestrogens in the blood. Ablation of circulating oestrogens, for example by oöphorectomy, would therefore decrease secretion of prolactin. Although this concept is still accepted today, in 1963 Kim et al. showed, paradoxically, that administration of very high doses of oestrogens may also lower prolactin secretion in rats, and this was adduced as one of the possible factors in the response of certain breast tumours to high dose oestrogen therapy. However, the vagaries of prolactin secretion are still far from understood, (as will be elaborated in the Discussion), and it is still not known, for example, whether high doses of oestrogen act mainly on the tumour cells directly, or by blocking prolactin uptake and utilisation by

the tumour cells, or via a feedback-effect on pituitary prolactin secretion. In 1962 McCalister and Welbourn strengthened the case for prolactin still further by showing that injection of ovine prolactin which caused an exacerbation in certain breast cancer patients, predicted a high likelihood of response to subsequent hypophysectomy.

In 1969, in a classic paper, further evidence of prolactin's role came from Pearson and his colleagues, who showed that the Huggins 7,12-DMBA-induced breast tumour in Sprague-Dawley rats was definitely a prolactin-dependent tumour, and in 1970 Nagasawa and Yanai in Tokyo, using similar techniques of appropriate endocrine-organ ablations followed by exogenous administration of the hormones, settled another long-debated point by showing that it was indeed prolactin and not growth hormone that maintained and stimulated these tumours. At the time these experiments were carried out, at the end of the 1960's, there was still no conclusive laboratory proof that prolactin existed as a pure entity separate from growth hormone in humans, although the implications these studies might have for breast cancer in women were obvious.

CONTROL OF PROLACTIN SECRETION AT PITUITARY AND HYPOTHALAMIC LEVEL

From this point onwards a considerable awakening of interest in prolactin took place, particularly in regard to the investigation of agents that could increase or diminish its secretion. Cassell, Meites and Welsch, (1971), found that certain ergot alkaloids inhibited secretion of prolactin, both by pituitary suppression and also via hypothalamic control. It had been shown as far back as 1956 by Polishuk and Kulesan that phenothiazine tranquillisers and also reserpine were able to stimulate breast growth and lactation in patients. It now appeared that of the group of "releasing factors" of the hypothalamus for the anterior pituitary, that for prolactin was inhibitory, and this has been called Prolactin Inhibiting Factor (PIF). Soon it transpired that a wide range of drugs which suppressed the pituitary, and particularly the phenothiazines, were thus able to switch off PIF production and secretion down the hypothalamo-pituitary axis, thereby allowing prolactin secretion to proceed at a higher level.

Hwang et al (1971) observed that levodopa markedly lowered prolactin secretion, while it raised GH and FSH levels. This is believed to operate via transformation of levodopa, a dihydroxyphenylalanine isomer, to dopamine in the brain, and this catecholamine is thought in turn to stimulate production of the various hypothalamic "releasing factors," which in the case of prolactin is - as mentioned above - an inhibitor. Stoll (1972) suggested that the hypothalamic centres might show a diminishing sensitivity with age, reflecting depletion of dopamine, (precisely as parkinsonism reflects a similar deficiency in the striato-nigral system). Since the hypothalamic centres are partly regulated by circulating steroid hormone levels, (the"long feedback" mechanism), he postulated that in order for levodopa to effect a lowering of prolactin secretion in certain older post-menopausal patients with a sub-threshold circulating oestrogen level, priming might be needed by giving oestrogens as well as levodopa. He showed that none of seven patients over two years past the menopause regressed on either levodopa or oestrogen therapy alone, but three of them showed some objective regression of tumour bulk with the two drugs together. He suggested that in younger patients levodopa alone might trigger the hypothalamic response without added oestrogen. No prolactin levels were measured in any of these patients. Minton and Dickey (1972) later showed in two patients that clinical remission did correlate with some lowering of the prolactin levels by levodopa, whereas in a third patient there was no clinical remission and the prolactin levels did not drop.

Although it appears at present highly unlikely that the progression of human breast cancer can be equated so simply and directly with the plasma prolactin levels, (as will be elaborated upon in the Discussion), and although the levels are usually within the normal range in these patients, clinicians are naturally wary of possible exacerbations being produced by administering phenothiazine tranquillisers and similar PIF suppressors. As this new experimental work is becoming more widely known, more and more clinicians are avoiding these drugs in breast cancer patients, at least until the whole matter has been more thoroughly elucidated.

In late 1972 and in 1973 results from our laboratory were

published (Salih, Flax, Brander and Hobbs, 1972; Flax, 1972; Flax, 1973; Flax and Salih, 1973) showing unequivocally that prolactin enhanced the survival of breast culture slices in vitro clearly above the controls in 30% of the cases studied. These results will be discussed in detail later on, but at this stage it may be said that they provided the first clear evidence that prolactin was stimulatory to a number of human breast cancers in vitro. Secondly, they showed that such stimulation took place at physiological concentrations of prolactin in the culture system employed, which supported the idea that this hormone probably promotes human breast cancer within the normal range of plasma concentrations.

PROOF OF THE EXISTENCE OF PROLACTIN AS A SEPARATE ENTITY IN MAN

Since it is literally only within the past two years that satisfactory proof of prolactin's separateness from growth hormone in humans has become available, it is relevant to summarise some of the laboratory and physiological evidence of the story. Ovine prolactin was prepared in a pure form free from growth hormone in the late 1930's, but it took until the 1970's to purify human prolactin, due to its considerable chemical, physiological and biological similarity to human growth hormone. When the latter was first isolated in the 1950's it was found to have a substantial amount of prolactin activity, which distinguished it from other previously purified mammalian growth hormones where prolactin was completely separate. Li and his colleagues (1969) were sufficiently convinced to conclude that human growth hormone and human prolactin were identical. However, Pasteels and his colleagues had noticed that cultures of human adult and foetal pituitary glands continued secreting prolactin or at least a prolactin-like substance into the culture medium over a long period, (measured by the classical bioassay of the pigeon crop sac effect), whereas growth hormone production declined. This suggested that two separate hormones might be involved (Pasteels et al., 1972). Addition of a hypothalamic extract to the culture reversed the pattern of secretion, thus confirming what is now the current concept, namely that the hypothalamus produces an inhibitory factor for prolactin release and a releasing factor for growth hormone. Pasteels (1972a) has reviewed his findings in detail.

Other lines of evidence suggesting that the two hormones were different included the production of different human "growth hormone" preparations of differing ratios of growth hormone: prolactin activities (Wilhelmi, 1961); the presence of cells in the human anterior pituitary with staining properties similar to those of prolactin-producing cells in other species, (Pasteels, 1963); the failure to find elevated growth hormone levels in the plasma of nursing mothers or of galactorrhoea patients, or to find any measurable growth hormone at all in certain lactating dwarfs (Geschwind, 1972); and the finding by Peake et al. (1969) of human pituitary adenomata rich in specifically prolactin-like activity.

The crucial requirement was now actually to differentiate the two hormones in the laboratory, and the key to the problem was that in its immunological properties human prolactin is related to ovine prolactin and not to human growth hormone (HGH), though it is chemically very similar to HGH (Forsyth and Edwards, 1972). Hwang, Guyda and Friesen (1971) and Friesen et al. (1972) studied the independent synthesis of prolactin and growth hormone by monkey and human pituitaries in short-term incubation, using the property, shown by Herbert and Hayashida (1970) that extracts of pituitaries of pregnant and lactating rhesus monkeys gave lines of partial identity with ovine prolactin when reacted on agar diffusion plates against an antiserum to ovine prolactin prepared in guinea-pigs. Ultimately they were able to produce primate prolactin of sufficient purity to use in a radio-immunoassay. In a more conventional technique Lewis et al. (1971a and b) found that prolactin was less readily extracted from fresh-frozen human pituitaries than growth hormone, possibly because of tighter binding within the cell, and were thus able to obtain a prolactin-rich fraction which could be further purified with a sephadex column.

These studies led to the development of several other radio-immunoassay systems, for example those of L'Hermite (1972) and Malarkey (1971), both using heterologous systems with anti-ovine prolactin antiserum. The classical pigeon crop assay method, as improved by Nicoll (1969) is still widely used and other bioassay methods are also available. As yet there are no definitive published comparisons between measurements made by the two types of assay.

Other problems include the fact that as yet highly-purified human prolactin is only available in very small amounts, though its existence is now established beyond doubt, and it is fair to state that at the present time all available assay methods are but steps towards the perfection of a homologous radio-immunoassay system for human prolactin.

PHYSIOLOGY OF PROLACTIN

The origin of prolactin in the human pituitary appears to be in certain cells of the acidophil group in the anterior lobe. Pasteels (1972b), the principal worker in this field, has clearly demonstrated that prolactin and growth hormone are secreted by different types of acidophil in both monkey and human pituitaries. He accomplished this using specific antisera against human growth hormone and against ovine prolactin and here again one sees another example of the use of the immunological cross-specificity between ovine and human prolactin. On light microscopy the prolactin cells stain orange-yellow with Orange G. On electron microscopy they are distinguished by large, varied and irregular secretory granules. These cells are rare in normal adult pituitaries but increase in number during pregnancy, becoming sufficiently abundant by term to have been recognised as "pregnancy cells" by Erdheim and Stumme as long ago as 1909 (cited by Forsyth and Edwards, 1972). Goluboff and Ezrin (1969) estimated that in late pregnancy and lactation more than 50% of all acidophils are prolactin-secreting.

Prolactin is phylogenetically the oldest of the polypeptide hormones secreted by the pituitary gland (Turkington, 1972). It has a molecular weight of 20-25,000, with 180-200 amino-acids and two or three disulphide bridges (Forsyth and Edwards, 1972). Its functions are numerous. Nicoll and Bern (1972) have described over 80 different actions in vertebrates, which may be considered in the following groups. Just how many of these are important in Man remains to be proved.

(a) It plays a major role in mammary growth and lactation in humans and animals, though the precise nature of the

interaction in the breast between prolactin and other pituitary and steroid hormones needs to be elucidated.

(b) It appears to play some part in maintaining the structure and function of the corpus luteum. How far this applies in humans is uncertain.

(c) It has some role in the development of accessory reproductive organs in male rodents, and very recent work on prolactin and prostatic carcinoma in Man has raised the suspicion, (though so far no definite proof), that it may be involved in that disease too (Farnsworth, 1972). Its role in the human male under normal physiological conditions is however still unknown.

(d) In certain animals, and probably also in Man, prolactin may reduce water, sodium and potassium excretion, thereby raising plasma osmolality, by acting directly on the proximal kidney tubule.

(e) Just as growth-hormone does have a certain small amount of intrinsic prolactin-like activity, prolactin has been found to have slight GH-like activity in various animals and in Man.

Like many other hormones, prolactin has a circadian rhythm of secretion, (Nokin et al., 1972) with a peak at 1 a.m. to 5 a.m. This time, being in the sleeping hours, would of course correlate with diminished hypothalamic activity, less PIF production and therefore greater prolactin secretion.

When we come to the question of stating normal prolactin levels, there are still considerable problems in that several different assay methods are in use, with different standards, and even the units differ greatly. The Brussels group uses a milli-unit system, the Cardiff group a milli-ampoule system, while the Montreal and St. Bartholemew's groups, who have at present probably the most reliable radio-immuno-assays for prolactin, use a nanogram/ml system. The latter will certainly become the substantive units. In normal adults, male and female, Friesen et al. (1972) have found a range of 0-28 ng/ml with a mean value of 10. As far as the ovarian cycle is concerned, there is an ovulatory peak in the rat, but apparently none in humans. During pregnancy there is a progressive rise, with a very high level

(mean of 200 ng/ml) at term, which falls rapidly in 2 - 3 weeks in non-nursing mothers. In lactating mothers the mean is about 100 ng/ml and doubles 30 minutes from the onset of suckling. The levels in 24 hour neonates are 40 - 400 ng/ml (mean 192) but in infants from the age of 6 weeks and older children they do not differ significantly from adult levels.

Not enough significant data is available yet to show whether prolactin secretion alters in post-menopausal women. Present indications are that it does not, although in rats it is known that ageing reduces hypothalamic activity, which would free the anterior pituitary from PIF and promote more prolactin secretion. If this were valid for women, it would correlate well with Stoll's "ageing hypothalamus" concept for breast cancer. In breast cancer patients there is so far no evidence that levels of prolactin are raised, even if the tumour is thought to be prolactin-dependent.

The different physiological, pathological and pharmacological influences on prolactin secretion and blood levels will be discussed in greater detail in later chapters.

Early research suggests the presence of receptor-like binding activity in normal mouse mammary gland cells, but the nature of these molecules has not yet been chemically identified (Turkington and Frantz, 1972). It is considered likely that some sort of prolactin receptor must be present in normal and possibly neoplastic human breast cells, but its nature is at the present time completely unknown.

ATTEMPTS TO PREDICT RESPONSE TO HORMONAL TREATMENT

It has been stated (Sellwood et al., 1968) that about 30% of breast cancer patients show significant regression of their tumours after endocrine environment changes. In spite of the multiplicity of trials and reports of each modality of endocrine treatment, there is a large measure of agreement on this figure. It is not surprising, therefore, considering the value of the remission if one is indeed attained, that there is a great need to select those patients who would be likely to benefit from hormonal treatment. There is an equal need to select and spare from fruitless treatment the two out of three patients who would not respond. This applies

particularly to the major endocrine organ ablations, attended as they are by a certain morbidity and mortality and the need to remain on permanent replacement medication.

Clinical Guidelines

Of the many attempts to select these patients, clinical guidelines have remained the most useful, though with a success rate no better than one in three. The menstrual status has been the main clinical feature considered, and although practice varies to some extent from one centre to another, most therapists have advised castration for premenopausal women with advanced or recurrent breast cancer, performed either by radiation or by surgery, (the main difference being that radiation takes 4 - 6 weeks for its effect to be complete). In the patient who is less than 5 years post-menopausal some authorities have also advised castration just as in the pre-menopausal group, while others have administered androgens as a form of anti-oestrogenic treatment, sometimes followed with castration if this has been successful. It is the patient over 5 years past the menopause who really enters the therapeutic maze. Adrenalectomy and hypophysectomy are the major ablative procedures in this group, while oestrogens have been the commonest exogenously administered hormone, especially in the older age-group. However, apart from the likelihood that a patient who has obtained a remission from a previous hormonal treatment, particularly castration, is more likely to benefit from a further endocrinal procedure, most clinicians would agree that there are quite simply no hard-and-fast rules at all. Preferences and indications for each form of treatment vary from clinician to clinician, as well as from centre to centre, and even the mode of performance of each treatment, (whether by surgery, irradiation, isotope implantation or drug administration), is hotly debated.

Apart from menstrual status, the second clinical guideline, namely the response to previous hormonal treatment, has already been mentioned. Even this is controversial. Dao et al. (1967) claim to have shown unequivocally that non-response to previous castration or other hormonal therapy does not diminish the chances of a response to adrenalectomy.

The next important guide to response is the disease-free interval, that is the period between removal of the primary tumour and the appearance of metastases. Atkins et al. (1968), in their review of 10 years of

experience, confirmed the findings of several previous workers that the longer the free period, the greater the likelihood of a response to endocrine ablation. A period of 2 years appeared to be fairly critical; the chance of responding to adrenalectomy or hypophysectomy if the free period was less than that became very small.

Some attempt has also been made to select hormonal treatment on the basis of the predominant set of metastases, that is visceral, soft tissue or osseous. For example, corticosteroids are said to yield relatively good results with brain, lung and liver metastases, whereas adrenalectomy is commonly thought to be ineffective for metastases in these sites yet very useful for bony metastases. However, every clinician knows that this type of differentiation is fraught with exceptions to the rule and does not offer a rational selection from the therapeutic lottery for the individual patient.

A very general working rule that is accepted by a majority of therapists is that it is reasonable to try first simple hormonal measures, that is administration of hormones, for the less urgently ill patients, whereas it is more logical to use surgical ablation first in patients who are in danger of rapidly losing condition or whose metastases are in dangerous sites.

Urinary Steroid Excretion Patterns

Clearly a prediction system was badly needed. With the advent of relatively better methods to determine urinary oestrogens than had been previously available, (Brown, 1955; Aitken and Preedy, 1956) several investigators tried to estimate these in the hope that a simple test to predict response to endocrine-ablative surgery would evolve. This hope failed to materialise because no consistent patterns of excretion were observed in these patients both before and after the various operations (Forrest, 1965). This finding also strengthened the conviction that the natural history of breast cancer was not simply determined by fluctuations in the total circulating oestrogens.

During the past decade, the Discriminant Function of Bulbrook, based on urinary steroid excretion, attracted the most attention. Bulbrook, Greenwood and Hayward (1960) measured the 24 hour excretion of aetiocholanolone and 17-hydroxycorticosteroids (17-OHCS). Applying the formula [80 - 80(17-OHCS in mg/24 hours) + aetiocholanolone in micrograms/24 hours], they claimed that those patients who had a

positive value for the "discriminant function" were far likelier to respond to adrenalectomy or hypophysectomy than those with a negative value. In view of the difficult laboratory techniques for measuring aetiocholanolone, several other investigators showed that alternative measurements were available which correlated well with the discriminant function and which were easier to perform. The "Alternative Discriminating Function" or "Miller Ratio" as described by Miller et al. (1967) was the most popular such alternative. It measured excretion of total 17-hydroxycorticosteroids and 11-deoxy-17-oxosteroids and derived a ratio of 0.11, above which the results corresponded to a positive Bulbrook discriminant and below which to a negative one. Juret at al. (1968) developed a similar prognostic approach by finding that objective remission was commoner in patients who excreted the greatest amount of androsterone and aetiocholanolone.

The cause of a negative discriminant is not known. All the approaches, in summary, suggest that an abnormally low androgen metabolite excretion in relation to 17-hydroxycorticosteroid excretion predicts a poor response to endocrine-ablative surgery. This may well be a genetically-transmitted enzymic or other metabolic defect and a very interesting prospective study was set up in Guernsey in 1961 by the Guy's group (Bulbrook et al., 1971), to test whether low androgen excretion might identify a group of women who would develop breast cancer in later life. However, interesting though these investigations are, the marked limitations of the discriminant function approach and the increasing realisation that there were multiple endocrine pathways involved in the disease, as opposed to a purely steroid hormone hypothesis, have unfortunately shown that this is not a satisfactory prediction system for the individual patient either.

Firstly, all investigators found small groups of patients who responded exactly contrary to the predictions. Secondly, it was clear that in patients over 50 years of age the number of negative discriminants rose steadily, such that over the age of about 60 the discriminant function had little predictive value at all. In addition, not all workers found the same proportion of negative discriminants in early breast cancer cases as Bulbrook. Further complicating factors were the knowledge that thyroid abnormality,, general illness, stress (such as a surgical operation or the prospect of one), and the steroid

metabolism of the tumour itself were all able to affect the discriminant function. The investigations of Wade et al. (1969), Ahlquist et al. (1968) and Fotherby et al (1970) showed quite clearly that the accuracy of prediction was not as good as the encouraging early reports had indicated.

Some workers have used a form of discriminant function combined with the disease-free interval in order to predict response, for example Wilson and Moore used a discriminant based on the response of adrenal steroid production to ACTH infusion coupled with the disease-free interval (1968). This appeared to improve the accuracy of prediction, but the disease-free interval seemed more significant than the bio-chemical measurements, and the value of the trial was somewhat diminished in that a number of the patients received 5-fluoro-uracil as well as adrenalectomy.

Oestrogen Uptake and Receptors

The next group of experiments aiming at a prediction system are those concerning oestrogen uptake and oestrogen receptors. In this context it should be remembered from the earlier discussion that it is by no means certain that oestrogen is the only or even the main hormone involved in promoting breast cancer. Radio-active studies with labelled oestrogen in vivo showed as long ago as 1951 that certain breast cancers showed selectively higher uptake of oestradiol than others (Lewison et al., 1951; Folca et al., 1961). This led to in vitro investigations of oestrogen receptors in target cells, and Jensen - a pioneer in this field - after originally showing that there were cytoplasmic receptors for 17-beta-oestradiol in the cells of the immature rat uterus, (Jensen et al., 1966) went on to confirm, as have several other workers, that oestrogen-binding sites are present in some human breast cancer tissues (Jensen et al., 1971). He also showed that there was some correlation between the presence of these receptors and the endocrine-responsiveness of the tumour. The cytoplasmic receptor is thought to be an 8S protein, (composed of two 4S fractions), which takes the oestradiol into the cell and transports it to the nucleus.

In 1973 Braunsberg, James et al. reported on an in vivo study of 35 patients whose oestradiol-uptake in vivo was correlated with clinical response. They found that patients with a low uptake tended not to respond favourably to endocrine ablation or to androgen therapy, but that

the converse was not true and that high oestradiol uptake was found in several patients who did not respond to treatment. As with all the oestrogen-uptake studies to date, both in vivo and in vitro, they were left with the conclusion that a good deal of further study was needed before its possible usefulness could be assessed.

Application of Culture Techniques to Mammary Cancer

Cammeron and Chambers (1937) were the first to culture breast cancer cells successfully using the coverslip technique, but extensive application of organ culture techniques to the mammary gland began in the 1950's (Fell, 1964). Once the technical problems of culture began to be overcome, an investigation of hormone effects in culture systems in vitro soon followed, again with the paramount aim of providing a prediction system for response to endocrine treatment. A number of different parameters were used to assess the response of the tissues, and these fell into four main groups: biochemical, histochemical, histological and radio-active. Hollander et al. (1959) found that 17-beta-oestradiol stimulated isocitric dehydrogenase and alpha-ketoglutarate production in homogenates of some breast tumours and that a few of these patients had remissions after oöphorectomy. Rienits (1959) reported effects of oestrone and testosterone on the respiration of tumour slices, using the Warburg method, but was not able to correlate any of the results with histology or the progress of the disease. Heuson and Legros (1963) reported that testosterone and 17-beta-oestradiol decreased ^{14}C-leucine incorporation by tumour cells, no clinical correlation being given.

In 1971 Burstein et al. described a cell culture technique in which they investigated the effects of cortisol, oestradiol, progesterone, testosterone and human chorionic gonadotrophin on 20 tumours. Response was assessed by ^{3}H-thymidine uptake. Ten of the 20 showed some alteration of uptake with various hormones, with some of these being increased by oestradiol and testosterone and some decreased. In the 6 patients in whom there was an opportunity to correlate the clinical course, there was some evidence that it accorded with the in vitro findings.

Ceriani and his colleagues (1972), assessing response on histology alone, showed that normal breast tissue could survive in a hormone-free medium for a short time, that insulin allowed complete maintenance,

and that addition of oestradiol and ovine prolactin to the insulin-containing medium showed some of tumour survival.

In 1968 Mioduszewska and her colleagues from Poland reported the effects of ovine prolactin, human growth hormone, 17-beta-oestradiol, progesterone, testosterone and cortisol on cell culture and organ culture systems of human breast cancer. The effects in trypsinised cell cultures and in the fragments of tissue in organ culture did not tally precisely and the number of patients studied, (34 in all), was too small to permit any conclusions about clinical correlation, but the authors felt that lack of response to prolactin and stimulation of growth by cortisol were probably associated with a poor response to hormonal treatment, while the converse was associated with a good prognosis. The validity of any conclusions drawn from this culture system is open to question as the media for both cell and organ cultures contained 20% human serum. The same objection applies to any culture experiments in which the media contain human or animal sera of unknown hormonal composition. Esber et al. (1973) have investigated this problem and have found that such sera, in which they measured oestrone, insulin, oestradiol, progesterone and testosterone, vary by up to 23 times in the concentrations of these hormones and there are in addition marked variations of ratio to each other. It was in order to obviate precisely this problem that the work to be described was based on a chemically-defined medium.

Using the coverslip technique, Barker and Richmond (1971) cultured breast tumours from 14 patients with stilboestrol, ovine prolactin and hydrocortisone. The response was assessed by glucose utilisation and lactic acid production. Unlike Mioduszewska they were unable to demonstrate any effect at all of prolactin and hydrocortisone, while in two cases stilboestrol appeared to decrease the activity of the tumour, a finding in agreement with Rienits (1959). The follow-up period was too short to evaluate any clinical correlation.

In 1970 Chayen et al. reported organ culture experiments in 16 breast cancers. It appeared as if they could be divided by a short-term culture system into a group that survived well without oestradiol added to the medium and a group that needed oestradiol in the medium. No clinical correlation was available. Assessment of response was based on a histochemical reaction which estimated the total production by the

pentose shunt of the co-enzyme dihydronicotinamide adenine dinucleotide phosphate (NADPH).

This work leads us to a description of the experimental method and results on which this dissertation is based. At this stage we may state, in general, that the emphasis in hormonal studies has shifted from urinary excretion patterns to the tumour itself, both in vivo and in vitro, but that no method hitherto developed has proved consistently reliable in the laboratory and acceptably accurate to apply clinically. Furthermore, we may state that the past two years have seen the whole subject become considerably more complex. There is now overwhelming evidence that - in addition to the steroid hormones - the anterior pituitary and the hypothalamo-pituitary axis play an important role, especially in regard to prolactin.

DESIGN AND METHOD OF THE EXPERIMENT

FUNDAMENTAL PRINCIPLES

It was decided to study human breast cancer tissue by an organ culture method both in the presence of various hormones and with appropriate controls. The following two fundamental principles were observed in the design of the experiment:

(1) The condition of the tumour was to remain as near physiological as possible. For this reason fresh, thin slices of tumour were used without any homogenisation, trypsinisation, centrifugation, fragmentation or other mechanical or chemical treatment. Other workers have also asserted that organ culture systems are closer to the in vivo situation than monolayer cell culture (Tchao et al., 1968; Röller et al., 1966). For the same reason the experiment was designed as a maintenance rather than a proliferative system, thereby avoiding substantial alterations in the biology of the tumour with successive generations of cells. The incubation period was therefore kept as short as was consistent with obtaining meaningful results; after appropriate trials a period of 24 hours was found to be optimal.

(2) A fully-defined chemical medium was used in order to obviate the host of unknown factors that must necessarily be involved in culture systems whose media include biological additives such as bovine or human serum. Esber et al. (1973) have confirmed the gross variability of hormone concentrations (up to 23 times) that exists in commerically-available animal sera.

THE PATIENTS

The data refer to biopsies of 150 patients taken between February, 1972 and late May, 1973. These were all females with various stages of breast cancer, most of whom were under the care of surgeons and radiotherapists at the Westminister Hospital, London, S.W.1., although a few patients were accepted from nearby hospitals provided that the biopsies could be properly collected and brought to our laboratory within an hour of removal. Six of these 150 patients had a second biopsy on which we were able to perform a repeat experiment during the period under review. Apart from being examined histologically in our own laboratory,

all these tumours had been histologically confirmed as part of the routine clinical management of the patients.

HISTOLOGICAL TYPING

All tumours in this series had been independently confirmed histologically by a pathologist, in addition to the routine histological assessment made in our laboratory as part of the project. However, several different pathologists in different hospitals had reported on these tumours originally. In addition, the appearances of a metastatic deposit more often than not fail to reproduce the particular histological variant of the primary lesion, so that no attempt was made in this project to relate any results to specific histological types of breast cancer. In 83 of the 150 biopsies the pathologist's report was specific enough to classify the tumour into sub-groups, and the distribution - according to the World Health Organisation "Histological Typing of Breast Tumours" (Scarff and Torloni, 1968) - was as follows:

Type I	(Intraduct and Intralobular Non-Infiltrating Carcinoma)	3%
Type II	(Infiltrating Carcinoma)	87%
Type III	(Special Histological Variants)	
	Medullary	3%
	Papillary, Intracystic and Intraductal	3%
	Mucous	3%
	Squamous	1%

THE BIOPSIES

All biopsies of primary and metastatic tumours were taken under strictly aseptic conditions, either under general anaesthesia or under local analgesia with 1% plain lignocaine, depending on the clinical circumstances of the particular patient and her treatment. A block of tumour tissue 1 cm in each dimension was more than ample for the test and it was usually possible to manage with half that amount. The block was preferably taken from as representative an area of the tumour as possible. It was immediately transferred to Trowell's T8 medium in a sterile container, which had been previously warmed to $37^{o}C$. The specimen was then taken to the laboratory. If a patient was to receive radiotherapy to a lesion, the biopsy was performed before irradiation

commenced. The only two significant problems in the technique of the entire experiment were related to the taking of the biopsy; one consisted of the taking of a biopsy with little or no tumour tissue in it and the other was due to contamination arising either from an initially infected lesion or from a lengthy delay before the specimen reached the laboratory. All such doubtful cases were discarded. The recognition of unsatisfactory biopsies and the numbers rejected will be mentioned in detail in the sections on Results and Discussion.

SELECTION OF HORMONES TO BE TESTED

I have indicated in the introductory chapter the close relationship between the steroid hormones and breast cancer, and clinical experience has shown how addition or removal of these may affect the disease adversely or favourably. There was also a great deal of evidence, much of it in animals, however, suggesting that prolactin was also involved in the growth of some breast cancers. Hardly any data had been reported on prolactin's role in human breast cancer. Such data as was available, for example that of Mioduszewska (1968), was not conclusive because of the small numbers involved and particularly because the culture media had contained as much as 20% of serum of unknown hormonal composition. Progestational hormones have been used with some success in the management of some patients with advanced breast cancer, but even to date their precise mode of action is ill-understood. They may operate directly on the tumour cell but a feedback mechanism through the pituitary is more probable. Furthermore, some progestogen metabolites have androgenic and others oestrogenic effects at target cell level. It therefore seemed reasonable to concentrate attention on the main human oestrogen - 17-beta-oestradiol, the main human androgen - testosterone, and prolactin. The first two are readily available but at the start of the project human prolactin was not easily obtainable in the United Kingdom in any satisfactory amount.

STANDARDISATION OF HORMONE CONCENTRATIONS

It is clearly impossible in an in vitro system to reproduce the in vivo conditions exactly, where a continuous and widely varying secretion of many hormones reach the tumour via the circulation. Furthermore, the extent of trapping and concentration of particular hormones by the tumour

is unknown, nor is it feasible to mimic this in vitro. Finally, in the culture system to be described the tumour slice does not lie permanently immersed in the hormone-containing medium, but relies on absorption up the lens paper covering the grid on which the slice rests. The concentrations used by other workers were of course available but it was decided, in view of the above reasons, not simply to follow those nor to attempt to imitate the physiological levels of these hormones in the blood. Instead, the concentrations to be used were determined by a series of trial experiments.

Ovine Prolactin

Ovine prolactin is known to behave very similarly to human prolactin and to be closely related to it (Forsyth and Edwards, 1972). This was the type of prolactin used in almost all the experimental work described in the introductory section. In 1953 the World Health Organisation Expert Committee on Biological Standardisation asked the National Institute for Medical Research, London, to prepare a Second International Standard for Prolactin, since stocks of the First International Standard were running low. This task was in due course completed using a freeze-dried powder prepared from sheep pituitary glands. At the time it was of course still not known whether human prolactin even existed as a separate entity. After an international collaborative assay the Second International Standard for Prolactin was eventually established, with a defined potency of 22 International Units per mg of powder. The International Unit Prolactin is defined as the activity contained in 0.04545mg of the Second International Standard. This material, which has been fully described by Bangham et al. (1963) was made available for this project by the courtesy of the Medical Research Council's Division of Biological Standards, London. It was provided in ampoules containing 10 mg of powder with an activity of 22 I.U. per mg.

Ovine and Human Prolactin Equivalence

I have previously alluded to the unsatisfactory state of affairs that existed between different laboratories with regard to the reference units and assay of prolactin. At the present time this is gradually being rectified and standardised and a system of nanograms per ml is universally emerging pari passu with refinement of the radio-immunoassay and purification techniques. Dr. A. S. McNeilly, of the

Department of Chemical Pathology, St. Bartholomew's Hospital, developed a radio-immunoassay system for prolactin at the time that this project was in progress and kindly investigated our ovine prolactin solutions. Using his radio-immunoassay and an ovine prolactin preparation of known strength for comparison, he found that in a sheep system our solution of 0.22 I.U. per ml was equivalent to 130 ng/ml and our solution of 0.022 I.U. per ml to 13 ng/ml. Now it has been generally agreed that sheep prolactin has roughly half the activity of human prolactin in a human system, so it seemed likely that the solutions used in our human breast system were equivalent to 65 and 6.5 ng/ml of human prolactin respectively. In other words, they were of a similar order of magnitude to the physiological range of concentrations in the plasma (0-30 ng/ml).

This finding was substantiated later in the project when a small amount of highly-purified (90%) human prolactin was obtained by courtesy of Dr. Henry Friesen of Montreal. A series of experiments was commenced using the highly-purified human prolactin in parallel with the ovine prolactin in our breast cancer culture system. Ten experiments have been completed to date and these have provided two important confirmatory findings. Firstly, it has been gratifying to note that there were no anomalous results; all tumours that were dependent on ovine prolactin were also dependent on human prolactin and vice versa. Secondly, dose-response curves comparing the pure Friesen preparation with concentrations of 0.22, 0.11, 0.022 and 0.011 I.U. per ml of ovine prolactin have confirmed that the potency of the human prolactin is about twice that of ovine prolactin. Our solutions of 0.22 and 0.022 I.U. per ml therefore do correspond to approximately 65 and 6.5 ng/ml of human prolactin respectively, as calculated previously.

Prolactin Concentrations

In selecting the concentrations of prolactin to be used, few precedents were available as this was the first large-scale investigation of human breast cancer tissue and prolactin in vitro, to the best of our knowledge. In the small series of Mioduszewska et al. (1968) it was stated that a concentration of 100 micrograms per ml of ovine prolactin was used, but no specific details of the preparation were given, and besides the culture medium consisted of 20% human adult serum. 47 experiments were accordingly conducted with 4 concentrations of the ovine prolactin used in this study; 0.22, 0.11, 0.022 and 0.011

per ml. As demonstrated above, these corresponded roughly to human concentrations of 65, 32.5, 6.5 and 3.25 nanograms per ml respectively. In the 47 experiments only one tumour showed better survival and higher activity at 0.011 I.U. per ml than at the higher concentrations. It was found that 0.022 and 0.022 I.U. per ml bracketed all the other dependent tumours and these were accordingly the concentrations chosen for the study. These concentrations are all of a similar order of magnitude to the normal plasma prolactin levels, which Dr. Friesen had found to be from 0 up to about 30 ng/ml. Prolactin dependence in our system is thus being demonstrated at concentrations approximating to physiological levels.

Oestradiol Concentrations

Twenty five experiments were conducted using 3 concentrations of 17-beta-oestradiol, namely 10^{-5}, 10^{-6} and 10^{-7} Molar. Not a single one showed better survival and higher activity at 10^{-7}M than at either 10^{-5} or 10^{-6}M. The last two were accordingly chosen as the two standard concentrations of oestradiol for this study. A higher concentration such as 10^{-4}M was found to be toxic to the cells and few or none survived. The two concentrations used are equivalent to 2.72 and 0.272 micrograms/ml respectively. In fact, on comparing these concentrations with those used by some other workers in breast culture systems, they are similar to those of Altmann and Chayen (1967) - 10^{-5}M and Heuson and Legros (1963) - 7 micrograms/ml. On the other hand, they are greater than those used by Mioduszewska et al. (1968) - 0.0015 micrograms/ml and Burstein et al. (1971) - 0.0015 micrograms/ml. Blood levels of oestradiol are of the order of 0.02 to 0.07 micrograms per 100 ml (Van de Wiele et al., 1970), with a rise around ovulation up to as high as 0.1 micrograms per 100 ml. These concentrations are of the order of 10^{-7} to 10^{-8}M.

Testosterone Concentrations

Twenty five experiments were conducted using 4 concentrations of testosterone; 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} Molar. Of these, only 3 showed better survival and higher activity at either 10^{-7} or 10^{-8}M than at 10^{-5} or 10^{-6}M. The last two were again chosen as the most suitable concentrations for use in this study, although it is conceded that a small number of cases might exhibit dependence on

testosterone only at these very low levels of 10^{-7} or 10^{-8}M. Testosterone has a molecular weight of 288.4 and the two concentrations used are therefore equivalent to 2.884 and 0.2884 micrograms per ml respectively. Here again these are similar to or substantially lower than those used by other workers, for example Burstein et al. (1971) - 5 micrograms per ml; Mioduszewska et al. (1968) - 5 micrograms per ml and Heuson and Legros (1963) - 11 and 33 micrograms per ml. Blood levels of testosterone are in the range of 0.03 to 0.10 micrograms per 100 ml in normal women (Gandy and Peterson, 1968), with Pearson Murphy (1969) finding a similar range though with a slightly higher mean (0.18 micrograms per 100 ml) in premenopausal women than in postmenopausal ones (0.09 micrograms per 100 ml). Once again these concentrations are of the order of 10^{-7} to 10^{-8}M.

Thus for the two steroid hormones experimental findings suggested that concentrations of 10^{-5} and 10^{-6}M bracketed the vast majority of the dependent cases and these were chosen for the study. They also turned out to be of the same order of magnitude as the concentrations used by many other workers. With respect to the blood levels of these hormones, it is impossible to mimic in vivo conditions precisely and in any case only two single hormone levels (oestradiol and testosterone) are being considered; under physiological conditions the blood contains several other active oestrogens (notably oestrone and oestriol) and active androgens (notably dehydroepiandrosterone and androstenedione). The total androgenic or oestrogenic activity in the blood is therefore considerably higher. Furthermore, the absolute blood levels of these hormones are not in themselves of prime importance, (as has been emphasised repeatedly), in view of the known ability of breast cancers to concentrate hormones selectively up to several-fold. Lewison et al. (1951), Folca et al. (1961) and Braunsberg et al. (1973) have amply demonstrated the ability of a proportion of breast cancers to exhibit a high oestradiol uptake, and Deshpande et al. (1963 and 1966) and Braunsberg et al. (1967a and b) have shown that a smaller proportion of these tumours can do the same for testosterone. Results from this study, as will be seen later, confirm that a certain percentage of breast cancers show enhanced activity specifically in the presence of testosterone and not of the other two hormones tested.

SETTING UP THE CULTURES

Slicing the Tumour

As soon as the biopsy arrived from the theatre it was placed in the Trowell's medium (Difco) in a 60x15 mm sterile disposable culture dish (Falcon Plastics). The whole setting up of the culture took place in a special sterile cabinet with positive pressure ventilation and an ultra-violet attachment to permit convenient sterilisation of the cabinet and its contents between experiments (Slee Laminar Flow Bench, HLF/H). Surrounding normal tissue and fat, if any, were then trimmed away and serial adjacent slices of the tumour were cut by hand, using a number 22 disposable scalpel (Gillette). The specimen was held by sterile dissecting forceps during the cutting, taking care not to traumatise it and avoiding taking a slice where the forceps might have crushed the tissue. The slices were as thin as could conveniently be cut by hand and about 2 - 3 mm in diameter.

Culture Method and Medium

It had been decided to use a maintenance culture system broadly similar to that described by Trowell (1959). His method enabled slices of normal organs and tissues to be kept alive in a chemically-defined, fully-synthetic medium for up to one week without either proliferation or de-differentiation. This proved to be a landmark in culture systems and has been used with minor modifications by a number of investigators. The details of his chemically defined medium, known as Trowell's T8 Medium, are given in the Appendix. It is entirely protein and hormone-free apart from 5 mg/100 ml insulin, (equivalent to approximately 10^{-5}M), the significance of which will be discussed later.

Trowell's method met the criteria which I outlined earlier in that (a) the tissues were permitted to remain very close to their physiological state in the body, without any proliferation, (b) no homogenisation techniques were used and (c) the medium was chemically-defined. Differences from Trowell's method included the fact that this study used malignant and not benign tissues and that they were maintained for only 24 hours instead of a week. Trowell found his medium to be perfectly adequate for a range of benign tissues but Chayen et al. (1970), in the breast cancer study to which I have already referred, found that the addition of glutathione and ascorbic acid, to concentrations of 10^{-4}M,

greatly improved the state of the stroma in short-term maintenance cultures. These were accordingly added to the T8 medium in this study.

The medium was measured into sterile plastic universal containers (Sterilin) in 10 ml amounts and glutathione and ascorbic acid were added from stock solutions to give the required 10^{-4}M concentrations. No further additions were made to the Medium Control container. The others all received appropriate amounts of 17-beta-oestradiol, testosterone and prolactin from stock solutions to give the required final concentrations. The steroid hormone and prolactin solutions had been prepared from the respective dry powders by dissolving them in 99.8% ethyl alcohol.

Sterility

Complete sterility was essential for setting up and incubating the cultures. I have already mentioned the special flow cabinet, the working surface of which could be sterilised by ultra-violet light and which was fitted with a positive pressure fan system. All plastic materials used in the experiments, including the culture dishes, the various containers and the syringes for making up solutions, were disposable and never re-used. Metallic apparatus such as dissecting forceps and the stainless-steel grids, were thoroughly washed after use, allowed to soak in Decon 90 detergent, and were than re-washed in water and autoclaved immediately prior to use. Disposable sterile scalpels were used for cutting the tumour slices.

Procedure

After appropriate labelling of a series of plastic culture dishes in the flow cabinet, (Fig.1) they were then dealt with as follows: a 25 x 25 mm stainless steel wire grid, with legs 2 mm high, was placed in each dish. This was more robust and cheaper than the tantalum used by Trowell in his earlier work. A piece of lens paper (Green's Lens Tissue No. C105) sufficiently large to cover the grid and the base of the dish was then placed over it. 5 ml of the prepared media were than poured carefully into each dish at the side of the grid. The lens paper rapidly became completely moist but the top of the grid, covered by the lens paper, remained above the surface of the fluid in the dish. The set of culture dishes consisted of: (i) a Medium Control (MC) containing Trowell's T8 medium plus 10^{-4}

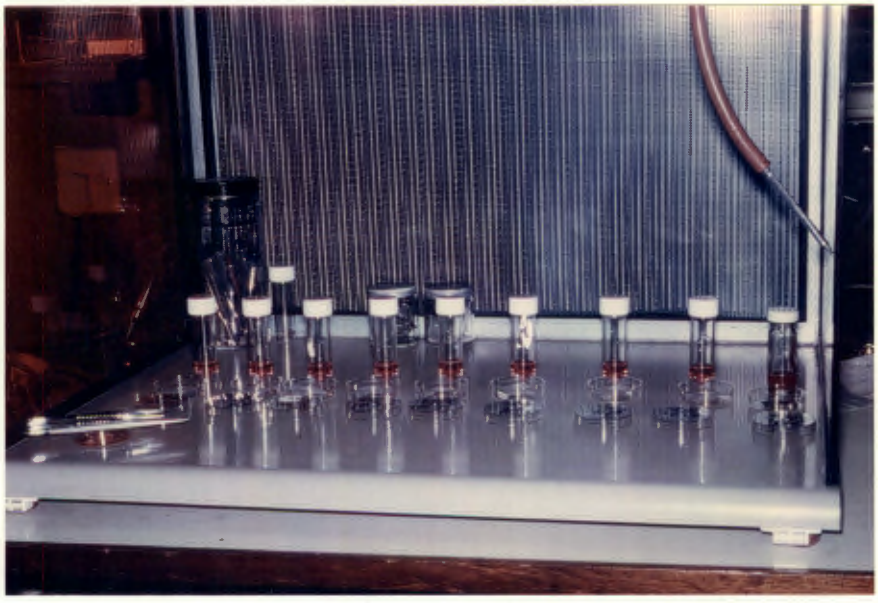


Figure 1: The set of culture dishes and prepared media in the flow cabinet. The tube for gassing the cultures is also seen.

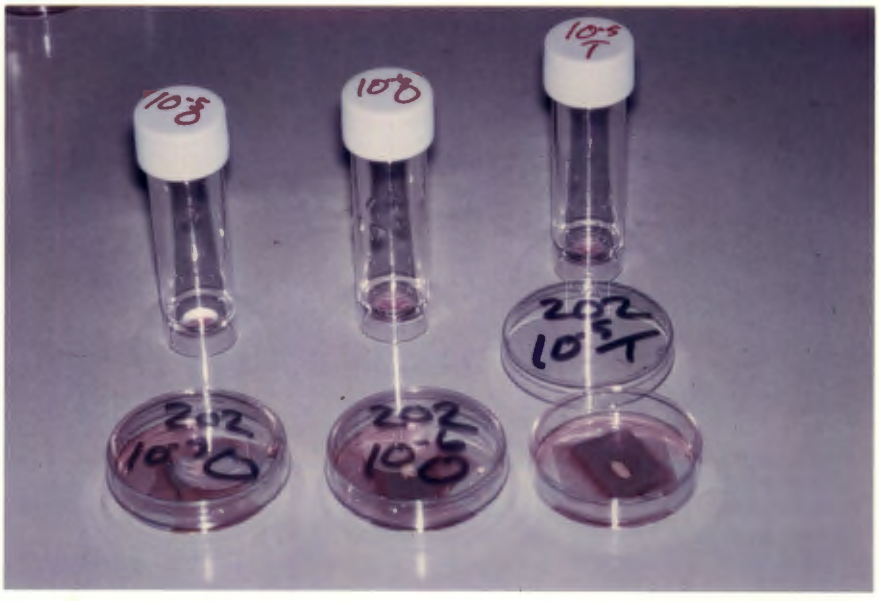


Figure 2: Examples of culture dishes with the tumour slices mounted on the lens paper on the grid.

glutathione and ascorbic acid, but without any added hormones;
(ii) Medium as above in 6 further culture dishes containing
respectively - 10^{-5}M 17-beta-oestradiol
10^{-6}M 17-beta-oestradiol
10^{-5}M testosterone
10^{-6}M testosterone
.22 I.U./ml ovine prolactin
.022 I.U./ml ovine prolactin.

A slice of the tumour was then carefully dipped into the medium and placed on the lens paper covering the grid in each dish (Fig.2). Care was taken to handle the tissue gently with fine dissecting forceps and not to transfer droplets of different media into each other.

The dishes were then covered with their lids and stacked in wire frames which were then placed in modified Kilner Jars (Fig.3). The jar lids had two vents leading out into soft plastic tubing controlled by a gate-clip for gassing. After closing the jars tightly they were gassed with a mixture of 95% oxygen and 5% carbon dioxide (British Oxygen). The gassing was controlled in such a way when closing the clips that all the air in the jars was replaced by the gas mixture but the pressure was not allowed to rise above atmospheric pressure. The jars were then placed in a Griffin Incubator at 37^{o}C and the time noted. At the same time as the dishes were incubated, the final slice of tumour was placed in a small screw-cap plastic specimen ampoule (Sterilin) for liquid nitrogen storage. This ampoule was placed in an ampoule rack (Arnold's Veterinary Products) which was then stored (Fig.4) in a liquid nitrogen refrigerator (Union Carbide). This specimen, stored with no added medium, hormones or fluid of any sort was called the Initial Control (IC). At the beginning of the project trials were conducted to see whether there was any difference between cooling the tissue in liquid nitrogen immediately as described and cooling it down gradually as is usually done for single cell suspension storage. A G.V. Planer Cooling Unit (Type R200), cooling at 2^{o}C a minute, was employed. No difference in the subsequent histochemical and histological assessments of the slices was noticed and accordingly they were routinely cooled immediately and not by the gradual method.

Repeat gassings were carried out in exactly the same manner as 8

Figure 3: The culture dishes are stacked in frames in the modified Kilner jars. One jar is being gassed.

Figure 4: Storage of tumour slices in ampoules in the liquid nitrogen refrigerator.

and 16 hours respectively and at 24 hours the incubation was ended. The tissues were then rapidly transferred from their dishes into labelled screw-top ampoules and these were immediately placed in racks in the liquid nitrogen refrigerator.

As far as the length of the incubation period was concerned, preliminary studies were again carried out. Chayen et al. (1970) had used varying periods up to 24 hours for their tumours in their small pilot study but it was clearly necessary to arrive at a standard period in order to make comparisons. After assessing survivals in the various hormones at periods of 6, 12, 18, 24, 30 and 36 hours it was considered that optimal differentiation of survival in the controls and the various media was evident at 24 hours. The differentiation was no better at 36 hours than 24 and in view of the clinical advantage of getting a result earlier a standard 24 hour incubation period was adopted.

ASSESSMENT OF SURVIVAL AND THE EFFECTS OF THE HORMONES

A suitable method was required to evaluate how the tumour had survived in the controls and the different hormones and whether in fact it had been enhanced by any of them.

Importance of Histology

Histological examination was clearly essential. Fell (1964) and Forsyth (1971) have both emphasised the importance of including histology side by side with biochemical studies, both as an aid to determine the response to substances administered, (in this case the hormones), but also to confirm that conclusions are based on satisfactory cultures and sections. One would hardly expect histological evidence of enhancement or proliferation in such a short-term maintenance system, but in fact it was easy to see when a tumour had deteriorated in a particular hormone or hormones or in the medium control. Vesiculation and vacuolation of the cytoplasm, pyknotic and deformed nuclei and areas of necrosis were readily apparent when present. Other slices had survived very well and appeared histologically just as intact as the fresh-frozen initial control (IC) which would approximate almost exactly to the state of the tumour as it was in the patient just prior to taking the biopsy. Chayen et al. came to similar conclusions with their histological assessments. In the present study there was in fact

excellent correlation between the histological and histochemical assessments, (the former being checked by an independent histopathologist). (Salih, Flax, Brander and Hobbs, 1972). However, histology was not nearly as sensitive as histochemistry in detecting minor differences in survival, nor so easy to read. Nevertheless, histology continued to be performed on all experiments both as a rough check against the histochemical results, and also for a more important reason; it was essential to confirm that each section did in fact contain tumour. Occasional biopsies, especially small ones from skin and subcutaneous metastases, were often far from homogeneous and consisted largely of collagen with very few malignant cells scattered through the specimen. If the histological check did not confirm that there was representative tumour tissue in any of the sections, and most particularly in the controls, then comparisons of activity and survival had to be considered invalid and that experiment discarded.

Selection of a Histochemical Technique

For the histochemical assessment a method based on measurement of the total dehydrogenase activity of the pentose shunt pathway was chosen. In 1953 Glock and McLean emphasised the importance of the recently discovered pentose shunt pathway in animal tissues. This - together with the classical Embden-Meyerhof glycolytic pathway - was one of the two prinicipal routes of carbohydrate metabolism (Fig. 5). As it was important in many situations to measure the relative importance and activity of these two pathways, they developed a method for assaying the two dehydrogenases of the pentose shunt, namely glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD). These enzymes catalyse reactions, as shown in the diagram, which produce large amounts of NADPH, which in turn provides energy for a variety of important cellular activities. One of the most elegant features of Glock's and McLean's method was that by using glucose-6-phosphate alone as a substrate, and working at a pH of 7.6, one was able demonstrate and measure the summation of both the G-6-P and the 6-PG dehydrogenases.

Other workers explored the role of the pentose shunt pathway in benign and malignant tissues. In 1956 Kit showed that the total dehydrogenase activity of the pentose shunt was 2 to 5 times greater in certain animal tumours than in a range of benign tissues tested,

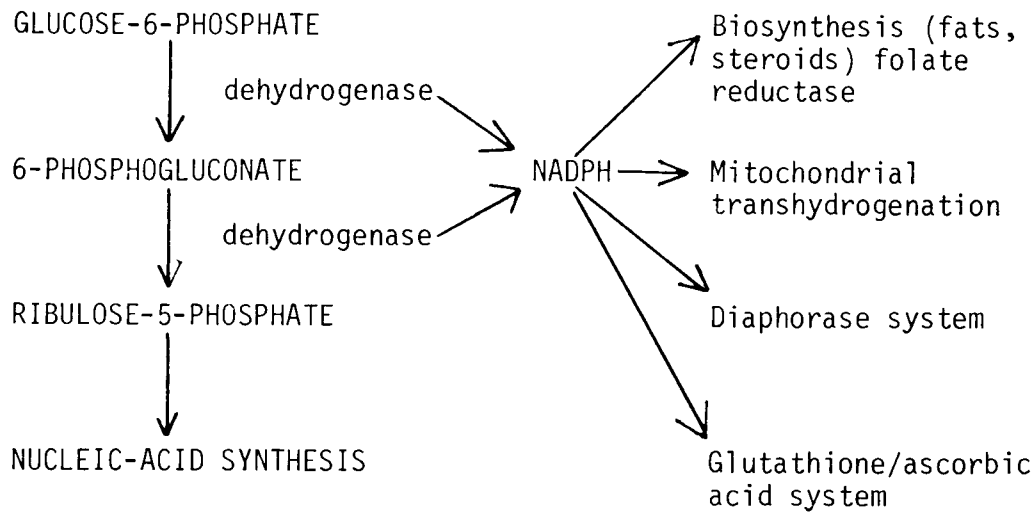


Figure 5: Simplified diagram of the pentose shunt pathway showing the routes which the NADPH, produced during the two dehydrogenations, can take.
(From: Altmann, F. P. and Chayen, J., 1967)

although activity was still relatively high in actively proliferating benign cells such as those of the reticulo-endothelial system. In 1962 Chayen et al. confirmed that 6-PGD was markedly elevated in rapidly proliferating cells, benign or malignant; it was very high indeed in tumours from several sites in humans and animals and strikingly higher than normal tissues from those sites. Considering normal tissues independently, the level was related to the degree of proliferative activity, for example it was fairly high in regenerating liver.

Following on these studies, a new test for gynaecological cancers was devised, (Bonham and Gibbs, 1962) measuring the 6-PGD in vaginal fluid. The authors claimed that this was easier to perform and more accurate than exfoliative cytology. Cameron and Husain (1965) confirmed the validity of the test in invasive genital cancer, but found that it had a serious limitation as a screening test in that many of the patients with carcinoma-in-situ did not exhibit a raised enzyme level.

Altmann and Chayen (1967), recognising the possibility of studying malignant tumours by these techniques of multi-phase biochemistry, as they were known, investigated the influence of 17-beta-oestradiol and also drostanolone (an androgen) in maintenance cultures of breast tumours and in 1970 published the short communication to which I have already referred. They suggested that certain tumours required oestradiol for complete or even enhanced survival while others did not. Altmann and Chayen emphasised the advantages of this type of approach over the conventional biochemical analyses, which involve the homogenisation and differential centrifugation of tissues. The hormones appeared to act by increasing the pentose shunt activity and their effects could then be measured directly or indirectly by appropriate techniques using one of the reactions illustrated in Figure 5 as a parameter. They themselves measured total NADPH using the diaphorase pathway which is actually a "wasteful" or unprofitable oxidative pathway for the cell in that it is not linked to any known mechanism of oxidative phosphorylation. Early work in the present project (Salih, Flax and Hobbs, 1972), confirmed that certain breast cancers required oestradiol for survival and even enhancement while others did not. In a small series of 14 cases it also appeared as if

this provided useful prognostic information for hormonal therapy. In our studies, as already mentioned, we decided - after testing both approaches - to use a direct technique, namely measuring the total dehydrogenase activity, rather than an indirect one such as the diaphorase pathway.

After our preliminary investigations of these multi-phase biochemical techniques, we then introduced a significant addition in that we began to study prolactin in this system as well as 17-beta-oestradiol and testosterone. As it turned out, in a substantial and highly significant proportion of the tumours prolactin was in fact demonstrated to enhance the dehydrogenase activity and the activity of the cells profoundly, and the technique proved to be a very convenient model to study the effects of the 3 hormones tested, (oestradiol, testosterone and prolactin). It was equally useful to study a number of other tumours and hormones.

Cutting the Sections

In order to ensure that no thawing, metabolic activity and consequent loss of enzymes occurred during mounting the blocks, a meticulous technique was observed. The block holder (No. 4035/S Slee), was placed in a small plastic well containing liquid nitrogen, surrounded by polystyrene insulation, and enough water was pipetted on to the top of the block holder to cover it. Only as the water began visibly freezing was the slice of tissue removed from its ampoule and rapidly mounted (Fig.6). Freezing was complete within 5 - 10 seconds after mounting the tissue and the block holder was then placed in the cryostat chamber.

Once all the slices, including the initial control (IC), were mounted and in the cryostat chamber, eight micron sections were cut in quadruplicate from each slice. The cryostat used was the Slee Retracting Microtome, Type HR, operating between -25^{o} and $-35^{o}C$. As each section was mounted on its appropriately-labelled slide, it was placed in a steel slide rack in the cryostat chamber, where it remained until the histochemical or histological reactions were performed.

On cutting each slice of tissue, care was taken that a sufficient depth in the tissue was reached and rapid preliminary methylene blue stains were performed to ensure that representative tumour tissue was indeed being cut before the definitive sections were taken.

Figure 6: After removal from liquid nitrogen, each slice is rapidly mounted on a block holder for cutting cryostat sections.

Of the four sections taken of each slice, two were set aside for histology and two for histochemistry.

Histology

On the one set of slides conventional haematoxylin and eosin staining was then carried out. I have already indicated that the prime aim of histology was to ensure the presence of satisfactory tumour tissue in each section, while it was also useful as a rough parallel with histochemical assessment of activity. Routine mounting under a coverslip with Canada Balsam was then followed by microscopic examination.

Histochemistry

A volume of 7 ml of the reagent mixture was found to be adequate for the number of sections to be treated. Accordingly, the following weights of reagents were prepared in flat-based plastic ampoules using an Instanton Electric Balance (Unimatic CL4D). (The concentrations are given in parentheses). Sources of the chemicals used are given in the Appendix.

0.021 grams Neotetrazolium Chloride (3 mg/ml): This served as the final electron acceptor, being reduced to highly-coloured insoluble formazan, which is the material seen under the microscope.

0.0105 grams Glucose-6-Phosphate (1.5 mg/ml): This is the substrate for the reaction and as mentioned previously, at pH 7.6 the use of this substrate alone permits measurement of the total dehydrogenase activity of both G-6-PD and 6-PGD.

0.007 grams Phenazine Methosulphate (0.1 mg/ml): This is the first electron acceptor from the reaction of the dehydrogenases on G-6-P; it then transfers the electrons to the neotetrazolium chloride.

0.014 grams NADPH (2 mg/ml): This serves as the co-enzyme for both stages of the electron transfer.

1.4 grams Polyvinyl Alcohol ("polyviol" or PVA) (25% w/v): Over the period of one hour's incubation it is obviously undesirable for the enzymes to diffuse widely from the cells. Altmann and Chayen (1965) showed that one of the problems of enzyme histochemistry is that if the tissue is not fixed, in order to avoid denaturation, then enzymes may dissolve out of the section into the incubation medium. On the other hand, if it is fixed to stop such a loss of soluble enzymes then activity

is inhibited and denaturation occurs. This applies strongly to dehydrogenase enzymes. They then showed that by using PVA, cytochemically a very inert substance, at a 20% w/v concentration, it was very effective in preventing loss of any nitrogenous matter out of tissue sections, and in particular it permitted soluble enzymes to be retained during enzyme histochemical reactions, without the use of any chemical fixative which would inactivate or denature the enzymes. In this study 25% w/v was found to be even more effective.

0.1M glycylglycine buffer was used to keep the mixture at a pH of 7.6, and for the number of sections studied it was convenient to use a volume of 7 ml.

Procedure

Five of the 7 ml of glycylglycine buffer, kept at 37°C in the incubator, were used to dissolve the polyviol, stirring with a glass rod. The G-6-P, NADP and neotetrazolium chloride were then poured into the dissolved polyviol. At this stage the sections were removed from the cryostat chamber and left at room temperature. The remaining 2 ml of buffer were added to the reagent mixture and the phenazine methosulphate, after being dissolved in .7 ml of distilled water, was also added to the buffered mixture. After thorough stirring the mixture was then ready for adding to the sections.

The slides were first placed in the incubator and a circular plastic ring half an inch in diameter was placed over each section. The mixture was then poured from the small 25 ml beaker in which it had been prepared into each small ring, about 3 - 4 drops being found to be sufficient to cover each section completely (Fig.7). The mixture was of a syrupy consistency, due to the polyviol, and this enabled it to remain confined to the area enclosed by the ring and not to leak away under it, thereby obviating the need for any form of sealing. Incubation at 37°C was then carried out for exactly one hour, after which time the slides were washed in distilled water for 10 minutes in a dark cupboard. Coverslips were then mounted over the sections using Farrant's medium. Time course studies at 15, 30, 45 and 60 minutes had been carried out initially. These had shown that incubation for one hour gave optimal results.

All the above chemicals were kept in the ordinary refrigerator

Figure 7: Pouring the histochemical reagent mixture into the ring chamber over the sections.

when not in use, apart from the glycylglycine buffer which remained in the incubator and which was regularly checked to ensure that its pH remained at 7.6 (pH Meter PHM 28, Radiometer, Copenhagen). One particular precaution to be observed was that the phenazine methosulphate, a chemical that is very sensitive to light, had to be kept in a dark place during storage and had to be exposed to light for as short a period as possible during weighing. Similarly, it had to be dissolved quickly on addition to the reagent mixture, which was then poured on to the sections in the incubator without delay.

Evaluation of the histology and histochemistry and of the overall results will be described in detail in the next chapter.

PATIENT FOLLOW-UP

I have indicated previously that this study was initially concerned with the development of a technique and not a clinical trial, but it was of course highly desirable to watch the clinical progress of each patient whose tumour had been tested. No attempt was made to conform to any method of patient selection or treatment protocol, although the results of the hormone-dependence studies were available to the clinician as an extra aid to selection of therapy if he so wished.

Documentation

As soon as a specimen was received by the laboratory a trained nursing sister employed by the Tumour Biology Group interviewed the patient and studied the clinical notes. A standard pro forma was completed for each patient, embodying all the relevant personal data as well as the specific details of the breast lump. On an attached sheet the whole of the clinical history was summarised, including all the management to date and all histological reports. These clinical notes were then brought up to date by the nursing sister or myself each time the patient attended a clinic.

In the case of patients who had not yet suffered recurrence of early breast cancer and who were under routine follow-up surveillance by the various consultant surgeons, arrangements were made for me to see the notes after each visit.

However, all those with advanced or recurrent breast carcinoma were seen by me personally, with the exception of a few private patients

and patients who had gone abroad subsequent to treatment. A special Breast Clinic was held every Wednesday afternoon where each patient was seen jointly by Dr K. Newton, Consultant Radiotherapist, Mr K. Robinson, Consultant Surgeon, and myself.

Response to Treatment

In the Results section data concerning the menstrual status, biopsy site and stage of the disease will be presented in relation to the hormone dependence studies. At the time of writing most of the Stage I and II patients tested have not yet required anything further than their primary treatment. Many of the others have either received non-hormonal treatment, or else hormonal plus other treatment. However, in a number of cases it has been possible to assess the correlation of the in vitro testing with the response to hormonal treatment. Illustrative examples of these will be given in the Discussion and an attempt will be made to evaluate the accuracy of prediction.

In assessing the response to hormonal treatment, I have insisted on objective improvement of all visible, palpable and radiological lesions before accepting it as a regression. Symptomatic relief alone has not been counted as regression, nor has mere arrest in the development of the lesions . Furthermore, the objective improvement has had to be maintained for at least three months. For the purposes of this study I have not included any intermediate partial regression group; situations such as the regression of some lesions and not of others will be specifically mentioned where relevant.

At the time of writing this thesis, it is gratifying to note that these early clinical correlations have proved sufficiently encouraging to warrant the institution of a formal prospective trial at the Westminster Hospital to test whether this prediction technique does indeed offer the patient a better chance of successful treatment than the 30% she is confronted with at present.

RESULTS

SURVIVAL SCORING ON A HISTOLOGICAL BASIS

After examination of each section by ordinary light microscopy under low and high-power objectives, it was given a score based on the absence or extent - if any - of degenerative and destructive changes in the tissue and cells, such as cytoplasmic vacuolation, pyknosis of the nuclei and frank necrosis. Perfect survival was assigned a maximum score written as ++++, while lesser degrees of survival received fewer plus symbols down to complete death of the tissue, which was assigned a minus symbol. A half was designated by a plus symbol in brackets.

SURVIVAL SCORING ON A HISTOCHEMICAL BASIS

The visible end-product of the histochemical reaction was a highly-coloured red-black dot of formazan, as seen by ordinary low and high-power light microscopy. The more active the tumour, (and therefore the total dehydrogenase activity of the pentose shunt), the denser the aggregations of formazan dots. It is possible to assess this density semi-quantitatively and at the start of the project a few experiments were thus assessed using the Quantimet 70 machine, which computed and compared the densities of the dots in a series of fields, but in nearly all cases direct visual assessment proved to be simple. If the method or some modification of it does eventually become widely-used, however, the availability of a means of automatic assessment might prove an attractive advantage.

Density of formazan was also scored with a system of +,(+) and - symbols, ++++ being the maximum. Total absence of any histochemical activity was assigned a minus symbol.

"DEPENDENCE" AND "INDEPENDENCE"

Generally speaking, the fresh-frozen initial control(IC) was relatively very active, the medium control (MC) was usually (but not always) much less active, often showing frank death of the tumour and zero activity histochemically, while the slices in the different hormones showed varying degrees of activity. There was good correlation

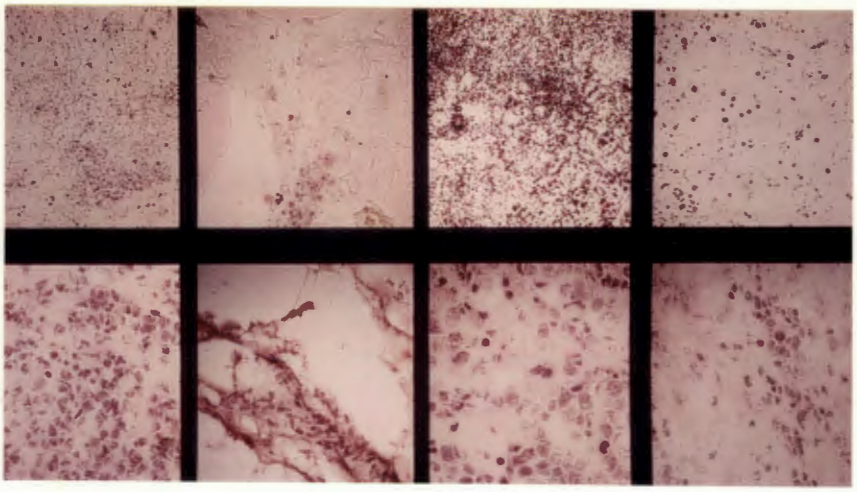
between histological and histochemical evidence of survival and degeneration (Salih, Flax, Brander and Hobbs, 1972), but when it came to detecting subtle differences or activity that was enhanced over that of the controls, histochemistry proved the main yardstick. In over half the 150 cases, the activity of the tumour in one and sometimes two of the hormones was clearly greater than that of both controls, (IC and MC), and these cases were defined as "Dependent." Examples are given in Figures 8, 9 and 10. The remaining cases, which had not shown enhanced activity compared with both controls, were defined as "Independent" (Figure 11), though they had often (although not always) fared better than the medium control, suggesting that these tumours were certainly not always unresponsive to the effects of these hormones in vitro.

Spectrum of Activity in the Independent Group

In this Independent group, then, there was a whole spectrum of activity; a particular tumour might have shown moderate activity in the IC yet poor survival in the MC and equally poor survival in all the hormones tested, suggesting that at least in vitro this tumour did not appear to be influenced by hormones. Another tumour might have been much more active than the MC in one or two of the hormones, (e.g. prolactin and testosterone), but if it was no more active than the IC it was deemed independent by our criteria. The same tumour might have fared so badly in oestradiol as to show signs of complete death of the tissue. It is thus important to bear in mind throughout the Results and Discussion that "independence" of a tumour does not necessarily imply that it is totally influenced by any of the three hormones tested. On the contrary, where a particular hormone causes striking tumour inhibition in vitro, its administration to the patient often met with a favourable response.

Hormonal Profile Concept

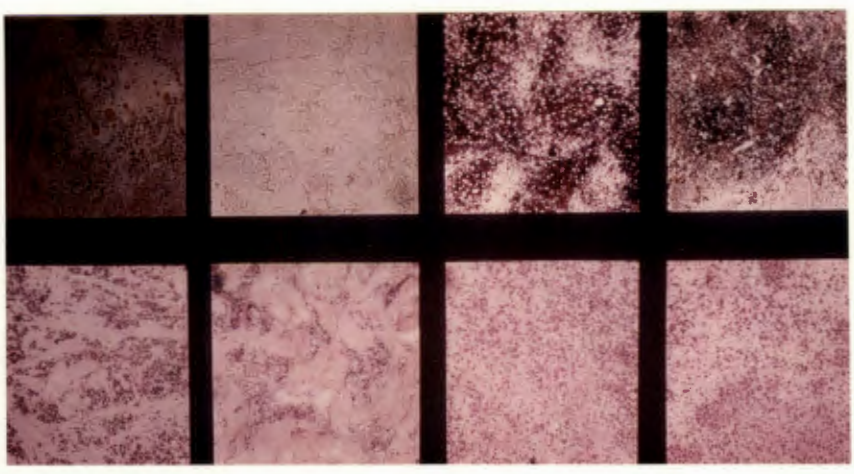
In view of the spectrum of response, the test could be regarded as giving a hormonal profile of the tumour rather than a rigid classification into "Dependent" or "Independent," which are not entirely satisfactory words for use in this disease, despite having been hallowed by long usage. They were only used in this study because it was realised that the results of the test might be taken into consideration when endocrine-ablative surgery, (a much more radical step than



(a) (b) (c) (d)

Figure 8: Composite histological and histochemical photomicrographs* showing a tumour that was dependent on 17-beta-oestradiol at 10^{-5}M.
(a) Initial Control (b) Medium Control (c) 10^{-5}M 17-beta-oestradiol (d) 10^{-6}M 17-beta-oestradiol

*All photomicrographs were taken at a magnification of 375x. In the composite pictures the upper line illustrates the histochemical results and the lower one the histology.



(a) (b) (c) (d)

Figure 9: Histology and histochemistry clearly demonstrate that this tumour is prolactin-dependent at both concentrations.
(a) Initial Control (b) Medium Control (c) 0.022 I.U. per ml ovine prolactin (d) 0.022 I.U. per ml ovine prolactin

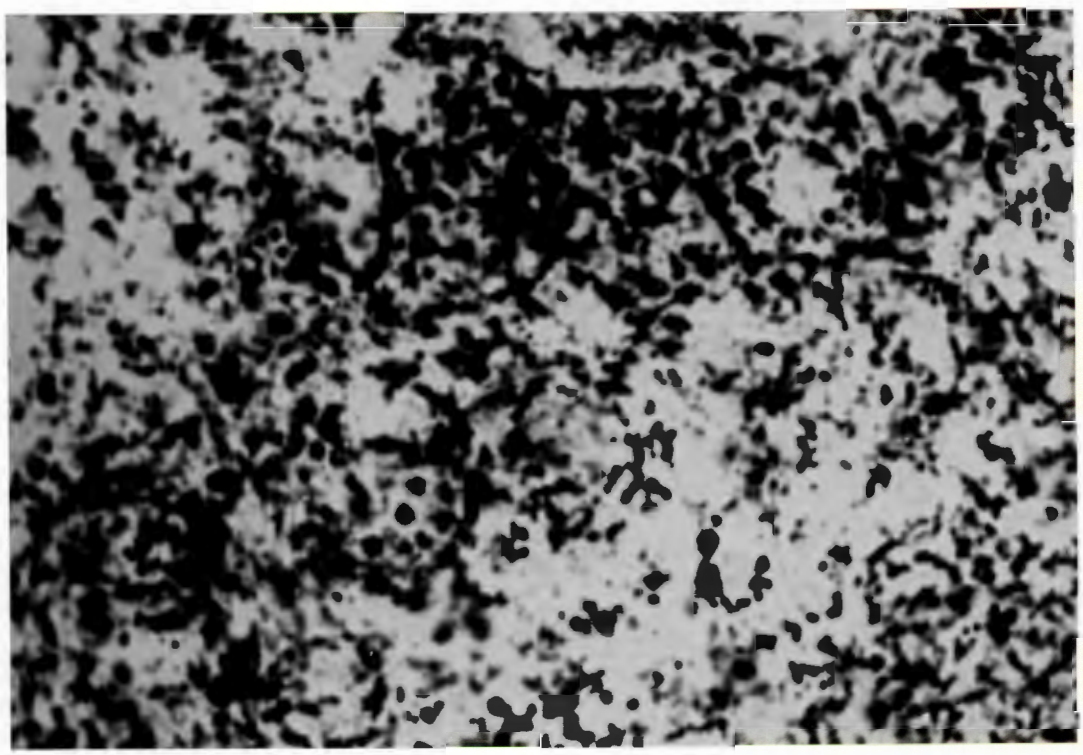
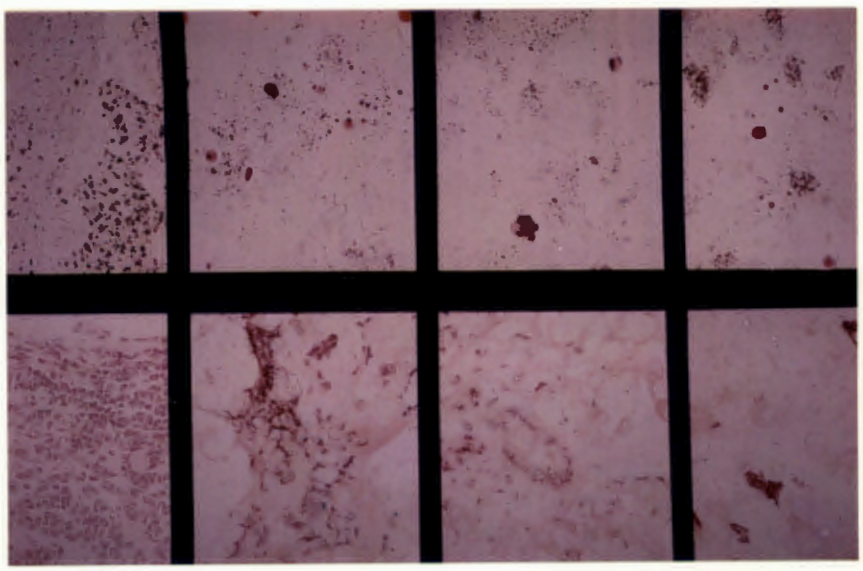


Figure 10: A black and white enlargement of a prolactin-dependent example to show detail of the formazan deposition, which is always related to the tumour cells only.



(a) (b) (c) (d)

Figure 11: This is an example of an independent tumour. For the sake of compactness only the survivals in the two concentrations of oestradiol are illustrated. The tumour survived equally badly in the other two hormones and the appearances were identical. (a) Initial Control (b) Medium Control (c) 10^{-5}M 17-beta-oestradiol (d) 10^{-6}M 17-beta-oestradiol

simple hormone administration), was being contemplated. It was thus desirable to have a strict, clear-cut definition so that one could be sure, (or at least as sure as possible within the limitations of an in vitro experiment), that the "dependent" cases stood a high chance of responding to such surgery. "Dependence" was accordingly defined as enhanced activity compared with both controls; the tumour not only had to be more active after culture in a particular hormone than after culture in medium alone, but it also had to be unequivocally more active than its state when freshly-removed from the patient.

It is therefore readily conceded that after long-term prospective evaluation of the test and its clinical correlation, it may be appropriate to draw a different dividing line or even to assess the results in a completely different way, for example, a hormonal profile instead of hormonal dependence. The fact that other as yet untested hormones, such as progestogens and growth hormone, may also have to be taken into account, could complicate the interpretation of results still further. For the present study however, it was deemed best to retain the clear-cut criterion of in vitro dependence described.

OVERALL IN VITRO HORMONE DEPENDENCE OF 150 FEMALE BREAST CANCERS

Dependent on Prolactin only	24	(16%)
Dependent on Oestradiol only	15	(10%)
Dependent on Testosterone only	14	(9%)
Dependent on both Prolactin and Oestradiol	16	(11%)
Dependent on both Prolactin and Testosterone	10	(7%)
Total Prolactin Dependent Tumours	50	(33%)
Total Oestradiol Dependent Tumours	31	(21%)
Total Testosterone Dependent Tumours	24	(16%)
Total Dependent Tumours	79	(53%)
Total Independent Tumours	71	(47%)

The table shows that 53% of the 150 tumours studied were able to thrive in one or two hormones to the extent that they were more active than both controls. The most dramatic finding is the high incidence of prolactin dependence. Thus 24 of the 150 tumours (16%)

were dependent on prolactin alone while a total of 50 (33%) were dependent either on prolactin alone or on prolactin as well as one of the steroid hormones. As indicated previously, the independent group covered a whole spectrum of hormonal responses in vitro, but no case in this group showed higher activity than both controls.

ANALYSIS ACCORDING TO MENSTRUAL STATUS

The patients have been considered in 3 groups; premenopausal, less than 5 years postmenopausal and over 5 years postmenopausal. Results were as follows:

	Premenopausal	Less than 5 Years Post-menopausal	Over 5 Years Postmenopausal
Dependent on Prolactin only	7	5	12
Dependent on Oestradiol only	4	4	7
Dependent on Testosterone only	1	1	12
Dependent on both Prolactin and Oestradiol	5	3	8
Dependent on both Prolactin and Testosterone	0	2	8
Total Prolactin Dependent Tumours	12	10	28
Total Oestradiol Dependent Tumours	9	7	15
Total Testosterone Dependent Tumours	1	3	20
Total Dependent Tumours	17	15	47
Total Independent Tumours	8	20	43

Considering the 150 as a whole, it is found that in the premenopausal group there were more dependent than independent tumours in the ratio of about 2:1. In the less than 5 years postmenopausal group more were independent than dependent, in the ratio of about 4:3. There was some increase in dependence again in the over 5 years postmenopausal

group, the proportions being approximately equal. Comparing these ratios in the 3 groups one finds that there is a tendency for dependence to be least common in the less than 5 years postmenopausal group, although the numbers are not highly significant (chi-square = 3.72 with 2 degrees of freedom; p = 0.16). This tendency is interesting in that it supports the well-known clinical experience that hormonal therapy is least likely to be successful in the period at and immediately following the menopause.

If we extract from the above table the total number of prolactin, oestradiol and testosterone-dependent results in each of the three groups, they may be compared overall and in pairs.

	Premenopausal	Less Than 5 Years Postmenopausal	Over 5 Years Postmenopausal
Prolactin	12	10	28
Oestradiol	9	7	15
Testosterone	1	3	20

Comparing the three overall to see whether there are any differences between the groups of dependent subjects, there is a significant probability that there are; chi-square = 8.19 (4 degrees of freedom), p = 0.08. This indicates that it is worth comparing them in more detail to see where the difference lies.

Comparing the premenopausal group with the less than 5 years postmenopausal group, there is no significant difference (p = 0.51). Similarly, there is no significant difference between the two postmenopausal groups (p = 0.31). However, if we compare the premenopausal group with the over 5 years postmenopausal group, there is a highly significant difference in the dependence ratios; chi-square = 6.93 (2 degrees of freedom), p = 0.03.

It can be seen at a glance that this difference in dependence ratios is due to (a) a greater incidence of prolactin dependence in the premenopausal group; (b) a greater incidence of oestradiol dependence in the premenopausal group, and (c) a considerably greater incidence of testosterone dependence in the over 5 years postmenopausal group (20 out of 63 as compared with 1 out of 22 in the premenopausal group). This difference is even more striking in that the 1 testosterone-dependent patient in the premenopausal group had had androgen therapy with durabolin

for several months before testing.

There would thus appear to be a very real trend of alteration in the distribution of in vitro hormone dependence of breast cancers from the premenopausal patients to those over 5 years postmenopausal. The group less than 5 years postmenopausal lies in a transitional zone in which all hormone dependence seems to be less common and in which profound re-adjustments are taking place. When the definitive post-menopausal pattern stabilises and the incidence of hormone dependence again begins to rise, the ratios of dependence have altered consider-ably from the premenopausal situation. The whole question of a tumour's capacity to adapt dynamically to an altered hormonal milieu will be discussed in a later Section.

EFFECT OF ENDOCRINE-ABLATION PRIOR TO TESTING

Thirty four of the patients had had an endocrine-ablative procedure at some stage (months or years) before tumour culture was carried out. Only 1 of the 34 had had an ablation (bilateral oöphorectomy) for gynaecological reasons before breast cancer became apparent. The others had all had the procedures for breast cancer. The details are shown below:

Radiation Menopause	11
Bilateral Oöphorectomy	14
Bilateral Adrenalectomy and Oöphorectomy	5
Bilateral Adrenalectomy alone	2
Hypophysectomy alone	1
Bilateral Oöphorectomy and subsequent Hypophysectomy	1

The table on the following page shows the dependences of the post-ablation group. Considering this group in terms of the number of years after the procedure, (i.e. after they stopped menstruating), the subgroups are too small for comparisons to be significant ($p = 0.58$), but it is interesting that we again see the same trend as in the series of 150 as a whole, namely that dependence appears to be less common in the 5 years after ceasing menstruation than

afterwards.

DEPENDENCES IN THE POST-ABLATION GROUP

	Overall	Less than 5 years After Procedure	Over 5 years After Procedure
Dependent on Prolactin only	6	3	3
Dependent on Oestradiol only	2	1	1
Dependent on Testosterone only	2	1	1
Dependent on both Prolactin and Oestradiol	3	2	1
Dependent on both Prolactin and Testosterone	2	0	2
Total Prolactin Dependent Tumours	11	5	6
Total Oestradiol Dependent Tumours	5	3	2
Total Testosterone Dependent Tumours	4	1	3
Total Dependent Tumours	15	7	8
Total Independent Tumours	19	14	5

By breaking down the post-ablation group further in terms of the three main specific procedures, as illustrated in the following table, the sub-groups become even smaller and less significant, but it is interesting that the patients who had had only ovarian function ablated, (either surgically or by radiation), nonetheless had a spread of dependences, including oestradiol, prolactin and testosterone. This might logically be expected since adrenocortical function would maintain at least a small amount of circulating steroid hormones. Of the 5 patients who had had both the ovaries and the adrenals removed, however, (i.e the bulk of the steroid-secreting tissues in the body), 4 were independent and 1 was dependent on prolactin, the secretion of which had not been interrupted, (although it could have been affected by feedback mechanisms). Following

the ablation the prolactin dependent case had originally had an excellent objective regression, while of the 4 independent cases 2 had regressed and 2 had not.

Two further postmenopausal patients had had bilateral adrenalectomy alone and their tumours were also independent, as was the tumour of 1 patient who had had a hypophysectomy a long time prior to tumour culture. Curiously enough, the recurrent tumour of a patient who had had a bilateral oöphorectomy and subsequently a hypophysectomy, both with good regressions, turned out to be testosterone-dependent and was in fact kept under control by oestradiol administration. In the context of this last case, it is interesting that Jantet et al. (1963) measured the degree of adrenocortical atrophy after hypophysectomy and found that it bore no relation to the efficacy of hypophysectomy in producing a regression. This is relevant in that if adrenocortical function persists to some extent after hypophysectomy, it could produce steroid hormones which might still sustain the tumour.

	Radiation Menopause	Bilateral Oöphorectomy	Bilateral Adrenalectomy and Oöphorectomy
Dependent on Prolactin only	1	4	1
Dependent on Oestradiol only	1	1	0
Dependent on Testosterone only	0	1	0
Dependent on both Prolactin and Oestradiol	1	2	0
Dependent on both Prolactin and Testosterone	1	1	0
Total Prolactin Dependent Tumours	3	7	1
Total Oestradiol Dependent Tumours	2	3	0
Total Testosterone Dependent Tumours	1	2	0
Total Dependent Tumours	4	9	1
Total Independent Tumours	7	5	4

INFLUENCE OF CLINICAL STAGE AT THE TIME OF BIOPSY

All patients were routinely assigned a clinical stage prior to biopsy. The widely-used convention was followed whereby Stage I referred to an uncomplicated mobile breast lump alone, less than 5 cm in its greatest diameter; Stage II implied mobile homolateral axillary nodes in addition to the breast lump; Stage III included any local, axillary or supraclavicular extension of the disease beyond Stages I and II, but without distant dissemination, and Stage IV referred to distant dissemination of the disease, regardless of the condition of the primary. The same convention was followed in cases of recurrence. The in vitro dependences were as follows:

	I	II	III	IV
Dependent on Prolactin only	3	2	8	11
Dependent on Oestradiol only	1	2	8	4
Dependent on Testosterone only	2	0	8	4
Dependent on both Prolactin and Oestradiol	2	4	3	7
Dependent on both Prolactin and Testosterone	4	1	3	2
Total Prolactin Dependent Tumours	9	7	14	20
Total Oestradiol Dependent Tumours	3	6	11	11
Total Testosterone Dependent Tumours	6	1	11	6
Total Dependent Tumours	12	9	30	28
Total Independent Tumours	10	7	26	28

The above table indicates that there does not seem to be any significant difference in the ratios of dependence in any of the clinical stages and this is borne out by statistical analysis ($p = 0.54$).

INFLUENCE OF THE SITE OF BIOPSY

Biopsies were taken from primary and metastatic tumour sites, the latter including lymph nodes, skin and subcutaneous deposits and also a few visceral metastases. These were classified in the two groups and the results were as follows:

	Primary	Metastatic
Dependent on Prolactin only	9	15
Dependent on Oestradiol only	8	7
Dependent on Testosterone only	4	10
Dependent on both Prolactin and Oestradiol	9	7
Dependent on both Prolactin and Testosterone	7	3
Total Prolactin Dependent Tumours	25	25
Total Oestradiol Dependent Tumours	17	14
Total Testosterone Dependent Tumours	11	13
Total Dependent Tumours	37	42
Total Independent Tumours	33	38

The table indicates that the site of biopsy did not affect the dependence ratios and statistical analysis confirms the lack of any significant association ($p = 0.69$). The dependences have been further analysed according to the specific origin of the biopsies, (i.e. skin, subcutaneous tissue or lymph nodes,) but this also revealed no differences. It should be noted that apart from a few experimental cultures, which have not been included in this series, this technique has not yet been evaluated for bony metastases.

D I S C U S S I O N

I propose to commence the Discussion with an assessment of the value and advantages of the technique itself.

(a) It is a relatively simple procedure that requires no elaborate apparatus and could be established in any conventionally equipped hospital laboratory.

(b) The time taken for the whole experiment consists of 24 hours of incubation of the culture plus about 2 hours for the various technical steps. The fact that a useful answer as to hormonal-dependence of an individual patient's tumour can be provided as soon as 26 hours after taking the biopsy is of great value to the clinician in a situation where he is anxious to start treatment for advancing disease. Equally, it would be very useful where one is considering employing adjuvant hormonal manoeuvres after primary surgical treatment of the early operable tumour.

(c) The tumour remains as close as possible to its natural condition in the patient. Since this is only a short-term culture with no proliferation and therefore no alteration of characteristics that daughter generations of cells might have, it enhances the value of conclusions drawn from these studies as compared with others lasting several days.

(d) The absence of any form of mechanical or physico-chemical preparation of the tumour before culture, as is the case with single-cell suspensions and other cell culture methods, is an obvious advantage tending to create more physiological conditions.

(e) A very important asset of this technique is that the histo-chemical reaction enables assessment of the carcinoma cells alone. Using techniques preparing single cell suspensions, one is unable to obtain a 100% survival of all the tumour cells and in addition it is impossible to be certain which of the surviving cells are malignant and which are from normal breast epithelium or the connective tissue element in the tumour. In this technique one can be absolutely certain that the formazan deposition is in relation to the cancer cells. Other assays in relation to a given mass of tumour are subject to errors arising from the varying content of stroma, oedema fluid and the allowances made for dry and wet weight.

(f) Parallel assessment of duplicate histological sections ensures that conclusions are based only on representative tumour.

(g) Oestrogen-binding to breast cancer lasts for only 5 hours (Deshpande, 1972), and therefore the present test allows time for the oestradiol level in the culture fluid to over-ride any pre-existing steroid bound in vivo at the time of the biopsy. Prolactin may bind for longer periods, (about 8 hours, personal communication from Dr. H. Friesen, 1973) but again it would seem that the culture fluid level can dominate by 24 hours. The test therefore offers the chance to see the true effect of chemically-defined medium upon the breast cancer tissue.

(h) Tumour culture methods in general offer one notable advantage over other hormonal prediction systems based on urinary or blood levels of hormones in that they measure, directly and unequivocally, the effect of a given hormone on the target tumour cells. In vivo the situation is considerably more complicated because of the multiplicity of hormones, feed-back mechanisms and fluctuations in secretions and levels. This is of course to some extent counter-balanced by the extreme caution with which one tries to extrapolate in vitro findings to the in vivo situation, the validity of which can in the final analysis only be established by carefully-controlled forward clinical trials.

(i) The technique has been extremely reliable. Pentose shunt dehydrogenase activity has accorded very well with the survival of the tumour and seems to be an important marker of neoplastic capacity. I have already discussed the two important technical problems, namely an inadequate biopsy and contamination, and these are both related to taking of the biopsy. As far as the technique itself is concerned, one can confidently state that given a good, sterile specimen a short time after biopsy, the experiment will reliably and reproducibly "work".

(j) Last but by no means least, this technique has made possible the first major study of the effects of prolactin on <u>human</u> breast cancer, and it has revealed, for the first time, that over 30% of human breast cancers are enhanced in vitro by prolactin.

REJECTED EXPERIMENTS

In addition to a number of experiments on other tumours, which will be mentioned later, and on breast biopsies that turned out to be benign or even secondaries from other primary tumours, 21 primary or metastatic breast cancer biopsies were rejected for technical reasons. Two tumours were non-viable on arrival, having been delayed $2\frac{3}{4}$ and $4\frac{1}{2}$ hours respectively after biopsy. They showed no histochemical activity on completion of the culture and marked deterioration histologically.

A further specimen that had been delayed a little over an hour before reaching the laboratory revealed a number of bacterial colonies on microscopy after culture and this experiment was accordingly rejected, although the enzyme reaction had worked. One other biopsy reached the laboratory in less than an hour and was taken from a clean-looking lesion, but this also showed some bacterial contamination after culture and was therefore rejected.

Of the remaining 17 biopsies, 11 were discarded because (a) there was no tumour discernable at all on histology, (b) there were so few tumour cells scattered sparsely within an abundant collagenous stroma (usually in skin or subcutaneous metastases) that it was impossible to compare the slices, or (c) the specimen was simply too small to permit the cutting of enough slices to carry out the complete experiment.

The final 6 were discarded because the biopsy came from a frankly infected or even ulcerating lesion, or from very near it. These specimens showed varying degrees of survival after culture but it was impossible to draw any valid conclusions from them.

On several occasions, after a first biopsy had proved unsatisfactory because of too little tumour in the specimen, a subsequent biopsy from a more representative lesion, or after a period of adequate dressings and topical or systemic antibiotic treatment, was successful.

In general, it was very easy to decide which experiments should be rejected. Histological examination permitted immediate rejection of any experiment in which each slice did not contain adequate tumour tissue. The same applied to any experiment where any of the sections revealed colonies of bacteria. Very often the decision could be made before the stage of microscopy by finding that the medium had changed colour after incubation from the normal bright-red to a pale yellow-brown.

This was because the phenol red indicator had changed due to a drop in the pH from the usual 7.6 to a more acidic level, as a result of bacterial activity. This gross change was usually associated with biopsies that were known to have been taken from infected lesions.

The technique thus has built-in safeguards, notably histological examination and the pH indicator, to prevent acceptance of any results based on unsatisfactory experiments.

SURVIVAL OF THE MEDIUM CONTROL

Although in the majority of cases the medium control showed markedly lower dehydrogenase activity than the initial control, and corresponding evidence of deterioration histologically, in 22 of the 150 cases it actually showed enhanced enzymic activity and no deterioration on histology compared with the initial control. There is reason to attribute the good survival in these cases to the insulin in the medium. Trowell's T8 medium contains 5 mg/100 ml insulin, (5 microgram/ml), and several workers have demonstrated the effect of insulin in mammary gland cultures. In mouse mammary glands insulin is absolutely necessary in vitro for the survival and maintenance of the cultures, and biochemically it has been shown to have a stimulatory effect on DNA and RNA synthesis, quite independent of its effect on glucose transport. (Forsyth, 1971). It also stimulates both glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) in the pentose shunt pathway (Leader and Barry, 1969), which are precisely the enzymes being measured in the present technique. Barker, Fanger and Farnes (1964) showed that normal adult human breast tissue slices could be kept alive in simple media for a period of up to about 5 days, without addition of serum or any hormones. However, addition of insulin produced striking proliferative changes in the morphology. Ceriani, Contesso and Nataf (1972) confirmed that for human breast cancer cultures as well as those of animal and normal human breast, insulin allowed more complete maintenance of the tissue and even some enhancement, which was more marked when oestradiol or ovine prolactin were added to the insulin-containing cultures.

In the 22 cases mentioned, therefore, it seems highly likely that these are examples of insulin alone providing a powerful stimulus to growth and metabolic activity, manifesting in this particular system

by increased dehydrogenase activity of the pentose shunt and by no evidence of deterioration after culture in the medium only. It is interesting that two of the patients among these 22 had repeat biopsies several months later and these again showed higher activity in the medium control than the initial control, thus supporting the reliability of the technique.

These examples of insulin enhancement of the medium control underline the need to compare the cultures in the 3 hormones under investigation with both the medium as well as the initial control before evaluating their effects.

PROLACTIN DEPENDENCE

Perhaps the most striking single result of the whole project has been the remarkably high incidence of in vitro dependence on prolactin. This has potentially sweeping clinical implications. It immediately offers some explanation, if prolactin dependence is indeed a valid concept in vivo, as to why a substantial percentage of patients fail to respond to hormonal measures involving oestrogen or androgen addition or subtraction only. It may also help to explain the clear demonstration by the Guy's Group (Atkins et al., 1960; Hayward et al., 1970), that transfrontal hyophysectomy gave the best remission rate of all ablative procedures; the difference may well be accounted for by complete removal of prolactin secretion in prolactin-dependent cases. Further indirect supporting evidence comes from the well-known clinical observation that some patients who have escaped from remissions induced by oöphorectomy or adrenalectomy may have a further remission following hyophysectomy. The experimental finding, in this relatively large series of cases, that prolactin, (both ovine and human), can enhance the activity of breast cancer cells above their control level in the fresh-frozen biopsy immediately after removal from the patient, provides powerful support for its role in human breast cancer.

Plasma Prolactin Levels

There is still a good deal of uncertainty about the plasma levels of prolactin. At present there is no clear evidence at all that human breast cancer patients have higher plasma prolactin levels than matched controls. The Cardiff group (Forrest, 1972), found no significant differences in prolactin levels in 48 patients of whom 6 had benign breast

disease, 10 primary breast cancer and 32 advanced breast cancer. There was also no significant difference in this series between pre- and postmenopausal patients. Although there is abundant evidence by now that increased prolactin secretion accelerates the growth of induced breast cancers (Meites et al., 1972), there is also evidence that in spite of the progressive growth of the DMBA-induced prolactin-dependent tumours in these animals, the plasma prolactin levels remained within the normal range and did not change significantly from month to month (Nagasawa et al., 1973). At this stage it appears highly likely that most human breast cancers whose growth is dependent on prolactin will be so at physiological levels. In one group of patients who are known to have grossly elevated prolactin levels, namely those with non-puerperal galactorrhoea, no increased incidence of breast cancer has yet been reported. Thus in Man we are as yet unaware of any evidence indicating a predisposition to breast cancer in patients with a high plasma prolactin level, although Meites et al. (1972) have shown that this is the case in rats. Whether prolactin may possibly play a part in the development of the rare but dreaded breast cancer of pregnancy or the puerperium, (when levels are high), is still not known.

However, evidence is steadily accumulating to show that prolactin is a powerful growth-promoter in a number of existing breast cancers. In the present study, as the results indicate, as many as 50 of the 150 tumours studied were dependent on prolactin in vitro. Of these, no fewer than 27 were dependent on the lower concentration used, (0.022 I.U. per ml, approximately 6.5 ng/ml), and 16 of the 27 were dependent at the lower concentration only. If the growth of these tumours is dependent on prolactin at physiological plasma levels, and indeed low physiological levels, then the problem of how to counter prolactin arises. The possibilities include suppression of its secretion at hypothalamic or pituitary level by surgical or pharmacological means and interference with its peripheral action at target cell level.

The search for a peripheral prolactin blocker is now being actively pursued, both by the pharmaceutical companies and by academic researchers, but to the best of our knowledge none has yet been reported. A good deal of attention has however been focussed on pharmacological and surgical attempts to suppress prolactin secretion. The pendulum

of opinion has also begun to swing away from explaining the beneficial effects of hypophysectomy in terms of adrenocorticotrophic and gonadotrophic hormones to attributing them to removal of prolactin. For example Ray (1972), in reviewing the experience of hypophysectomy for disseminated breast cancer at Cornell and the New York Hospital, emphasises the importance of prolactin above the trophic hormones. Ray goes as far as to give prolactin abolition quite unequivocally as the rationale for the success of the operation. He also mentions the importance of a complete ablation of the gland, as leaving even a small fragment of the anterior lobe could compromise the result. Jantet et al. (1963) actually showed that even when hypophysectomy was performed by the transfrontal route, 16 out of 22 subjects had residual anterior pituitary tissue at autopsy.

Hypophysectomy as a Suppressor of Prolactin Secretion

There are only very scanty data in the literature correlating the response of anti-prolactin measures of any sort with plasma prolactin levels. In three cases Dickey and Minton (1972) showed that some lowering of the prolactin level by levodopa was associated with a clinical response. We have so far had the opportunity of studying the plasma prolactins of 6 breast cancer patients and the results are of some interest. The first two sets of estimations were performed by Dr. Boyns and Mr. Cole, of the Tenovus Institute, Cardiff, who assayed human growth hormone (HGH) in the plasma samples as well. Their prolactin units are in "milli-ampoules/ml" and growth hormone is measured in the conventional micro-units/ml. Three further sets of prolactins were assayed by Drs. Friesen and Del Pozo, of McGill University, Montreal and the sixth set by Dr. McNeilly of St. Bartholomew's Hospital.

Patient 48, 65 years of age, had originally been treated by radiotherapy in April 1969 for a Stage III carcinoma of the left breast. Local recurrence developed in 1970 and she had subsequently been treated with norethisterone, oestradiol, cytotoxic chemotherapy and finally, in June 1971, the oestrogen antagonist ICI 46474 ('Tamoxifen'). The latter produced some degree of regression in the local disease but bony metastases developed a few months later. In June 1972 biopsy and in vitro testing of the breast lesion showed clear-cut prolactin dependence, at both concentrations. On 7th July, 1972

a trans-ethmoidal hypophysectomy was performed. Plasma prolactin levels were monitored and these showed a transient fall (Fig.12) soon followed by a climb back to pre-operative levels. Parallel growth hormone assays remained low, suggesting that even a near-complete ablation may not be enough to stop prolactin-secreting cells from maintaining a normal plasma prolactin level. The patient obtained no remission of her disease and despite chemotherapy died in November, 1972.

Patient 70, who presented in July, 1970 at the age of 42 years with a right breast destroyed by tumour, a pleural effusion and a lung metastasis, illustrates the opposite picture. She initially had a good regression from a radiation menopause, but by April, 1971 there was again active disease in the right breast and skeletal metastases were present. Norethisterone was administered without success. By June, 1972 the patient was rapidly losing condition in the face of progressing local and bony disease. Biopsy and testing of the breast lesion at this time showed it to be independent but with good survival in prolactin and marked inhibition by oestradiol. The latter was accordingly administered with definite regression of the breast disease but no effect on the bony metastases. A repeat breast biopsy and culture were performed on 27th August, 1972, at which stage the patient was in unrelieved agony and bedridden because of pelvic and vertebral bony metastases. Local radiotherapy to several of the bony deposits had not alleviated the pain at all. The new culture revealed oestradiol inhibition just as before, but this time the tumour had survived so well in prolactin as to be classified as dependent on it. In the face of a hopeless clinical situation it was decided to resort to hypophysectomy, which was performed by the trans-frontal route on October 25th, 1972. This produced a remarkable result; the patient rapidly lost all her pain, regained normal mobility and was in due course able to return to her work as a music teacher. The local breast disease resolved completely and to date, some 8 months post-operatively, she remains well and symptom-free, with no signs of active disease on clinical examination. The bony metastases are showing early signs of re-calcification on X-ray. Figure 13 shows that her plasma prolactin was at a level undetectable by the Cardiff assay both pre- and post-operatively, but the HGH level did drop immediately post-operatively to very low levels and stay there. This patient's hypophysectomy was thus probably

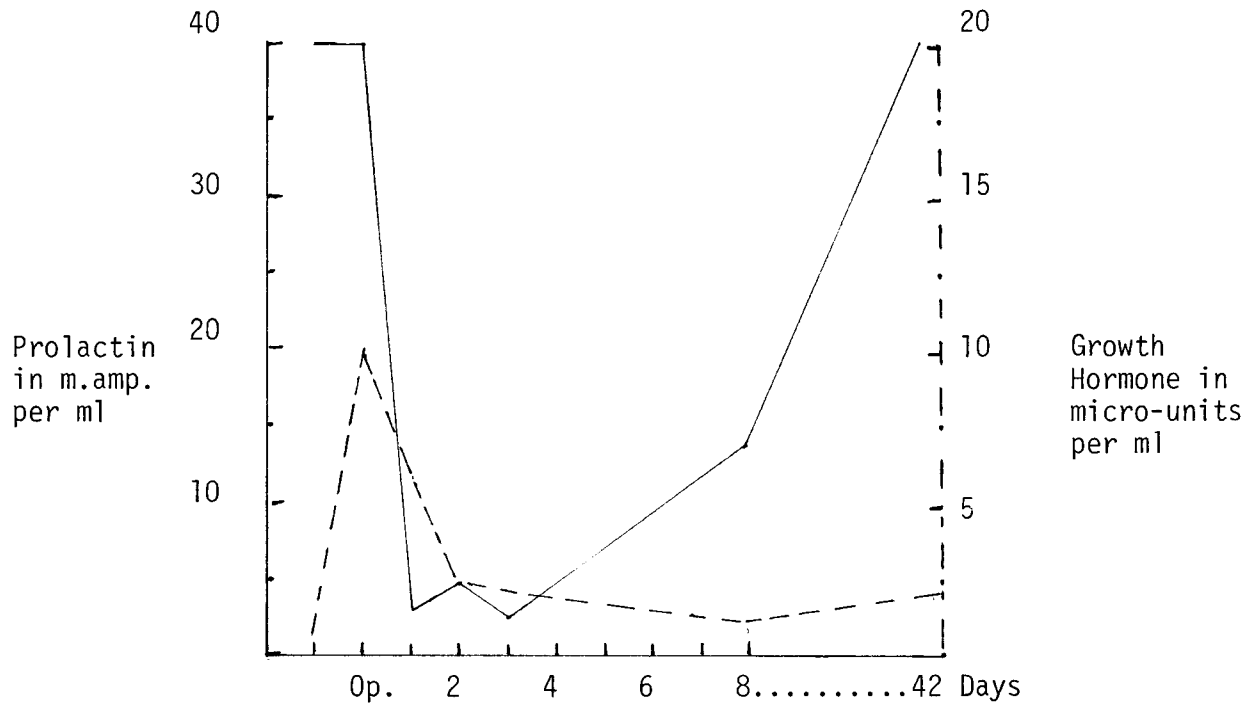


Figure 12: Plasma prolactin and growth hormone levels of Patient 48 following trans-ethmoidal hypophysectomy.

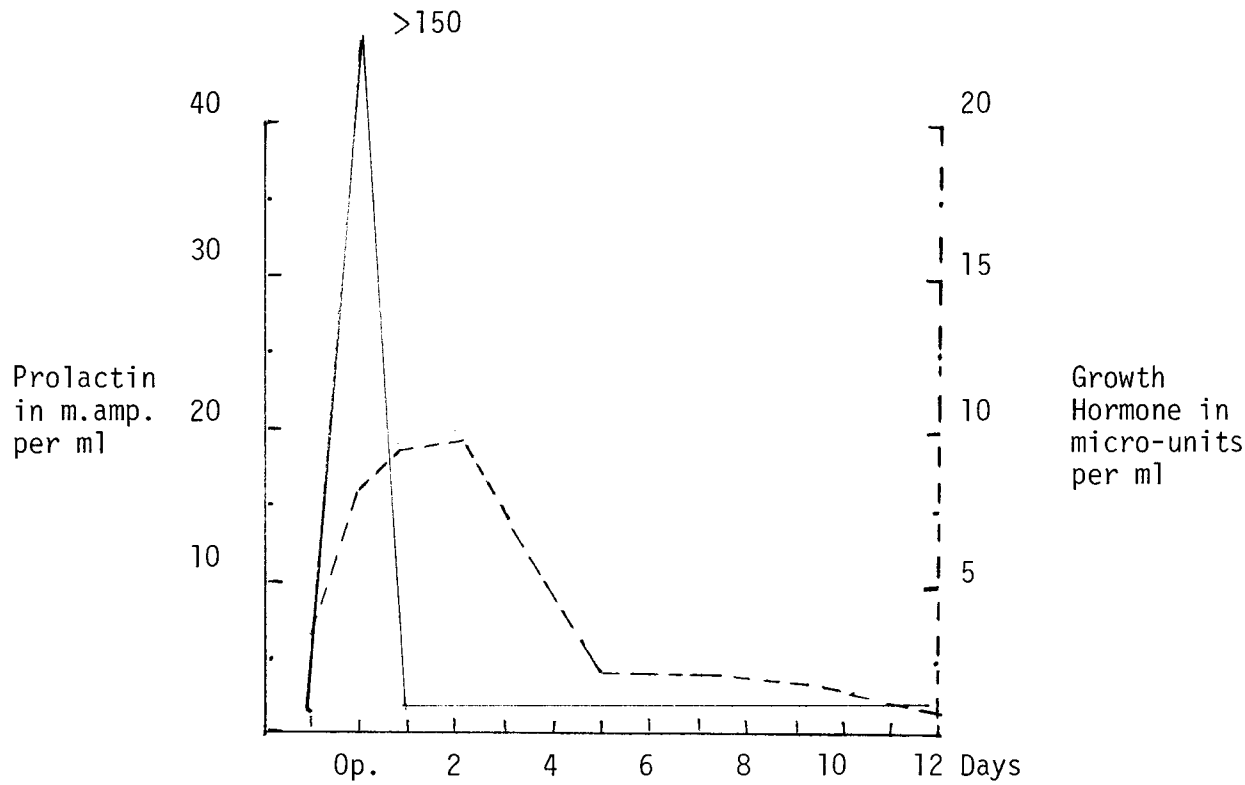


Figure 13: Plasma prolactin and growth hormone levels of Patient 70 following trans-frontal hypophysectomy. The very high prolactin peak is in a specimen taken under general anaesthetic, which is known to produce such elevation.

complete and went hand in hand with an excellent regression.

Mrs. E. S., 48 years of age, had not had in vitro testing of her tumour since she had no soft-tissue lesion that could be biopsied, but her history and response to hypophysectomy are relevant to the present study. In January, 1972 she had been treated by simple mastectomy and radiotherapy for a right breast carcinoma. Six months later vertebral deposits appeared. In January, 1973 she was seen at the Westminster Hospital with multiple bony metastases, including ominous deposits in the first and second cervical vertebrae. These were treated with local radiotherapy and a collar to try to prevent cervical subluxation. She was also given levodopa (500 mg six-hourly) in addition to the cytotoxic chemotherapy she was already receiving and which was not controlling the pain or the lesions. The levodopa did seem to produce some purely subjective improvement in her bone pain and as it is known that levodopa does lower prolactin secretion and has been suggested as a possible predictive test for pituitary ablation (Minton and Dickey, 1972; Stoll, 1972), trans-frontal hypophysectomy was recommended. This was performed on March 23rd, 1973, with an outstanding result. In addition to rapid symptomatic relief, X-rays taken less than two months later showed clear evidence of healing and re-calcification of vertebral, rib and pelvic metastases, (Figs.15 and 16) which is in fact unusually early to detect radiological signs of regression of bony metastases. Figure 14 shows that her plasma prolactin levels dropped down to 4 ng/ml post-operatively and stayed down. Dr. Del Pozo (1973) has recently informed me that the lower limit of sensitivity of the McGill prolactin assay is presently about 4 - 5 ng/ml and that they are rarely finding levels below this. This patient's hypophysectomy would then appear to have been virtually complete, and about 4 months post-operatively her excellent regression is being maintained.

Patient 183, 36 years of age, had had a left modified radical mastectomy abroad in November 1970, followed by an oöphorectomy. In January 1973 she came to the Westminster Hospital with Stage IV disease, (scar recurrence and bony metastases). Biopsy and testing of the scar recurrence revealed prolactin dependence and on 13th April trans-ethmoidal hypophysectomy was performed. Figure 17 shows that her plasma prolactin levels dropped and remained down post-operatively and the patient

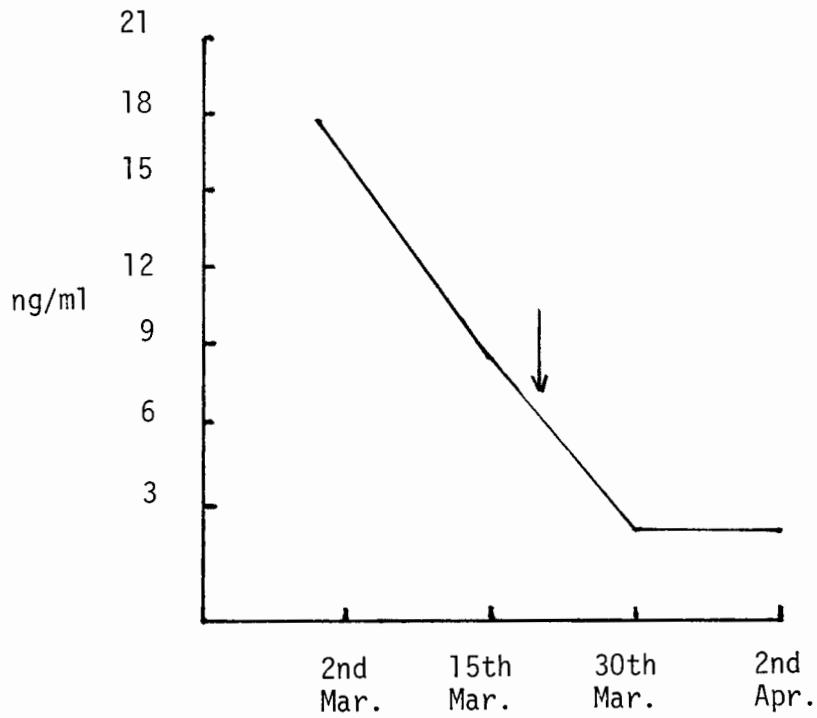


Figure 14: Plasma prolactin levels of Mrs. E.S. following trans-frontal hypophysectomy.

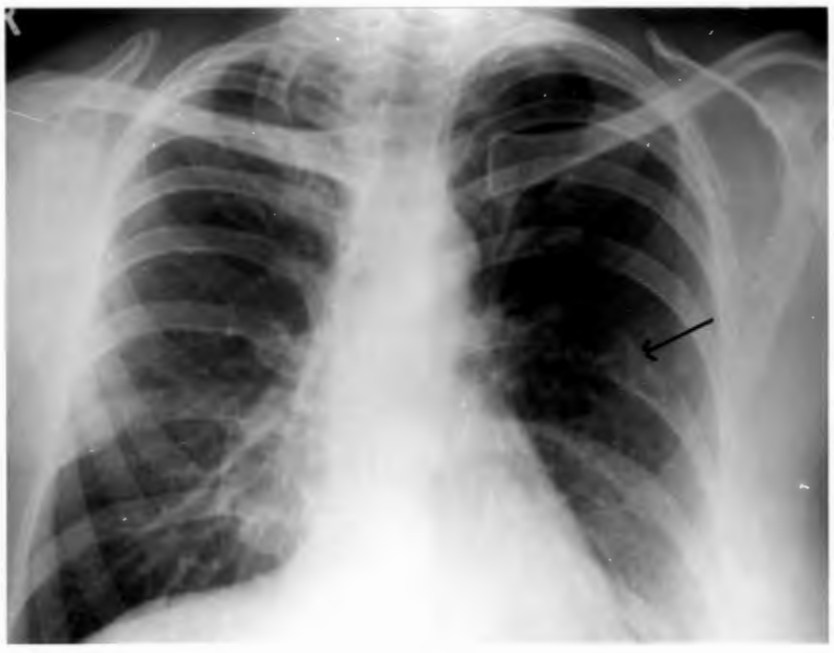


Figure 15: Mrs. E. S. Chest X-ray on 4th January, 1973 shows rib metastases.

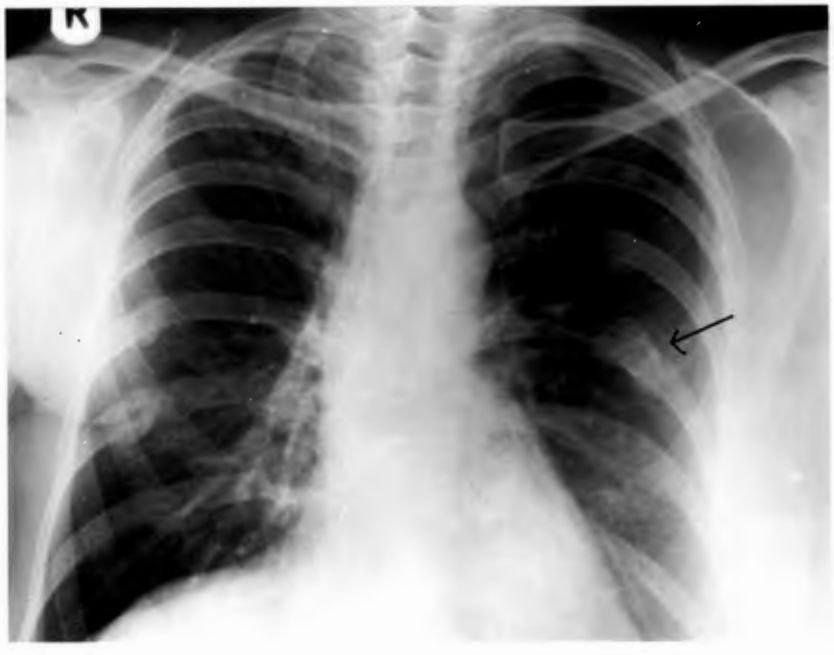


Figure 16: On the 21st May, 1973, 2 months after trans-frontal hypophysectomy, re-calcification is clearly occurring. A later radiograph taken in early July shows even more impressive bone healing.

has had objective regression of her disease, with radiological resolution of the metastases and a return of alkaline phosphatase levels to normal.

Patient 69, 51 years of age, had been treated by radiotherapy for a left breast carcinoma in February 1968. Local recurrence in September, 1969 was treated by a radiation menopause. After initial regression the disease advanced and by July 1972 she had been successively treated with cyclophosphamide, norethisterone, combination chemotherapy and durabolin, none of these having achieved a regression. Biopsy and testing in July, 1972 showed independence with equally poor survival in all hormones. She was maintained on prednisone and 5-fluoro-uracil but by this time had progressing vertebral metastases. A repeat biopsy of the breast lesion was taken in April, 1973 and this time it showed prolactin dependence. All other forms of treatment had been exhausted and on 11th May, 1973 a trans-frontal hypophysectomy was performed. After an initial symptomatic improvement which only lasted a few days, it soon became clear that the progress of the disease had not been arrested. It will be seen from Figure 18 that her plasma prolactins failed at any stage to reach a sufficiently low level and oscillated right up to pre-operative levels. It is very significant that the operation had been complicated in this patient by the finding of an internal carotid aneurysm and thus in spite of it having been performed by the trans-frontal route, there was some doubt about the completeness of ablation.

Pharmacological Attempts to Suppress Prolactin Secretion

Mention has been made earlier of the finding of Cassell et al. (1971) that ergocornine and ergocryptine inhibited the growth of 7,12-DMBA-induced tumours in Sprague-Dawley rats, this effect being due to suppression of pituitary prolactin secretion, mediated both via hypothalamic PIF and a direct anterior pituitary effect of the drugs. In 1971 Lutterbeck et al. described the use of the ergot alkaloid 2-brom-alpha-ergocryptine (CB 154) to terminate galactorrhoea in 3 non-puerperal women and in 1972 Varga et al. showed that it also inhibited puerperal lactation. No prolactin levels were measured in the blood of these patients but the drug was believed to act by specifically suppressing prolactin secretion. Later in 1972 Besser et al. showed that the syndrome of galactorrhoea plus impotence in men or

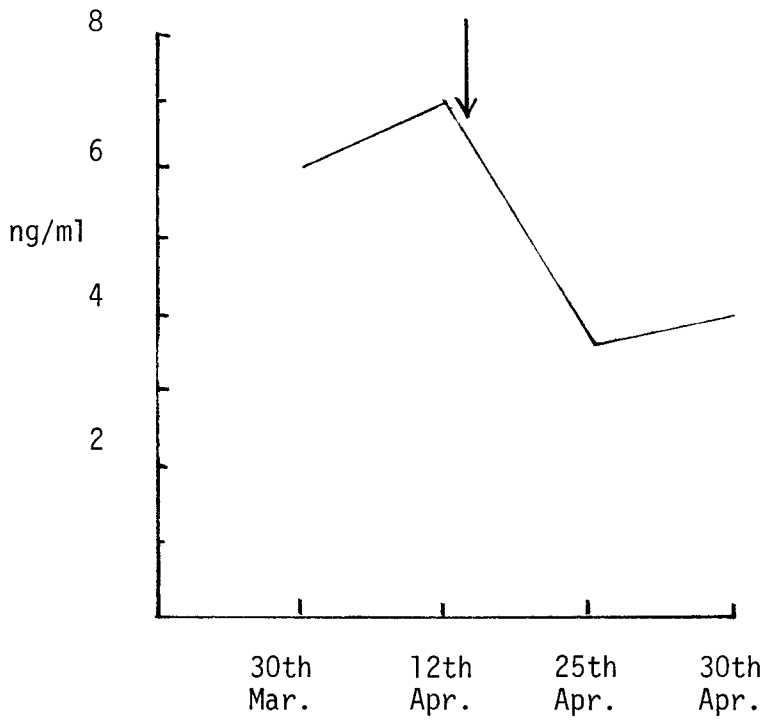


Figure 17: Plasma prolactin levels of Patient 183 following trans-ethmoidal hypophysectomy.

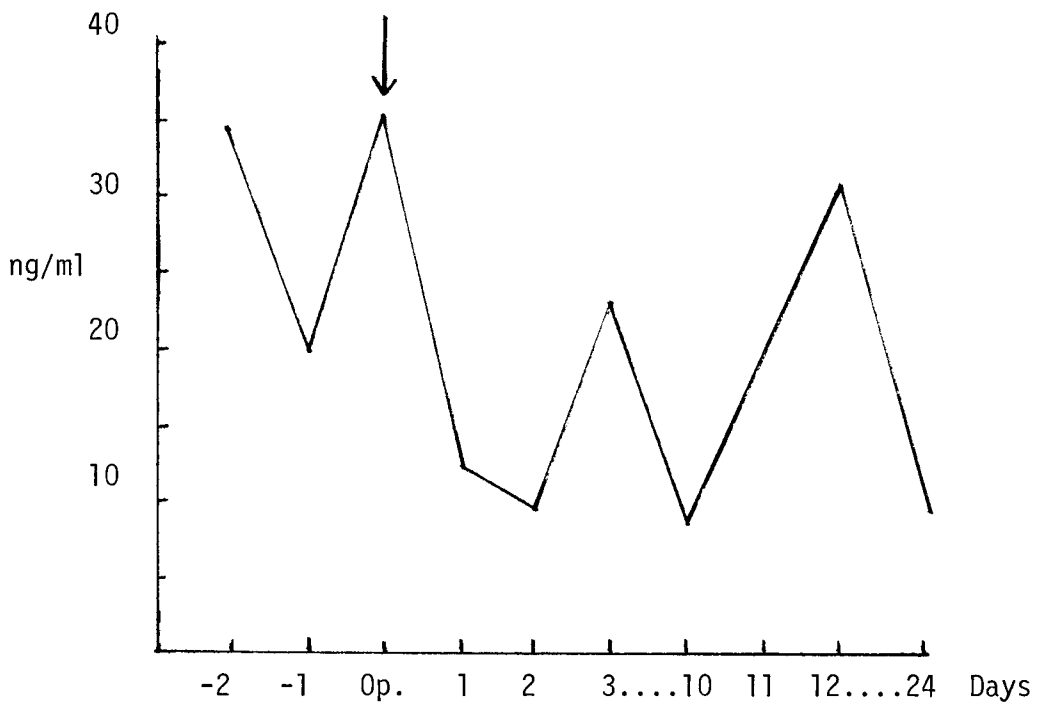


Figure 18: Plasma prolactin levels of Patient 69 following trans-frontal hypophysectomy

amenorrhoea in women could be successfully treated with CB154. Plasma prolactin levels were measured in these patients and were often greater than 1,500 ng/ml, which is over 500 times normal. They fell dramatically in response to treatment although it is not known how near zero they reached because the assay in use at the time had a lower limit of sensitivity of 22 ng/ml of prolactin, which is near the upper limit of the physiological range. Besser et al. found that the response to treatment, that is the cessation of galactorrhoea and the return of normal gonadal function, suggested that there was an inverse relationship between prolactin secretion and gonadotrophin secretion in Man. This is precisely what we would expect in view of the previously-discussed evidence that the hypothalamic "releasing factors" are indeed "releasers" for GH and the trophic hormones, whereas for prolactin the effect is inhibitory (PIF). Del Pozo et al. (1972) showed that levodopa could transiently depress prolactin levels further than CB154, but this could not be sustained for more than 4 hours, even on regularly-spaced medication. Boyd et al. (1970) had shown that levodopa increases GH secretion, and Sherman et al. (1971) showed that chlorpromazine, which increases prolactin secretion, reduces GH release. Thus all these drugs would appear to be acting non-specifically on the hypothalamus, either stimulating or depressing the secretion of the "releasing" factors down the hypothalamo-pituitary axis, the net effect on the gonadotrophins, ACTH and growth hormone being inverse to that on prolactin. However, there is good evidence that the ergot alkaloids act directly at pituitary level as well, (Flückiger, 1972) and at the present time the whole complex subject of the primary site of action of all these drugs remains controversial.

Cyclicimide, (CG603), a thalidomide derivative and also an inhibitor of prolactin secretion in the rat, produced a clinical remission in only one of 23 patients in whom it was tried in a European trial, (Heuson, 1972), the dose being the maximum tolerable one of 2 gm daily. However, the drug is toxic, produces weight loss, which itself can produce mammary tumour regression (Heuson, 1972), and is to our knowledge not being further tried in humans.

Is Human Breast Cancer Simply Related to Prolactin Levels?

In considering the treatment of breast cancer by lowering prolactin

secretion, the situation in humans would thus appear to be considerably more complex than in the rat, in whom tumour regression may be obtained by any of the methods that lower prolactin secretion, though not necessarily to complete shut-off. In 1972 the European Breast Cancer Trial published its results on treatment of 19 patients with CB154 at the recommended dosage of 2.5 mg three times daily; not a single remission was obtained (Heuson et al., 1972). No plasma prolactins were measured in their study and it is thus not known how effectively the levels were lowered.

One patient in this present study, number 52, a lady of 62 years, presented in November 1970 with a Stage III right breast carcinoma that was treated by radiotherapy. This regressed but did not disappear. In March, 1972 it re-activated and she was treated first with norethisterone and then with ICI 46474, with no effect. In January, 1973 a biopsy revealed prolactin dependence, at high and low concentrations, and she was given a course of CB154, 2.5 mg three times a day. No objective signs of regression were seen and after 6 weeks the treatment was stopped and chemotherapy instituted. (The latter has also not succeeded in controlling the disease.) Figure 19 shows that her plasma prolactin levels were certainly lowered, though by no means abolished. This does not reflect an inability to register low levels on the part of the assay method, which Dr. McNeilly believes to be accurate down to 1 - 2 ng/ml.

We have now seen that there appears to be some correlation between the success of hypophysectomy and the sustained depression of plasma prolactin levels post-operatively. At the same time, there is the problem of completeness of the operation by either route and of the extent of prolactin secretion by the residual fragments. CB154, which does produce a sustained depression of prolactin levels, although never below 3 ng/ml (Del Pozo et al., 1972), has not yet been shown to produce any objective regressions in breast cancer, whereas levodopa, which usually <u>does</u> drop prolactin levels to below 3 ng/ml, but always transiently, has achieved temporary regressions in a few patients (Minton and Dickey, 1972). It may well be, as the present study suggests, that a substantial proportion of these prolactin-dependent tumours are able to thrive in a very low concentration of the hormone, presumably taking it up selectively as required. The various pharmacological

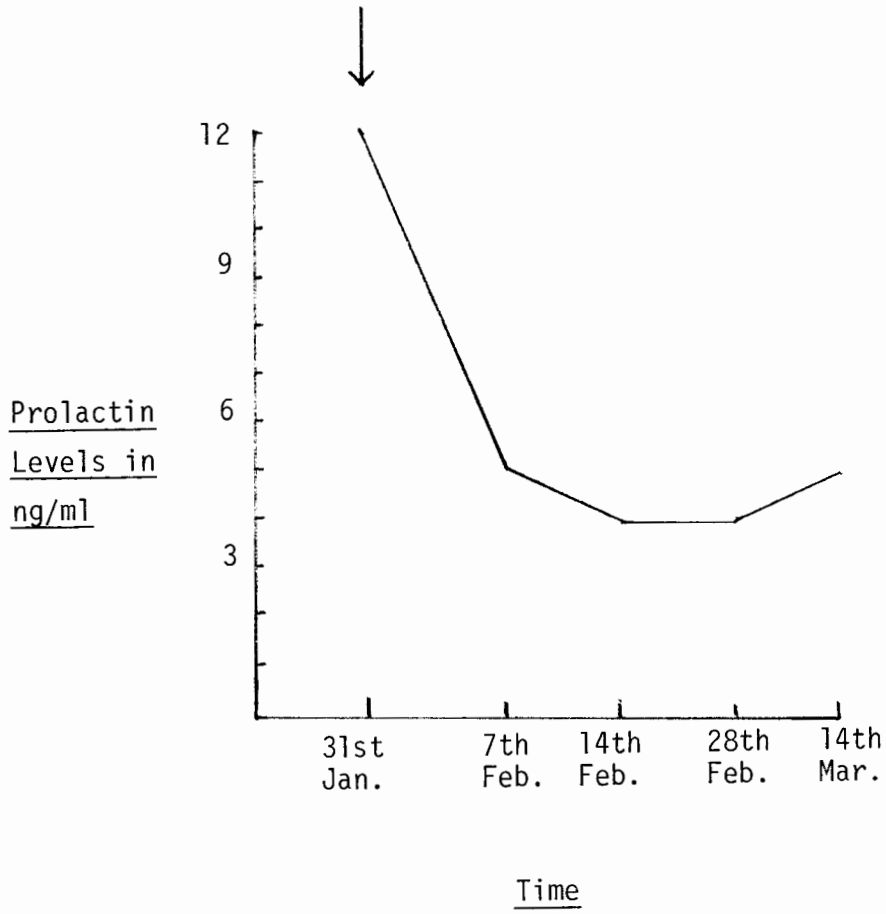


Figure 19: Plasma prolactin levels of Patient 52 after administration of CB154.

manoeuvres, in the present state of knowledge, may simply not be depressing prolactin secretion and levels sufficiently or for long enough.

It is now known that intravenous administration of synthetic thyrotrophin-releasing hormone (TRH) in Man is able to stimulate prolactin as well as thyrotrophin secretion (Bowers et al., 1971; Jacobs et al., 1971). Whether under physiological conditions there is a hypothalamic releasing mechanism for prolactin as well as an inhibitory one is unknown. Even if there were it is not known whether this would be a function of TRH or of some hitherto undiscovered hormone.

Another aspect of the complexity of the problem is the knowledge that pituitary stalk section, which could be expected and in some cases has been confirmed to raise plasma prolactin levels, (by interrupting PIF secretion down the hypothalamo-pituitary axis,) (Turkington et al., 1971), nonetheless results in a number of remissions in disseminated breast cancer. One possibility is that stalk-section may operate by interrupting the releasing factors for the trophic hormones, which may result in switching off ovarian and adrenocortical steroid production in cases that are steroid hormone and not prolactin-dependent. Another suggested rationale has been that stalk-section may result in virtual abolition of function of the anterior pituitary by some mechanical or vascular derangement. The paradox still remains difficult to explain and is yet another facet of the intricate relationship between the growth of human breast cancer and plasma prolactin levels.

Treatment of Prolactin Dependent Cases by Inhibitory Hormones

The prolactin-dependent cases did not necessarily need to receive anti-prolactin measures in order to gain a remission. In several instances excellent remissions were obtained by adminstering the hormone which appeared to be strikingly inhibitory to the tumour in culture. For example, patient 46, 62 years of age, was treated by radiotherapy for a right breast carcinoma in 1969. In 1970 local recurrence developed and she was treated successively with norethisterone and then a combination of durabolin and prednisone. By August 1971 chemotherapy with 5-fluoro-uracil had to be started. In May, 1972 it was decided to biopsy and culture the local recurrence in view of

failure to control it with chemotherapy. The biopsy turned out to be prolactin dependent but was also inhibited markedly by oestradiol. Ethinyl oestradiol was then administered and good objective regression ensued.

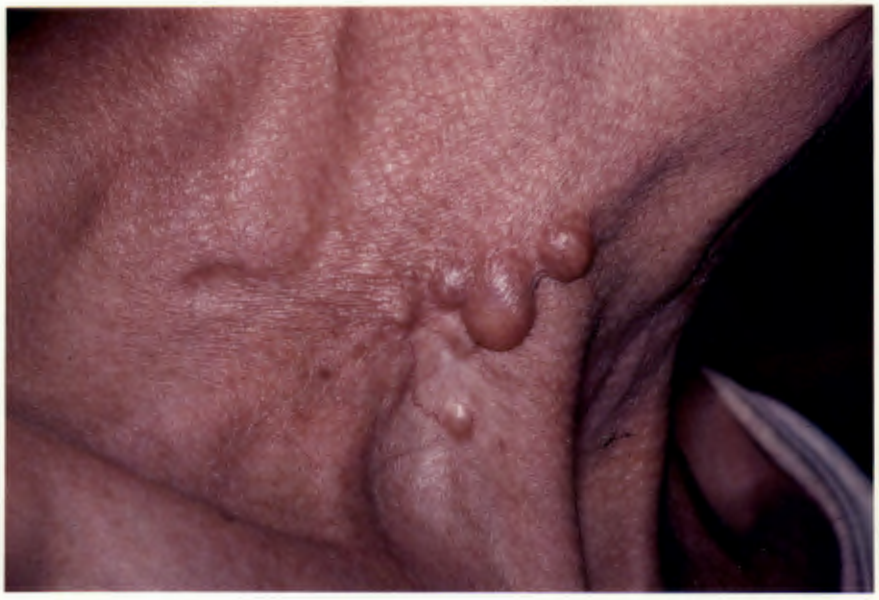
INFLUENCE OF OESTROGENS ON PROLACTIN SECRETION

Meites (1972), one of the principal workers in this field, has clearly shown that apart from the "short-loop feedback" whereby high circulating prolactin or implantation of prolactin in the median eminence of a rat reduces prolactin secretion by the in situ pituitary, several other hormones, including oestrogen , thyroxine (T4) and tri-iodo-thyronine (T3) all have a positive "long-feedback" influence on prolactin secretion. Oestrogens seem to increase prolactin secretion both by hypothalamic and direct anterior pituitary effects, while thyroxine apparently acts only at pituitary level. Other workers have confirmed that oestrogen administration raises prolactin levels in humans as well as in animals, (L'Hermite et al., 1972 b). Pasteels et al. (1972) demonstrated in humans that the number of "prolactin cells" in the pituitary were increased as a side-effect of administration of various hormones like oestrogens and androgens, (including to breast cancer patients,) and in conditions like hepatic cirrhosis where androgens and oestrogens are not catabolised properly. In a small series of patients Forrest (1972) has shown that oestrogenic therapy may enhance and anti-oestrogenic therapy may depress prolactin secretion.

Anti-Oestrogenic Therapy in Prolactin-Dependent Cases

Certain of the prolactin-dependent cases in this study were given anti-oestrogenic treatment by the clinicians concerned and underwent a good remission. Patient 38, 69 years of age, had radiotherapy to a left breast carcinoma in 1953 followed in 1954 by a toilet mastectomy and bilateral oophorectomy. In 1963 she received local radiotherapy to recurrent supraclavicular disease. In April 1968 she developed hepatomegaly and progressive supraclavicular disease. Durabolin and norethisterone failed to control the disease and 5-fluoro-uracil, cyclophosphamide and finally combined chemotherapy were tried. In May, 1972, the disease still not under control, a biopsy of the supraclavicular mass was cultured and showed dependence on both oestradiol and prolactin. The patient was started on the anti-oestrogen ICI 46474 and on this treatment she had an excellent

(a)



(b)

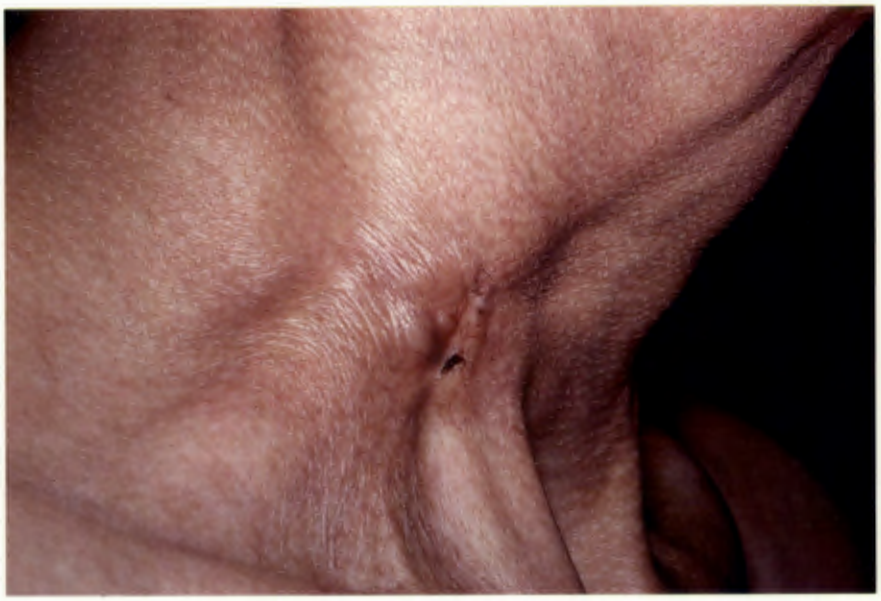


Figure 20: Patient 38. (a) Biopsy of one of these cervical nodules on 1st May, 1972 showed prolactin and oestradiol dependence. In addition to these nodules hepatomegaly was also present. (b) After treatment with ICI 46474 the patient was still in good health when last seen in March, 1973. The remaining nodules in the neck had regressed, as had the hepatomegaly.

objective remission and remains well with no active disease 7 months later (Fig. 20). Patient 47, 71 years of age, presented with a Stage IV carcinoma of the left breast in June, 1972. Biopsy and testing showed dependence on prolactin only. The primary lesion was treated with radiotherapy and the patient was then given ICI 46474 for the disseminated disease. There has been good objective regression and 11 months later she remains well on the drug.

The Effect of an Oestrogen-Blocking Drug

ICI 46474 ('Tamoxifen') is the trans-isomer of 1(p-beta-dimethyl-amino-ethoxy-phenyl)-1,2-diphenylbut-1-ene, a non-steroid preparation which is a potent anti-oestrogen in several mammalian species including Man. It is believed to act by competitive interference with oestradiol uptake at various sites. ICI 46474 has been shown to be useful in the management of disseminated breast carcinoma (Cole et al., 1971; Ward, 1973). Although its main action is that of a peripheral oestrogen blocker, the possibility that it will also inhibit oestrogen production cannot be excluded on present evidence. Similarly, it is very likely that by blocking oestrogen receptors in the pituitary and hypothalamus, ICI 46474 may be able to interfere with feedback mechanisms for the secretion of prolactin and other pituitary hormones (Walpole, 1973). This is therefore another tantalising glimpse of potential hormonal manipulations at a level far more sophisticated than would have seemed possible a few years ago.

Complex Interactions of Prolactin and Oestrogens

Further difficulties in understanding the precise nature of prolactin's and oestrogen's effects on breast tumours need to be mentioned. For ill-understood reasons, believed to involve stimulation of differential growth of the mammary epithelium induced by prolactin, elevation of prolactin levels immediately around the time of administering a carcinogen like DMBA to a rat can protect the mammary gland from the action of the carcinogen (Meites, 1972). Similarly, administration of oestrogen (Huggins, 1965) at this crucial stage also protects the rat mammary epithelium against DMBA's carcinogenic action, undoubtedly acting at least in part by stimulating prolactin secretion. Each of these treatments, perplexingly enough, has been shown to promote growth of mammary cancers already present as a result of DMBA induction.

Hilf et al. (1971) have raised another problem by showing that in

the R3230AC mammary tumour in Fischer rats, a different model from the DMBA tumour in Sprague-Dawley rats, treatment with fluphenazine, (a phenothiazine), which increases prolactin secretion, is accompanied by marked growth and secretion in the normal mammary tissue, yet regression in the mammary tumours. This effect was enhanced by oestrogens. Thus, in this animal model, at least, a hormonal milieu which stimulates normal mammary gland growth does not of necessity stimulate growth of neoplastic mammary tissue. Huseby and Thomas (1954) in fact reported that some women with breast cancer experienced secretion responses in the normal breast tissue with administration of oestrogens, yet a simultaneous decrease in the size of the breast carcinoma.

Although in the case of rats the majority of authorities feel that the 7,12-DMBA tumour is essentially a prolactin-dependent one, Dao and Sinha (1972) stated at the Tenovus Symposium on prolactin and carcinogenesis that median eminence lesions, which greatly elevate prolactin secretion, were nonetheless unable to stimulate tumour growth unless ovaries were present, and if they had been previously removed then ovarian grafting allowed further progression of the mammary tumours. Dao felt that both oestrogen and prolactin are needed for these DMBA tumours in rats and that it is difficult to assign primary importance to one or the other. In the ensuing discussion Pearson disagreed, since with perphenazine administration alone in ovariectomised rats, (i.e. raising the prolactin secretion), the tumours did progress, but Meites took a middle course stating that they did go on for a while but that after 15-18 days they stopped growing and at that stage required oestrogen as well. Pearson admitted this possibility and it was further agreed that there might be some differences in the liability of different strains of Sprague-Dawley rats to develop the DMBA-induced tumour.

The Effects of High Doses of Oestrogen

There is considerable controversy about the manner in which large doses of oestrogens may produce a remission in some cases of breast cancer in humans. In 1963 Kim et al. stated that although physiological concentrations of oestrogens stimulated prolactin secretion in rats, administration of high doses depressed it. He therefore suggested that its success in some breast cancer cases was due to a central inhibition of prolactin secretion. Meites et al., (1971), however, claim that it does not depress prolactin secretion but actually increases it, and feel

that the effect is by interference with the direct peripheral action of prolactin on the mammary tumour tissue.

Pearson et al. (1972) supported this hypothesis by ovariectomising female rats bearing the DMBA tumour, then re-activating the tumour with perphenazine injections, (which produces high prolactin levels by PIF suppression), and finally adding high doses of oestradiol benzoate to this regime, which produced striking tumour regressions. They felt that this was very strong evidence that the action of high doses of oestrogens was a peripheral prolactin-blocking one rather than a central effect on prolactin secretion.

One additional new factor needs to be taken into account, namely the demonstration by the present study (Flax et al., 1973) that 14 human breast tumours were dependent on testosterone alone and a further 10 on testosterone as well as prolactin. This raises the possibility that administration of oestrogen to these patients may succeed by antagonising testosterone action at tumour cell level. This will be discussed in greater detail in the section on testosterone dependence.

Value of the Present Technique in the Human Situation

There are thus clearly still many gaps in the understanding of the control of prolactin secretion and of the mechanism and site at which administration of other hormones or drugs may influence prolactin-dependent tumours. The dearth of data on factors influencing prolactin secretion in humans with breast cancer is not likely to be rapidly rectified because the elegant and ingenious controlled situations that have been devised in experimental animals cannot be duplicated in patients, for obvious reasons. A technique such as the one described in this study is therefore all the more valuable because it offers an opportunity to study the effects of prolactin on the target tumour itself, at a chosen range of concentrations, without grappling with the complexities of prolactin secretion in vivo. Furthermore, it indicates which hormone or hormones, if any, are directly inhibitory to the tumour itself, without involving a debate about central or peripheral sites of action. This enabled successful regressions to be achieved by administration of the inhibiting hormone, as in patient 46 mentioned previously, without attempting to interfere with prolactin secretion.

The human situation seems to be more complex and more critically dependent on concentrations than the DMBA tumour in rats, which is readily

stimulated or suppressed by raising or lowering prolactin secretion in a simple linear manner. Suppression may certainly be achieved in the rat without lowering the levels to anywhere near complete shut-off point, which it appears may well be necessary in humans, and which this study tends to confirm by demonstrating that prolactin dependence often exists at very low concentrations.

It may be this very factor which has enabled the goal of a "selective medical hypophysectomy" for prolactin to elude us up to now, whereas in animals this is more easily achieved. It would seem highly desirable to pursue vigorously a pharmacological agent that will interfere with or block prolactin's action at peripheral tumour cell level, rather than influence its secretion via the pituitary or hypothalamus. Here again it is suggested that the present technique offers an excellent approach to investigate such agents, which could be combined with prolactin in the culture medium in various concentrations and compared with cultures in prolactin alone.

DEPENDENCE ON PROLACTIN AND A STEROID HORMONE

It will have been noticed in the Results section that a number of tumours were dependent on prolactin as well as one or other of the two steroid hormones. Interestingly enough, there has not been a single tumour over the past 15 months that has exhibited dependence on all three hormones tested. This is pertinent in that it suggests that those breast cancers which are promoted by a steroid hormone, may require an androgen or an oestrogen but not both. The fact that some breast tumours may be dependent on more than one hormone goes some way to explaining the failure of endocrine therapy in 2 cases out of 3 selected on the usual clinical guidelines.

This is a new concept and raises interesting problems in treatment. In some patients counter-measures to one of the two hormones may produce a good regression, and this can sometimes be rationalised in terms of a known feed-back mechanism. For example, patient 38, whom I have mentioned earlier, had a tumour which was dependent in vitro on oestradiol and prolactin. ICI 46474 alone produced a good regression, probably operating as a peripheral oestrogen blocker and possibly also by interfering with feed-back stimulation of prolactin secretion centrally. In other cases it is more difficult to postulate a mechanism.

Although the precise nature of the interaction between prolactin and the steroid remains uncertain, it is suggested that when one learns from the test that two hormones are capable of enhancing the growth and activity of the tumour, one has the option of employing manoeuvres which will counter or remove one or both. It may well be that simultaneous attack on both hormones will produce a higher regression rate than concentrating on only one, which could permit a tumour cell population dependent on the other to flourish. Conversely, in the event of the third hormone being strikingly inhibitory to the tumour, the test offers the chance of administering that to the patient. It is thus hoped that a more rational approach to hormonal therapy may result from this type of in vitro technique.

OESTRADIOL DEPENDENCE

A relatively small proportion of patients were dependent on oestradiol alone. This may be to some extent related to the preponderance of postmenopausal patients studied. It should also be borne in mind that we now know that patients who respond to oöphorectomy, anti-oestrogen drugs or a radiation menopause cannot automatically be considered oestrogen-dependent, in that the same treatment may - by lowering oestrogens - reduce although probably not abolish prolactin secretion by the feedback mechanism. This may affect prolactin-dependent tumours.

In the oestrogen-dependent group there were again several examples of clinical progress bearing out the results of the test. Two will be illustrated here. Patient 179, 45 years of age and premenopausal, had a left radical mastectomy in 1965 and radiotherapy to a recurrence in the scar in 1970. In March, 1973 she presented with a malignant right pleural effusion and active left axillary disease. Biopsy on 14th March showed oestradiol dependence and a radiation menopause was performed with good objective remission.

Patient 181, 49 years of age and premenopausal, presented in February, 1973 with a right breast lump and vertebral metastases. Bilateral oöphorectomy was performed and the breast lump biopsied at the same time. Oestradiol dependence was found and it was therefore considered that a regression was likely to be achieved, which turned out to be the case. At the end of May she was still completely free of pain and with no clinical signs of active disease. X-rays show early recalcification of

the vertebral metastases.

VALUE OF THE TEST IN AVOIDING FRUITLESS ABLATIVE SURGERY

I have made clear in the introductory section that in addition to the need to select the patients who will benefit from endocrine-ablative surgery, one of the chief aims of a predictive system is to demarcate those who are unlikely to respond. It has already been emphasised that the present study was concerned with development of a technique rather than a clinical trial, and the patients were freely treated by the clinicians concerned on the basis of their own conventional practice, not on the results of the test. Nevertheless, follow-up of the patients showed several situations where the test had very clearly suggested that the ablative procedure to be used would fail.

A typical example was patient number 87, who presented as a pre-menopausal lady of 47 years with a Stage III carcinoma of the left breast in August, 1972 and was treated by radiotherapy. In November, 1972 a toilet mastectomy was performed. Hormone dependence testing on that specimen showed it to be independent, but with poor survival in oestradiol and good survival in testosterone, suggesting that oöphorectomy was contra-indicated and that - if anything - oestrogens were inhibiting the tumour. By March, 1973 widespread dissemination of the tumour occurred, there having been no other treatment in the interim. Since the patient was premenopausal it was decided to perform bilateral oöphorectomy in accordance with the conventional clinical guidelines. A skin metastasis was biopsied and re-tested at the same time. This confirmed independence with very poor survival in oestradiol and good survival in testosterone, i.e. precisely the same result as before. As predicted, no remission occurred and indeed the downhill course accelerated, with the patient dying of fulminating dissemination of the disease a few days after oöphorectomy.

This and other examples suggested that clinical guidelines based on the menopause were by no means always correct. As indicated in the Results, only about two-thirds of premenopausal patients were hormone-dependent, and not all of those on oestradiol. Conversely, although only about a half of the patients over 5 years post-menopausal were hormone-dependent, some of those were oestrogen-dependent. The potential value of the test, if its early promise is substantiated in

forthcoming clinical trials, is obvious. It may suggest a therapeutic approach which at times seems to be wholly at variance with the convention based on age and menstrual status. For example, in the young, premenstrual patient discussed above, oestrogen administration rather than removal would have been suggested by the result, and certainly the rapid decline after oöphorectomy hints that this procedure did more harm than good.

TESTOSTERONE DEPENDENCE IN HUMAN BREAST CANCER

An interesting facet of these studies has been the demonstration that 14 patients in the series had tumours which were dependent on testosterone only in vitro in addition to 10 which were dependent on testosterone as well as prolactin. This is not a concept that has been explored in any depth up to now in humans, though there is a well-known Shionogi androgen-dependent rat breast tumour, described by Minesita and Yamaguchi, (1965) which was produced by passaging a mammary adenocarcinoma, originally from a female, from one male rat to another. It was completely suppressed by oestrogens and Minesita and Yamaguchi attributed its androgen dependence to adaptation of the tumour cell in parallel with modification of the hormonal milieu interieur. They suggested that some cases of recurrence in humans might arise by a similar sort of mechanism.

In human breast cancer, it is well-known that oestrogen administration may produce some clinical remissions. In considering bilateral adrenalectomy, the conventional view has been that it produces a remission in the postmenopausal patient by removing adrenocortical oestrogen production, but one could also argue that it removes androgens and androgen precursors. Nonetheless, androgen-dependence in the human situation has hitherto not been seriously evaluated. In 1942 Farrow and Adair showed that carcinoma of the human male breast was an androgen-dependent tumour, and Farrow and Woodard (1942) claimed exacerbation of bony metastases in female breast cancer patients by administration of large doses of testosterone propionate, (as assessed by the development of hypercalcaemia). It is however now well-known that oestrogen as well as androgen administration can produce hypercalcaemia in these patients, and Jessiman et al. (1963) have clearly shown that it may arise spontaneously in the course of the disease without administration

of any steroids. Thus development of hypercalcaemia per se cannot be taken as evidence that actual tumour growth has been stimulated.

Although interest in steroid uptake by breast tumours centred on oestrogens in the 1960's, Deshpande et al. (1963, 1966) did show that certain breast tumours concentrated testosterone, and Braunsberg et al. (1967a and b), though they found only 1 breast tumour in 10 that concentrated testosterone rather than oestradiol, conceded that there might be a small proportion of human breast cancers that did so. Adams and Wong (1968) clearly demonstrated the ability of breast cancers to metabolise testosterone actively and recently Jenkins and Ash (1973) showed that the formation of 5-alpha-dihydrotestosterone was greatest in the most undifferentiated breast carcinomas but did not occur in normal breast tissue. Even more recently Miller et al. (1973) have claimed that this conversion occurs, albeit to a very minute extent, in normal breast tissue as well.

Clinical Examples Supporting A Testosterone Dependence Hypothesis

It was therefore very interesting to observe that in the present study 14 tumours specifically showed dependence on testosterone only and not on the other two hormones tested (Flax et al., 1973). It is relevant that 13 of the 14 patients were postmenopausal at the time of testing, while the fourteenth had been treated with an androgen for several months before testing. Six of the 14 had been treated with androgens or some other form of anti-oestrogenic therapy, (such as a surgical or radiation castration or an anti-oestrogen drug), prior to testing their hormonal dependence. This was highly suggestive that the tumours had adapted themselves to live in an altered hormonal milieu, much like the Shionogi rat tumour model. A further 6 of the 14, all post-menopausal, had tumours which were testosterone-dependent without any previous hormonal treatment, which may indicate that a certain proportion of breast cancers in postmenopausal women may be androgen-dependent ab initio, and that the menopause itself, like the administration of artificial anti-oestrogenic measures, may bring about precisely the sort of modification in the hormonal milieu that we are discussing.

Strong support for the testosterone-dependence hypothesis also came from the clinical progress. Patient 111, 76 years of age, for example, had had her menopause at 40. In September, 1970 she underwent simple mastectomy and post-operative radiotherapy for a right

breast carcinoma. In October, 1972 several small nodules recurred near the scar. On biopsy these were shown to be testosterone dependent with complete death of the tumour in oestradiol. Ethinyl oestradiol 1 mg daily was commenced as anti-androgenic treatment and there was excellent objective regression. The patient remains well with no signs of recurrence at the time of writing in June, 1973.

Patient 42, 62 years of age and over 5 years post-menopausal, was a similar example. She presented in May, 1972 with a large Stage III carcinoma of the left breast. Biopsy revealed the carcinoma to be testosterone dependent and also to be markedly inhibited by oestradiol. She was treated only with ethinyl oestradiol 1 mg daily as anti-androgenic treatment. No radiotherapy was given. There was an excellent objective remission, with the residual lump and the axillary nodes shrinking and disappearing completely. She remains well with no signs of disease in June, 1973.

Patient 92, a 62 year old lady who had had the menopause at 50, illustrated a different form of successful anti-androgenic therapy. She had had a simple mastectomy followed by radiotherapy for a left breast carcinoma in August, 1969. In October, 1971 a mass appeared in the supraclavicular fossa and the anti-oestrogen ICI 46474 was administered. In due course the mass disappeared completely. In July, 1972 it recurred together with an axillary node, which was biopsied and showed testosterone dependence, though with fair survival in oestradiol as well. Adrenalectomy was performed as an anti-androgenic measure and this brought about excellent objective remission which is still maintained.

Adaptation After Previous Oestrogen Dependence

The last patient, no. 92, like several others in the group, illustrates a further important aspect of testosterone dependence. There are good clinical grounds for suspecting that these tumours had adapted themselves after having been originally oestrogen-dependent. Patient 92's tumour had shown an excellent response to anti-oestrogenic therapy for many months before recurring and being shown to be testosterone-dependent. Similarly, patient 24 was a lady whose tumour growth had previously been stimulated by stilboestrol and controlled by durabolin administration. When the tumour subsequently escaped from control after several months of

treatment with durabolin, testing showed testosterone dependence. Two more patients, numbers 82 and 31, had had previous excellent objective responses to radiation and surgical menopause respectively, before the disease later escaped from control and was found to be testosterone-dependent. Patient 82 was interesting from another point of view as well, in that after her successful radiation menopause she had then had a very successful trans-ethmoidal hypophysectomy, which gave her 2½ years free of active disease. It was only when the disease flared up yet again that it was biopsied and shown to be testosterone dependent. (A further excellent remission was obtained by ethinyl oestradiol, but since local radiotherapy was also given to the lesion it cannot be counted as a success of purely hormonal treatment.)

It is interesting and relevant that in this culture system the effects of oestradiol and testosterone were not necessarily diametrically opposed, though sometimes they were, as illustrated in the first two examples above. Patients 103 and 31 showed testosterone dependence of their lesions but they also survived moderately well in oestradiol. It was accordingly anticipated that oestradiol administration would not benefit either patient. As it transpired, oestradiol was among the hormonal treatments tried by the clinicians concerned in both patients and neither achieved a remission.

Most androgen production in the female is derived either directly or via precursors from the adrenal cortex, although in the case of breast cancer patients there may be some para-endocrine activity and androgen synthesis by the tumour itself. Although thus far only one patient in this group has undergone adrenalectomy with the deliberate intention of reducing androgen production, (followed by a successful remission,) the findings lead one to wonder whether this may not be the principal underlying mechanism of at least some successful adrenalectomies?

Certain testosterone antagonists such as cyproterone acetate are now available and it is intended to test these by our in vitro technique to investigate their effects on testosterone-dependent breast cancers.

We are left, then, with the impression that at a given time a human breast cancer may well be androgen-dependent, that this is

invariably after the menopause, (which naturally diminishes oestrogen production,) or at least after anti-oestrogenic treatment, and that it may even be a feature of a tumour which earlier in its natural history had shown an excellent response to anti-oestrogenic measures. This leads persuasively to the concept that these tumours have a dynamic capacity for adapting themselves to the prevailing hormonal milieu, and that this may explain the common clinical phenomenon of a particular hormone producing a good regression, followed by further successful control of a recurrence with an opposing hormone. It must obviously be remembered that the actions of all hormones are central via intricate endocrine feedback pathways as well as peripheral, and we have already seen how complex and controversial is the interaction of oestrogens and prolactin. Furthermore, testosterone may well be converted to some other final active metabolite for use by the cell. However, this in vitro technique strongly supports an androgen-dependence hypothesis by showing that only testosterone and neither oestradiol nor prolactin have enhanced the activity of these tumours, and this has tallied fairly closely with the clinical progress.

HORMONAL TREATMENT IN THE "INDEPENDENT" CASES

I have explained earlier that "independence" of a tumour, i.e. failure to show higher activity of the pentose shunt pathway and good survival compared with both controls, did not necessarily preclude some hormone-responsiveness in vitro nor a clinical response to appropriate hormonal treatment. An independent tumour could survive very well in all three hormones, very badly in all three, or variably. It was in the tumours that had responded variably in vitro that there appeared to be the greatest likelihood of applying the information usefully, either by attempting to suppress or oppose a hormone in which the tumour had fared a good deal better than the medium control, or by administering the hormone in which the tumour had fared even worse than the medium control.

Correct Prediction of Unresponsiveness to Hormones

Typical examples of patients who might have been expected not to respond on the basis of the vitro studies and who in fact achieved no regression on trials of hormonal therapy were numbers 26, 68, 86 and 114. In all these the tumours had survived more or less equally in all

the hormones tested, and in no case had any particular slice survived much better or much worse than the medium control. These patients received a variety of hormonal treatments, including oestrogens, norethisterone, ICI 46474, oöphorectomy and androgens, but none of them achieved any remission. All had to be treated with chemotherapy and as a matter of fact two of the four were dead of disseminated disease within less than a year after biopsy and culture, while the remaining two (114 and 68) are poorly-controlled and deteriorating in spite of cytotoxic drugs within 6 months of testing. It will only emerge from a prospective clinical trial whether those cases that fail to respond to hormones in vitro have a much worse prognosis than those that do, but these examples suggest it.

Anticipated Failure of Incorrect Treatment

As in the case of dependent cases who did not respond clinically when given inappropriate treatment, there were several independent cases who behaved in the same way. Patient 29's tumour was found to be independent but inhibited markedly by oestradiol. The selected hormonal treatment was norethisterone, which failed to produce a remission. The patient died 4 months after testing. Patient 54 had an independent tumour which was inhibited by testosterone. Androgens were not given, however, although norethisterone was. This failed to produce a remission and 6 months after testing the patient developed a hemiplegia due to a cerebral metastasis. I have already referred to patient 87, who was tested twice with an intervening interval of 4 months. Although independent on both occasions, both tests showed that oestrogens were if anything inhibiting the tumour while it survived well in testosterone. Despite this, the patient was subjected to oöphorectomy because she was pre-menopausal, and deteriorated and died remarkably rapidly post-operatively.

Correct Prediction of Successful Treatment

Finally, there was a number of examples of patients with independent tumours who responded very well indeed to treatment that corresponded to the in vitro testing. Patient 59's tumour showed complete death in testosterone and good survival in oestradiol. This lady of 76 had multiple advancing carcinomata in each breast. She was given norethisterone and prednisone with complete failure to obtain a remission, but on the strength of the test the norethisterone was replaced with the

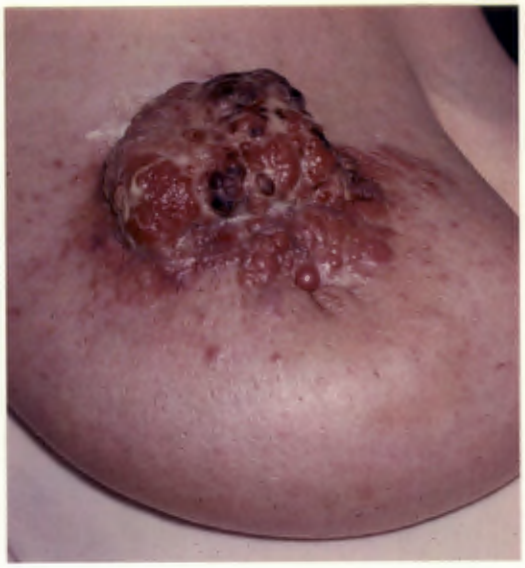
androgen deca-durabolin and the patient achieved an outstanding remission. After presenting in Stage IV in May, 1972 and undergoing biopsy in July, 1972, at which time her treatment was changed to an androgen, she remains well and symptom-free in June, 1973.

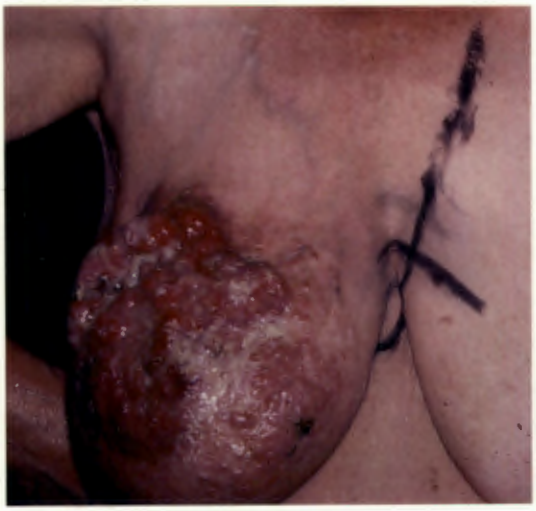
Patient 78, 83 years of age, presented in August, 1972 with an ulcerating carcinoma of the right breast. On testing this appeared to be independent but completely inhibited by oestradiol. This was administered and she had an excellent objective regression lasting from August, 1972 to February, 1973, when pain due to a metastasis developed in the sacro-iliac area. It is emphasised once again that in the clinical examples I have been mentioning, only cases where hormonal therapy on its own can be evaluated are included. No examples of regression or failure of regression have been used where there has been any form of adjuvant therapy like irradiation or cytotoxic drugs.

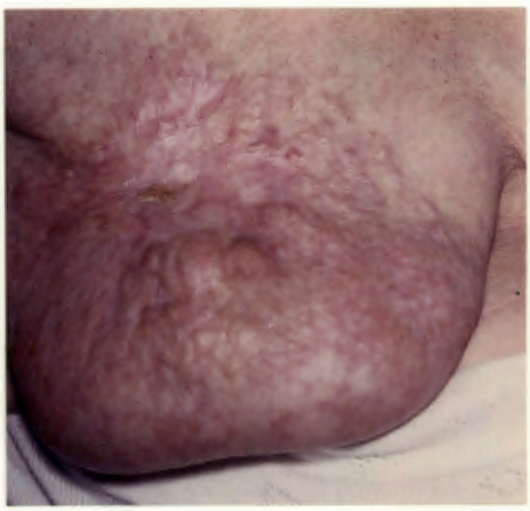
Patient 30, 79 years of age, had noticed a right breast lump in January, 1972 and by the time she presented in April, 1972 there was gross fungation. Biopsy and testing showed the tumour to be independent but to be inhibited by androgens. A full course of radiotherapy between the 8th and 26th May completely failed to control the tumour or stop the fungation and durabolin was commenced on 31st July in view of the result of the test. An outstanding remission was obtained and by the beginning of October, 1972 the florid ulcerating lesion had completely healed. (Fig.21) This regression was maintained on durabolin therapy until the end of April, 1973, when satellite lesions began to appear round the original tumour.

Patient 140, 59 years of age, had received radiotherapy to a Stage III right breast carcinoma in August, 1970, followed by a toilet mastectomy in October of the same year. By December, 1972 there was advancing local disease on the chest wall and radiological evidence of pulmonary metastases and a pleural effusion. Biopsy and culture showed that although the tumour was independent, it had been inhibited by oestradiol while it had survived fairly well in prolactin. She was accordingly given stilboestrol and also prednisone, which might possibly have lowered prolactin by a pituitary suppressing effect. An excellent objective regression of her disease ensued; by May, 1973 there were no nodules of carcinoma at all on her chest wall, while her

(a)







(b) (c)

Figure 21: Patient 30.

(a) On the 19th May, 1972 this fungating tumour was biopsied and shown to be independent, but also to be inhibited by testosterone.

(b) Despite treatment with radiotherapy from 8th to 26th May the lesion advanced, as this photograph taken at the end of the course shows. More than 2 months later there was still no delayed response to radiotherapy.

(c) In view of the result of the test, treatment with durabolin was commenced on 31st July, 1973. An outstanding regression was achieved and maintained, as this photograph taken on 26th March, 1973 shows.

pulmonary lesions also appeared to be regressing.

I feel that the above examples illustrate my earlier statement that the Independent group of tumours covers a spectrum ranging from those who are very unlikely to benefit from any form of hormonal treatment to those who may well achieve outstanding objective regressions. Furthermore, the test has seemed to indicate a specific successful line of treatment based on a rational experimental method. It may turn out to be more convenient to express the results as a "hormonal profile" rather than in terms of dependence. At the same time, in spite of many examples of success, I cannot emphasise too strongly that the pit-falls in translating an in vitro test to an in vivo situation are legion, and that final proof of the usefulness of the test in clinical practice must await the outcome of formal rigorously-controlled prospective clinical trials, which are now being undertaken at the Westminster Hospital.

PATIENTS TESTED TWICE AT DIFFERENT STAGES OF THEIR DISEASE

Up to the time of writing 6 patients who have been included in the series of 150 have had second biopsies and tests, the results of which are pertinent in relation to the treatment they had received in the interim. For the purposes of the Results Tables, only the first biopsy was included, so that they apply to 1 biopsy each on 150 separate patients. The results of the second biopsies are described below.

Patient 69 has already been mentioned in the section on prolactin dependence. After testing in July, 1972 had showed independence of the tumour with no useful variation in survival to suggest possible hormonal treatment, she was treated for the rest of the year with prednisone and 5-fluoro-uracil. This failed to control the local and bony disease and by January, 1973 the lesions were progressing rapidly. Other combinations of chemotherapy also failed to produce a regression and the biopsy was repeated in March, 1973. This time the tumour turned out to be dependent on testosterone and on prolactin. We have already seen that hypophysectomy produced only a transient subjective improvement, followed by further inexorable progress of the disease. Prolactin levels, however, showed that the hypophysectomy had been incomplete. In this case it is difficult to see why the tumour should change from being independent to prolactin and testosterone-dependent. It is conceivable that the characteristics of her

tumour might have altered as a result of 6 months of cytotoxic treatment or that more than one line of cells existed.

Patient 70 has also been discussed in detail in the section on prolactin dependence. In her case there had been no qualitative difference in the result of the test, with inhibition by oestradiol and good survival in prolactin in both instances. However, there was a quantitative difference in that in the second test the survival in prolactin had been so good as to have been classified as dependent. This enabled a more confident recommendation of hypophysectomy to be made. The operation was in due course carried out with an outstanding result.

Patient 24 was discussed in the section on testosterone dependence. After having had a right mastectomy in 1955, axillary disease recurred in 1963 and a series of hormonal manoeuvres followed, including oöphorectomy in 1964, stilboestrol in 1971, (which definitely stimulated the tumour,) and a year of durabolin up to March. At this stage biopsy of the tumour, which was out of control, showed testosterone dependence but with fair survival in prolactin as well, though not good enough to be deemed dependent. The axillary disease was treated by extensive resection and grafting and the durabolin was discontinued. She then remained well until March, 1973 when further metastases developed on the right arm. At this stage repeat biopsy and testing were carried out, confirming that the tumour was again testosterone dependent, but this time it was clearly prolactin-dependent as well. A trans-ethmoidal hypophysectomy was performed in May, 1973 and at the time of writing an evaluation of the result is awaited.

Patient 87 has previously been cited as an example of the use of the test in avoiding fruitless ablative surgery. Her first biopsy suggested that oestrogens were inhibiting the tumour and testosterone permitting it to survive well, despite the fact that she was pre-menopausal. This would have tended to contra-indicate oöphorectomy. When her disease spread, however, oöphorectomy was nonetheless performed, on the usual clinical guidelines, and a repeat biopsy of a skin nodule was taken at the same time. (There had been no other treatment in the interim 4 months.) The result was identical to the first and seems to have been vindicated in that the operation appeared to accelerate the patient's downhill course and early demise.

Patient 95, a lady of 70 years of age, had had a right radical mastectomy in July, 1967. From November, 1970 she was under treatment for recurrent chest wall and axillary disease. In April, 1971 she was given the anti-oestrogen ICI 46474 with possibly slight diminution in the size of the nodules but no sustained regression. In September, 1972 testing showed dependence on oestradiol and prolactin. Treatment was changed to prednisone and durabolin, but after a transient improvement more nodules began growing. In January, 1973 repeat biopsy showed prolactin dependence only. It is tempting to speculate whether several months of treatment with durabolin might have altered the hormonal milieu in such a way as to modify the tumour's original dependence on both prolactin and oestradiol to prolactin only. Hypophysectomy is at present being considered for this patient.

Patient 106, 57 years of age, was treated by simple mastectomy and radiotherapy for a right breast carcinoma in October, 1969. After excision of a skin recurrence on the right shoulder in June, 1971 she was put on to ICI 46474. In September, 1972 a further recurrence appeared on the left shoulder. On excision biopsy and culture this showed oestradiol and prolactin dependence. The ICI 46474 was continued as anti-oestrogenic treatment (and possibly as anti-prolactin treatment as well by the feedback mechanism), but over the next few months a further nodule developed in the biopsy scar. Repeat excision biopsy and testing were carried out in January, 1973 and this time the tumour was dependent on testosterone and prolactin, instead of oestradiol and prolactin as it had been 5 months previously. Had the prolonged course of anti-oestrogenic treatment caused the tumour to modify its characteristics? At the time of writing the patient is being considered for hypophysectomy.

In this small group of 6 patients, therefore, only one (no. 87) had had no hormonal treatment between the two tests and the result on both occasions was identical. In the other patients the results - while reasonably similar - showed some signs that hormonal treatment between the two tests had altered the characteristics of the tumour in what would seem a fairly logical manner, although in the case of patient 69 it is difficult to explain why the tumour changed from being independent to prolactin-dependent. As the follow-up period of the whole group of 150 patient lengthens, there should be further opportunities to perform

repeat biopsies and in vitro testing. If the results correlate with the response to treatment then this would offer the prospect of monitoring the natural history of the disease and changing to the appropriate hormone pari passu with any alteration of dependence of the tumour.

LABILE RESPONSE OF THE TUMOUR TO AN ALTERING HORMONAL MILIEU

Since hormonal treatment was first used in breast cancer patients, it has been observed that successive regressions could sometimes be obtained with opposing hormones, or even by stopping the administration of a particular hormone once the disease had escaped from its control after a period of worthwhile regression, (the "rebound" phenomenon). This has been observed in several of the 150 patients in the present study and the phenomenon is made more interesting by the availability of in vitro hormone studies.

I have already indicated in detail in the discussion on testosterone dependence how 4 of the patients described there had in fact had good objective regressions on anti-oestrogenic therapy earlier in the natural history of their disease. Later, when the disease was again out of control and testing was carried out, it was found to be testosterone-dependent and responded to anti-androgenic measures. It was also pointed out that all the patients whose primary tumours were testosterone-dependent ab initio were in the post-menopausal age-group, suggesting that the menopause itself had altered the relationships of the various hormones in the milieu intèrieur. Finally, the preceding section indicates that in the few patients whose tumours were tested twice, there were signs that a few months of treatment might modify the hormone-dependence according to the hormone administered.

In addition to the above observations, the results of the present study show that the lowest incidence of hormone-dependence was around the menopause, which correlates with clinical experience. The menopause is a phase when profound hormonal re-adjustments are taking place before there is a stabilisation to a definitive post-menopausal pattern. Five years and more after the menopause the incidence of hormone-dependence again rises.

One is thus clearly led towards the conclusion that a given breast cancer cannot be assigned a permanent immutable label of dependence on

a particular hormone once and for all. It appears instead much more likely that the tumour is readily able to adapt itself to changes in the hormonal milieu. This lability would explain many of the apparent paradoxes that have been observed by clinicians treating these patients with hormonal therapy. One of the most interesting aspects of the present study is that the in vitro findings seem to correlate well in a number of cases with the vagaries of the clinical response. The changes in the hormonal milieu could come about through hormonal treatment or through the menopause, and there might in addition be further alterations with progressive ageing, related to hypothalamic and pituitary function. Furthermore, these changes would operate within a physiological range, and not at raised levels.

One further fascinating speculation that might be mentioned here is the effect of cortical influences, stress and emotion on breast tumours. Every clinician has come across patients who insist that the initial appearance, or the sudden aggravation of their disease, was closely related to physical or more especially emotional trauma. We have been trained to treat these assertions with a good deal of reserve, to say the least, which has been very proper in view of our imperfect understanding of the disease. However, there is now indisputable evidence that the hypothalamus does affect anterior pituitary function, and hence prolactin and other polypeptide hormone release, including the gonadotrophins. In view of the equally convincing evidence that cortical (i.e. conscious) influences can control hypothalamic function, may there not occasionally be a grain of truth in claims of exacerbation of the disease by stress? Could cortico-hypothalamic pathways explain the heretical claims of apparent tumour regression through faith alone?

CLINICAL USEFULNESS OF THE TEST

Monitoring the Natural History of the Disease

The beauty of the technique described, if forthcoming clinical trials unequivocally support its applicability to the in vivo situation, is that it will afford us for the first time an opportunity to monitor the course of the disease scientifically in the laboratory. Furthermore, instead of providing a broad non-specific result like oestrogen receptor or urinary discriminant techniques, which aim simply to predict whether the tumour is likely to respond to hormonal treatment in general, this

test indicates a specific choice of treatment. Being simple to carry out and giving a result within about 26 hours, it would be reasonable to perform a minor biopsy under local anaesthetic whenever the disease escapes from control with a particular hormone.

Studies on Metastases

The method also affords us an opportunity of studying metastases at different sites. It is well-known that on a particular treatment metastases may respond well in one site yet continue growing in another. Patient 70 was one such example in this series, where the breast disease regressed strikingly on oestradiol therapy, yet the bony disease progressed. However, a hypophysectomy to remove prolactin, as indicated by the test, resulted in excellent regression of all metastases.

There are certain aspects to this problem of metastases which have not yet been investigated in this system. In the first place, since the technique depends on the culture of intact slices of tumour, no evaluation of in vitro hormone dependence has been made for malignant pleural and peritoneal effusions. Secondly, although the technique has been successfully applied to primary tumours and metastases in skin, soft tissues, lymph nodes and viscera, it has only been used in 3 cases (not included in this series) of bone metastases. The technique was successful in these 3 cases, where the bone had been largely replaced by tumour, but there is a problem in evaluating results for bone in that the marrow contains rapidly-proliferating haemopoietic and lymphoid system cells which also have a very active pentose shunt pathway. There would therefore be difficulty in distinguishing neoplastic and other normal bone-marrow cells with the histochemical technique used. This difficulty would become proportionately less the more completely an area of bone is replaced by tumour. In one such patient, not included in the series, culture of a rib segment almost completely replaced by tumour showed very good survival in testosterone. The clinician elected to perform a bilateral adrenalectomy on the strength of this result and an excellent objective regression lasting 7 months up to now was obtained. However, at present the use of the technique for bone biopsies still needs further evaluation and possibly considerable modification.

Adjuvant Hormonal Treatment

A further advantage of an accurate prediction system would be its

use in considering appropriate adjuvant hormonal treatment at the time of primary surgery or radiotherapy. Although at the present time it is thought that adjuvant treatment such as prophylactic castration may possibly delay the appearance of metastases, it is generally agreed that it is not likely to increase the overall life-expectancy of the patient. It is conceivable, if an accurate prediction were available, that tailoring the hormonal therapy to the individual tumour might improve the overall situation. Again, only long-term clinical trials will give the answer to this question. Being able to pinpoint the exact hormonal therapy might also enable us at some future date to lessen the scope of the present mutilating surgery for breast cancer, particularly now that there is a better understanding of the hypothalamic and pituitary factors involved, which may lead to specific pharmacological antagonists.

CLINICAL CORRELATION

I have emphasised previously that this project was concerned with the development of a technique to study hormone dependence of breast cancer in vitro, and that although the progress of the patients involved was kept under surveillance, this could under no circumstances be considered a clinical trial. There were no protocols of selection or treatment and a variety of clinicians employed a wide range of therapeutic manoeuvres, including combinations of hormones. If the technique is to be proved valuable in predicting response to specific hormonal therapy, a long-term prospective evaluation will of course be necessary. Before deciding whether to embark on such a formal trial, it is therefore relevant to attempt to assess, albeit retrospectively, the clinical correlation in these first 150 patients.

In the first place, all patients in the series who have hitherto had no hormonal treatment at all following the test must obviously be excluded from the evaluation. The same applies to patients on no treatment at all and to those who received other treatment such as irradiation or cytotoxic drugs concurrently with hormonal treatment. The vast majority of the patients are therefore excluded for these reasons. Forty-six patients remain in whom it is possible to assess the results of hormonal therapy alone, whatever its nature.

Since the mode of action of a number of hormones on a tumour and

on feedback mechanisms is ill-understood, it is impossible to interpret the effects of progestogen and corticosteroid administration in terms of oestradiol, testosterone or prolactin dependence. The same problem also arises in the case of experimental new drugs such as CB154. Finally, since the result of the test was available and was often used by the clinician as an additional aid to selecting treatment, it is clearly impossible to analyse the figures as one would a random trial.

Results

Twenty-two of the 46 patients had achieved an objective regression on hormonal therapy and 24 had not. In 6 of the 46 it was difficult to correlate the clinical response with the test, for example, where prednisone had been used alone or in combination with another hormone. In the remaining 40 it was possible to evaluate treatment in terms of the test. As explained previously, the test could either indicate which of the three hormones to remove or counter, or which inhibitory hormone to administer. Bilateral oöphorectomy, radiation menopause, administration of androgens and administration of ICI 46474 were accepted as anti-oestrogenic measures; oestrogen administration and bilateral adrenalectomy as anti-androgenic measures, and hypophysectomy as an anti-prolactin measure.

"Positive Correlation"

Nineteen of the 40 patients achieved a good objective regression on treatment which corresponded with that indicated by the test.

"Negative Correlation"

Seventeen of the 40 patients fared badly and failed to obtain a regression on treatment which was contra-indicated by the test. In 5 of these cases the test result had shown that no hormonal treatment was likely to be effective, whereas in the remainder the indicated line of treatment was different and often opposite to that chosen by the clinician.

Thus in 36 of this small group of 40 cases there appears to have been good correlation with the prediction of the test. In the remaining 4 patients the prediction of the test turned out to be wrong,

For the reasons mentioned, this preliminary retrospective evaluation of the test is obviously unsuitable for statistical analysis, but the extremely high correlation rate (90%) is certainly promising. On the

strength of these very encouraging results, a formal prospective trial is now commencing at the Westminister Hospital.

USE OF THE METHOD FOR OTHER TUMOURS

Benign Breast Lumps

Before giving some examples of the use of the technique for tumours other than in the breast, it is interesting to take note of the results in a few benign cases not included in the series, that were studied at the beginning of the project. Six benign breast lesions were cultured by exactly the same technique, three fibroadenotic lumps and three fibroadenomata. The initial controls of all three fibroadenotic lumps showed zero activity on histochemistry, confirming that the pentose shunt pathway proceeds at a much slower rate in normal or slowly-proliferating tissues than in malignant, rapidly-proliferating ones. One of these fibroadenosis cultures showed considerably enhanced activity in the presence of oestradiol.

Two of the three fibroadenomata showed little and no activity respectively in the initial controls, though one of them was again enhanced by oestradiol. The third raised a very interesting problem; the patient had presented with what was thought to be a Stage III carcinoma of the breast and was treated by radiotherapy. She was later referred for toilet mastectomy for the residual lump and the histology resembled a fibroadenoma more than carcinoma, although there was some controversy about the definitive diagnosis. The clinicians still thought that this was very likely to have been a carcinoma and have kept the patient under regular surveillance. To date she has not yet had a recurrence, the mastectomy having been performed in February, 1972. The interesting feature of this tumour on testing was that it showed very good survival and activity in the initial control, no activity in the medium control, and even higher activity with oestradiol; in other words, it was oestradiol-dependent by the criteria used. The very good survival of the initial control suggested that the pentose shunt was operating vigorously in the tumour in vivo, and therefore to some extent supported the clinical impression that this may have been a malignant tumour after all.

Male Breast Carcinoma

One male breast carcinoma has been studied by this technique,

(also not included in the 150). This 60 year old man had a right radical mastectomy for a Stage II infiltrating papillary carcinoma in September, 1972. This was followed by radiotherapy. In vitro studies on the specimen showed it to be dependent on both testosterone and prolactin, although it also survived fairly well in oestradiol. No adjuvant hormonal therapy was added and at present in June, 1973 the patient has not developed signs of recurrence. I think that this is an extremely important result in that it confirms the finding of Farrow and Adair (1942) that the human male breast cancer was an androgen-dependent tumour, but it also suggests that prolactin may play a role as well. Kennedy and Kiang (1972) reported two males with disseminated breast cancer who each had a good response to orchidectomy and who then each had a further excellent objective regression following trans-frontal hypophysectomy. The crucial observation they made was that oestrogen administration had failed to help both patients. The findings in the one male case studied by this technique dovetail remarkably well with the two described by Kennedy and Kiang. In view of fair survival of this tumour in oestradiol, it would be expected that our patient would be unlikely to respond to oestrogen administration either, although he ought to respond to orchidectomy if the need arises. It will be recalled that in this section on testosterone dependence two cases were described where the tumour had survived fairly well in oestradiol and therefore failure to respond to oestrogen therapy had been correctly anticipated. The point was made that the in vitro effects of oestradiol and testosterone were not necessarily diametrically opposed, though they often were, and that knowledge of the precise nature of the hormone response in vitro would therefore be very advantageous.

It would also be expected that our patient would respond well to trans-frontal hypophysectomy, in view of the prolactin dependence. Kennedy and Kiang found that there were relatively few reports of hypophysectomy for disseminated male breast cancer in the literature, but in spite of the bad reputation of the disease there was probably a 50% objective regression rate. They felt that males stood a better chance of regression from ablative procedures than females.

Malignant Melanoma

Bodenham (1972) has pointed out various features which suggest that malignant melanomas may - in some cases - be influenced by

hormonal factors, including the rarity of the disease before puberty, alterations in growth pattern at puberty, the menopause, pregnancy and menstruation, the significantly longer survivals of females with the disseminated disease, and finally the favourable response of a small number of patients to alterations of the hormonal environment, including hypophysectomy, androgen administration and oestrogen administration. He pointed out the need for a widely-applicable method to detect such patients, although they probably comprise only a small proportion of melanomas. His technique was based on oral administration of radio-phosphorus and measurement of tumour uptake by a Geiger probe inserted into the lesion. After several days of baseline readings the effects of oestrogen administration were then observed (Hale, 1961). This technique involved practical problems and is not suitable for screening a large number of patients.

It was decided to test the present technique on melanoma patients and to this end melanoma biopsies from 11 patients were collected in exactly the same way as the breast biopsies and processed in an identical manner, excepting that they were each cultured with 4 concentrations of ethinyl oestradiol and testosterone respectively, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}M, but not with prolactin. Four of the tumours were also cultured with concentrations of adrenocorticotrophin (ACTH) respectively.

Melanomas proved to be an extremely easy tumour to work with in this system. Being relatively firm and homogeneous, they were very easy to slice by hand and yielded highly cellular cryostat sections. The histochemical reaction worked very satisfactorily in all the 11 cases and confirmed the impression that total dehydrogenase activity of the pentose shunt pathway is an excellent marker of neoplastic activity. Three tumours showed increased activity in testosterone and few in oestradiol, while there was no significant difference between the two hormones in the remaining 4 tumours (Flax, Salih and Westbury, 1973).

Other Tumours

A selection of other tumours has been tested in this system and all of the experiments have worked successfully. All of them showed either moderately high or very high activity of the pentose shunt in

the initial controls, with variable activities in the different hormones tested, confirming the usefulness of this pathway as a marker for studying neoplastic activity in vitro. The tumours included: 1 hypernephroma, 1 ovarian adenocarcinoma, 1 arrhenoblastoma, 1 endometrial stromal sarcoma, 1 soft tissue metastasis of a bronchogenic carcinoma, 1 soft tissue myeloma deposit and 1 soft tissue metastasis of a rectal carcinoma. A very interesting potential application of the method is to prostatic carcinoma; the steroid hormones are known to influence the progress of some cases and recent research suggests that prolactin may play a role as well.

USE OF THE TECHNIQUE FOR OTHER HORMONES AND PHARMACOLOGICAL AGENTS

This study has concentrated on the influence of 17-beta-oestradiol, testosterone and ovine prolactin in maintenance culture systems. However, a few other hormones and drugs have also been tested and these preliminary surveys have supported the usefulness of the system for investigating pharmacological agents. The use of human as well as ovine prolactin in a small number of cases has already been described. Six breast tumours were also cultured with the anti-oestrogenic agent ICI 46474 by exactly the same technique, at concentrations corresponding roughly to the recommended dosage of 10 mg twice daily; these tests confirmed that at those concentrations the drug did indeed behave as an anti-oestrogen. In the case of melanomas 4 tumours were also shown to survive very well in various concentrations of ACTH, and the system would also be interesting to study the effect of beta-MSH, the circulating fraction of the melanocyte-stimulating hormone of the pars intermedia. There is every reason to expect that the technique could be useful to study the effect of a wide range of hormones, hormone antagonists and other drugs on a variety of tumours. The technique also offers the opportunity of studying the effects on the tumour of two or more hormones or a hormone and its antagonist, in a single culture dish. This has not yet been investigated in the present study.

USE OF THE TECHNIQUE FOR HORMONE UPTAKE STUDIES

Radioactive studies on tumour uptake of various hormones,

especially oestrogens, have hitherto been based on single cell suspension systems, in which (as discussed earlier) only a proportion of the cells remain viable. Furthermore, one cannot be certain which of the survivors are normal and which are tumour cells. It will be of considerable interest to label the hormones used, (especially prolactin), with radio-active isotopes, and to study the reactions on the intact slices both qualitatively and quantitatively. This has just been commenced in the present study, using ^{14}C-leucine-labelled prolactin.

SUMMARY

(1) It is well-known that certain cases of human breast cancer are profoundly modified in their behaviour by various hormonal influences.

(2) The actual initiating agent in human breast cancer is probably not a hormone and a virus appears to be the likelier carcinogen. However, once the malignant change has been initiated, hormonal factors play a predominant role as promoters.

(3) Following the observation in 1896 that bilateral oöphorectomy benefited some women with disseminated breast cancer, a period of some 30 years elapsed before attention was again focussed on the role of hormones in the disease.

(4) Bilateral orchidectomy was noticed to produce regressions in some male patients with breast cancer and bilateral adrenalectomy and hypophysectomy were in due course shown to have similar beneficial effects in a proportion of women with the disseminated disease.

(5) At the time, and until very recently, the effects of these ablative operations were thought to be produced purely via the gonadotrophic, adrenocorticotrophic and steroid hormones, but a satisfactory rationale was never produced. Modern work suggests that the situation may be more complex and that the role of other pituitary and hypothalamic factors, particularly the prolactin secretion mechanism, may be important.

(6) At the same time as regressions were being produced by the ablative operations, some successes were being obtained by the administration of exogenous steroid hormones, both oestrogenic and androgenic.

(7) Experimental methods of tumour induction in animals were also being actively investigated and of these the 7,12-DMBA - induced breast tumour model in rats was particularly useful.

(8) Although the understanding of the hormonal mechanisms involved was imperfect, and although a prediction system for deciding which patients were likely to respond to hormonal therapy was not available, a set of clinical guidelines based mainly on the menstrual status of the patient evolved.

(9) The conventional approach relied on ovarian ablation in the

premenopausal patient and on either an ablative operation (adrenalectomy or hypophysectomy) or a wide range of administered hormones (oestrogens, androgens, progestogens, corticosteroids) in the postmenopausal patient. The patients in the early post-menopausal years seemed less likely to respond to hormonal treatment.

(10) Experimental animal work in the 1950's showed that prolactin might promote the growth of certain rodent breast tumours and a few early observations suggested that this applied to human breast tumours as well.

(11) In 1969 it was demonstrated that the DMBA-induced tumour in rats was definitely prolactin dependent and a great deal of experimental work was carried out to investigate the role of prolactin and the control of its secretion in animals. A hypothalamic substance called Prolactin Inhibiting Factor (PIF) appeared to be the most important controlling mechanism.

(12) Attention naturally turned to considering whether a similar situation might exist in humans and how prolactin secretion might be controlled. Many pharmacological agents were found to influence prolactin secretion, mainly via PIF, but also by direct pituitary action. Of these the most important groups were the phenothiazines, which increased prolactin secretion, and certain ergot alkaloids and levodopa, which decreased it.

(13) In 1972 data from the present study showed that in vitro the activity of over 30% of human breast cancers was enhanced by prolactin. Ovine prolactin, which behaves very similarly to human prolactin, was used for most of the experiments, but later tests with a small amount of purified human prolactin that became available confirmed the findings.

(14) The evidence for the separate existence of human prolactin is summarised; it was only in 1971 that unanimity was reached that it did exist independently of human growth hormone, although it had been isolated as a separate hormone in other mammals several decades previously. The physiological role of prolactin is discussed.

(15) Data on human prolactin levels in different conditions are still scanty and several different assay systems are in operation. From data so far available the levels do not appear to be increased in

women with breast cancer.

(16) Only about 1 in 3 patients shows a favourable response to hormonal treatment for advanced breast cancer and there is a great need for a prediction system to select these.

(17) Studies on urinary steroid excretion patterns, and particularly the Discriminant Function, have been extensively investigated, but these have not proved to be sufficiently accurate for use with individual patients.

(18) Important research indicated that certain breast tumours showed selective higher uptake of oestrogen and there was some evidence that this might correlate with the clinical response. However, these studies do not as yet take into account other hormones that may be involved and have so far not proved effective as a prediction system.

(19) In spite of attempts to predict response, management of hormonal therapy of the patient at present still relies mainly on clinical guidelines such as the menstrual status, distribution of metastases and disease-free interval.

(20) The development of cell and organ culture techniques permitted in vitro studies of the action of hormones and drugs on various tissues, including breast tumours. This approach continues to be extensively investigated but as yet no method has been shown to have reliable clinical applicability. In general, cell suspension techniques have certain major disadvantages in this approach as compared with organ culture techniques.

(21) A technique was developed which utilised short-term maintenance cultures of intact breast cancer slices in a fully-defined synthetic culture medium. Evaluation of survival of the tumours was both by histology and by a histochemical technique which measured the total dehydrogenase activity of the pentose shunt pathway.

(22) It was decided to test the tumours against two experimentally-selected concentrations each of 17-beta-oestradiol, testosterone and prolactin. The concentrations selected, after appropriate standardisation experiments, were similar to those used by other investigators.

(23) The techniques of handling the biopsies, setting up the

cultures and carrying out the histological and histochemical examination are described in detail.

(24) The system of scoring the survival and activity of the tumours is described. It was found convenient to classify a tumour as "dependent" on a particular hormone if it had shown higher activity in that hormone than both a medium control and a fresh-frozen uncultured control of the tumour immediately after removal from the patient. The "independent" cases covered a spectrum ranging from those that had shown no differential responses in the hormones tested to those that had either survived very well or been strikingly inhibited by a particular hormone.

(25) No attempt at a clinical trial was made at the start of the project, but the progress of all the patients was carefully followed and documented in order to evaluate whether there was any useful correlation with the result of the test.

(26) Seventy nine of the 150 tumours tested satisfied the criteria for dependence on one or two of the hormones tested. The most dramatic feature was the finding of prolactin dependence in 50 (33%) of the tumours.

(27) Analysis of the results in relation to menstrual status shows that there is a significant change in dependence around the menopause. Prolactin and oestradiol dependence are markedly commoner before the menopause and testosterone dependence afterwards.

(28) Hormone dependence is least common in the five years immediately following the menopause, which supports the clinical observation that hormonal therapy is least effective in this period.

(29) In the patients whose tumours were tested subsequent to an endocrine-ablative procedure, the dependence seemed to be related to the nature of the ablation.

(30) The site of the biopsy and the clinical stage of the tumour did not appear to influence the distribution of dependence.

(31) The advantages of the technique used are enumerated. In particular it avoids drastic changes of the tumour from its physiological conditions, and it also measures the effect of a given hormone on the tumour cell specifically. It offers the opportunity to study a particular hormone's action at target cell level without involving other variables such as feedback mechanisms and interactions with other circulating hormones.

(32) The only two serious technical problems were contamination of the culture and an inadequate biopsy. The method has built-in safeguards to detect and eliminate these.

(33) A small number of tumours fared better in the medium control than in the initial control. This is almost certainly due to the stimulating effect of insulin in the medium.

(34) The remarkably high incidence of prolactin dependence is discussed. There is much controversy about the factors controlling prolactin secretion and it appears as if many forms of other hormonal treatment may influence it by a feedback mechanism. It does seem, however, as if prolactin-dependence of human breast cancers operates as physiological and not excessive levels of circulating prolactin.

(35) Investigations are in progress to find a specific prolactin-suppressing pharmacological agent. Bromergocryptine and levodopa have attracted the most attention so far, although no striking results have hitherto been obtained from their use in women with breast cancer.

(36) It is demonstrated that hypophysectomy may well operate, at least in some cases, by removing prolactin secretion, and that inadequate hypophysectomy may fail to bring about a regression because of continued prolactin secretion.

(37) A number of other hormonal and anti-hormonal treatments may well work via a prolactin-suppressing effect, and several possible examples are cited, but it is clear that the situation in humans is a good deal more complex than in the rat, where the relationship between prolactin secretion and breast tumour growth is a relatively simple and direct one.

(38) Examples are also given of correlation of the clinical progress with in vitro dependence of the tumours on oestradiol.

(39) The concept of testosterone dependence is introduced. Although well-known in rats, this concept has never been explored in depth in humans. Clinical examples are given which support the in vitro findings. There is some evidence to suggest that at least some adrenalectomies may produce regressions by removing testosterone.

(40) The test also appears to be of potential use in avoiding fruitless endocrine-ablative surgery.

(41) Many of the dependent and independent cases were also amenable to successful hormonal therapy by administering a hormone that was inhibitory

to the tumour in vitro, as an alternative to countering one in which the tumour had survived well.

(42) Evidence from this study, including from several patients whose tumours have been tested at different stages of their disease, strongly opposes any suggestion that a breast cancer can be assigned one unchanging hormone dependence which applies throughout its natural history. The tumour appears to be very labile and to be able to adapt itself dynamically to changes in the hormonal milieu, for example those occurring at the menopause or as a result of treatment.

(43) The potential uses of this technique as a prediction system and for monitoring the course of the disease are discussed, but it is emphasised that there are many difficulties in translating in vitro results to an in vivo situation, and that only long-term, prospective, controlled trials will determine its validity.

(44) In a group of 40 patients in whom it was possible to make a comparison between the results of the test and the response to hormonal therapy there was good correlation in 36 (90%). This highly-encouraging finding has resulted in the establishment of a formal trial of the technique at the Westminister Hospital.

(45) In addition to its use in breast cancer, the technique has been tried for a variety of other tumours, notably malignant melanoma, and with a number of other hormones and drugs. It appears to be a good research tool for tumour studies of this nature.

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APPENDIX I

COMPOSITION OF TROWELL'S T8 CULTURE MEDIUM

	mg/100 ml
NaCl	610
KCl	45
$CaCl_2$	22
$MgSO_4.7H_2O$	25
$NaH_2PO_4.2H_2O$	45
$NaHCO_3$	282
Glucose	400
L-arginine HCl	2.1
L-cysteine HCl	4.7
L-histidine HCl	1.0
L-isoleucine	2.6
L-leucine	2.6
L-lysine HCl	3.6
DL-methionine	1.5
DL-phenylalanine	3.3
DL-threonine	4.8
L-tryptophan	0.4
L-tyrosine	1.8
L-valine	2.3
Thiamine HCl	1.7
P-aminobenzoic acid	3.5
Insulin	5.0
Chloramphenicol	3.0
Phenol Red	1.0

APPENDIX II

DETAILS AND SOURCES OF MATERIALS

Slee "Pearse" Cold Retracting Microtome Cryostat (Type HR), Slee Block Holders, Simple Tubular Pattern, No. 4035/S and Slee Laminar Flow Bench, HLF/H, with ultra-violet attachment, from South London Medical Equipment Ltd., London S.E.13.

Instanton Electric Balance Unimatic CL4D, from Stanton Instruments Ltd., London S.W.17.

PHM28 pH Meter, Radiometer Copenhagen, from V. and A. Howe and Co. Ltd., London S.W.6.

Griffin Incubator, operating at 37^{o}C.

"Little Sister" Autoclave, operating at 30 lb. per square inch at 135^{o}C.

G.V. Planer Cooling Unit Type R200, cooling at 2^{o}C per minute.

Chance Propper Pre-Cleaned Select Microscope Slides, 76 x 25 mm., from Solmedia Ltd., London E.17.

Steel Slide Racks, Staining Troughs and Chance Coverslips 22 x 32 mm., from A.R. Horwell Ltd., London N.W.6.

Ampoule Racks from Arnold's Veterinary Products, Reading, Berkshire.

Screw-Cap Specimen Ampoules for liquid nitrogen storage and Sterile Plastic Universal Containers (P128/A) from Sterilin Ltd., Richmond, Surrey.

Plastic Sterile Disposable Culture Dishes, 60 x 15 mm., from Falcon Plastics, London W.C.1.

Stainless-Steel Sharp-Point Dissecting Forceps (MA2478, 6 inches) and Stainless-Steel Spatulas (MC2332, Nuffield pattern), from C.E. Payne and Sons Ltd., London S.W.4.

Stainless-Steel Grids cut from "expanded metal," (mesh 1.3 mm., 0.0005 inches thick), from The Expanded Metal Co., London S.W.1.

Green's Lens Tissue C105, from J. Barcham Green Ltd., Maidstone, Kent.

Gillette Scimitar Sterile Disposable Needles No. 1 (21g) and Sterile Disposable Scalpels No. 22 from Gillette Industries

Ltd., Isleworth, Middlesex.

B & D Plastipak Sterile Disposable Polypropylene Syringes, from Becton, Dickinson and Co. Ltd., Republic of Ireland.

Liquid Nitrogen, from Nitrochill Ltd., Wembley, Middlesex.

Liquid Nitrogen Refrigerator and Dewar Flasks of liquid nitrogen, from Union Carbide, Darlington, Durham.

Decon 90 Detergent, from Decon Laboratories, Brighton, Sussex.

Trowell's T8 Culture Medium, from Difco Laboratories, East Molesey, Surrey.

17-beta-oestradiol, testosterone and L-glutathione (reduced), from Koch-Light Laboratories, Colnbrook, Buckinghamshire.

Sheep Prolactin, 22 I.U. per mg., in 10 mg. ampoules, from the Medical Research Council Division of Biological Standards, London N.W.7.

Human Prolactin (90% pure), from Dr. H. Friesen, McGill University, Montreal, Canada.

Ethyl Alcohol 99.8% v/v, from James Burroughs Ltd., London S.E.11.

Neotetrazolium Chloride, from SERVA, Heidelberg, West Germany.

Phenazine Methosulphate, from Sigma Chemical Company, St. Louis, U.S.A.

Glucose-6-Phosphate (di-sodium salt) and NADP, from Boehringer, Mannheim, West Germany.

Polyvinyl Alcohol, from Bush, Beach and Segner, London W.1.

Glycylglycine, Ascorbic Acid and Xylene from B.D.H. Chemicals Ltd., Poole, Dorset.

Ehrlich's Haematoxylin, Eosin, Canada Balsam and Farrant's Medium, from Raymond A. Lamb, London N.W.10.

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IN-VITRO ŒSTROGEN SENSITIVITY OF BREAST-CANCER TISSUE AS A POSSIBLE SCREENING METHOD FOR HORMONAL TREATMENT

H. SALIH H. FLAX
J. R. HOBBS

Tumour Biology Group, Westminster Hospital and Medical School, London S.W.1

Summary Fourteen malignant and five benign breast tumours were cultured in Trowell's T_8 medium containing different concentrations of 17β-œstradiol. At least two distinct classes of breast tumours were found—one class required œstrogens for survival, the other did not. These classes were divided on the bases of their histological appearance and total dehydrogenase activity before and after culture. Clinical follow-up of the malignant cases suggests correlation with the in-vitro steroid dependence of the breast tumour. It is hoped that this approach may provide a rational basis for deciding a hormonal therapy for individual patients.

Introduction

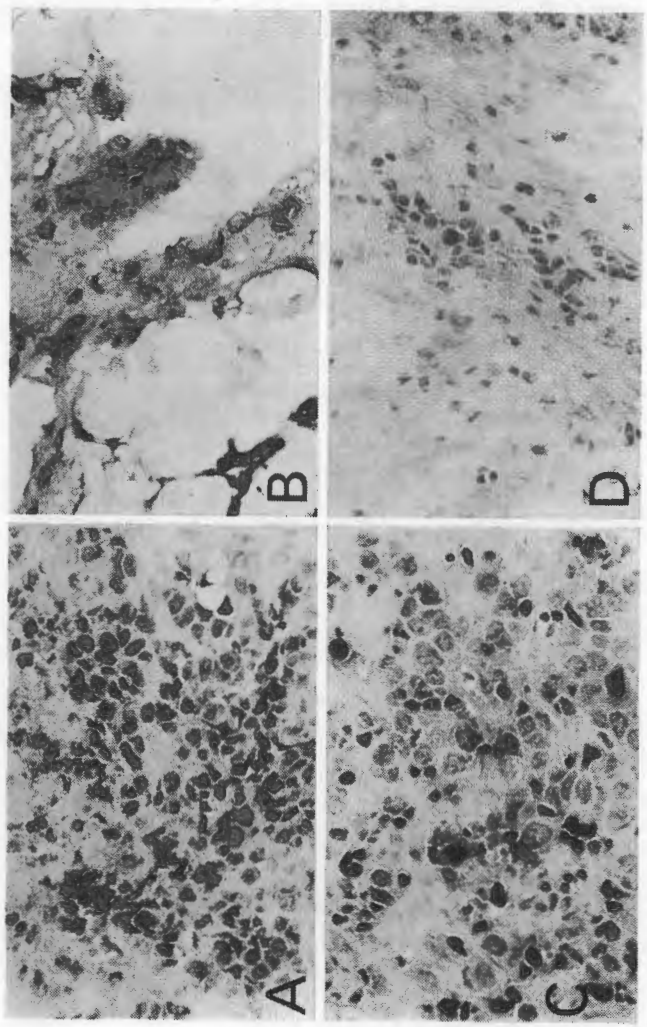
IN 1961 Hayward and his co-workers [1] combined urinary 17-hydroxycorticosteroid and ætiocholanolone levels in a discriminant function which might, they suggested, predict the response of patients with advanced breast cancer to hypophysectomy or adrenalectomy with oophorectomy. Since then this group has done a lot of work on correlating discriminant values with response to treatment.[2] However, this type of approach has not permitted response and non-response to be distinguished completely, and in individual cases more information than the discriminant function provides is required. Chayen et al.[3] reported some preliminary results on the in-vitro response of human breast-cancer tissue to steroid hormones. They showed that breast tumours could be divided into two distinct classes. One class required œstrogens for survival in vitro and the other did not; however, no clinical correlations were given.

We describe here an improved method for assessing the in-vitro survival of individual breast cancers and the clinical follow-up to see whether or not steroid dependence was demonstrated.

Materials and Methods

Thin slices of tissue were obtained from fourteen malignant cases (and five patients undergoing local excision for cystic mammary dysplasia). This was done under aseptic conditions at the time of operation and the slices were placed on a sterile cloth in a sterile bottle containing Trowell's T_8 medium. Some of the slices were chilled immediately in n-hexane at −70°C and others were maintained for 24 hours in Trowell's T_8 medium alone, and also in Trowell's T_8 medium containing various concentrations of 17β-œstradiol and/or testosterone. The maintenance culture technique used was that developed by Trowell.[4,5] Since the nature of the container was unimportant,[6] a modified Kilner jar served as the chamber. This contained a wire frame supporting four tissue-culture dishes (60×15 mm.). In each of these dishes stood a stainless-steel grid, and over the grid a lens paper was placed. The slices of tissue, which were teased and cut into small pieces, were then placed on to the lens paper and the culture medium was poured into the dish such that it just made contact with the tissue. The atmosphere inside the chamber was adjusted by short periods of gassing and the chambers were incubated at 37°C. The gassing mixture used was 5% carbon dioxide and 95% oxygen. After the maintenance period the tissues were chilled quickly in n-hexane at −70°C, and 8 μ sections were cut from cultured and uncultured specimens, in a cryostat at −25°C to −35°C. The histological appearances of the sections were examined in duplicate and each treatment was compared with uncultured biopsy. To support these findings total dehydrogenase activity of the pentose-shunt pathway was determined [7] in intact tissue sections after the reduction of neotetrazolium chloride (with phenazine methosulphate as the first hydrogen acceptor) to highly coloured formazan.[8] This insoluble salt was examined qualitatively in all patients, but some preliminary semiquantitative determinations were made by Dr. S. Bradbury (department of human anatomy, University of Oxford) in two cases using ' Quantimet 720 '; one case showed conclusively in both clinical and biochemical findings that œstrogen was essential for growth, and the other was independent of œstrogens.

Histological types of tumours studied were scirrhous, mucoid, and medullary. In some cases histological information on cases initially operated on elsewhere was not available. Nine of the fourteen cases had advanced breast cancer and, in some cases with metastases, biopsy samples were obtained from skin, liver, and cervical nodes. Types



Cryostat sections of human secondary breast-cancer tissue taken from axillary node and stained with haematoxylin and eosin (reduced to about half of ×375).

(A.) Tissue taken at operation.
(B.) Same specimen after maintenance in T_8 medium for 24 hours, showing deterioration compared with A.
(C.) Same specimen after 24 hours in T_8 medium containing $10^{-9}M$ 17β-œstradiol; histological appearance better than that in B (no œstradiol).
(D.) Same specimen after maintenance in T_8 medium containing $10^{-9}M$ 17β-œstradiol for 24 hours; histological appearance better than in B but worse than in C.

Cryostat sections of the same human secondary breast-cancer tissue as in A-D.

The photographs show total dehydrogenase activity of the pentose-shunt pathway deposited as coloured formazan after reduction of neotetrazolium chloride when phenazine methosulphate is used as the first hydrogen acceptor (reduced to about half of ×375).
(E.) Tissue taken at operation.
(F.) After maintenance in T_6 medium alone for 24 hours; the few whorls of cancer cells show very little activity.
(G.) After maintenance in T_6 medium containing $10^{-6}M$ 17β-oestradiol for 24 hours; very high activity of the pentose-shunt pathway which ultimately produces reducing power for biosynthetic purposes essential for survival of cancer tissue.
(H.) Tissue maintained in T_6 medium containing $10^{-6}M$ 17β-oestradiol for 24 hours. As in E, cells are still showing activity.

4

5

TABLE I—SUMMARY OF HISTOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF TISSUE CULTURE OF ŒSTROGEN-DEPENDENT AND ŒSTROGEN-INDEPENDENT BREAST-CANCER TISSUE

No. of cases	Concentration of 17β-oestradiol	Histological appearance	Total dehydrogenase activity	No. surviving, based on:	
				Histology	Dehydrogenase activity
Œstrogen-dependent 7	0	− (7)	− (7)	2	
	$10^{-8}M$	++ (7)	↑ ↑ (7)	7	
	$10^{-6}M$	+ (7)	↑ (7)	7	
Œstrogen-independent 7	0	− (4) ++ (1) Same (2)	↑ (1) Same (6)	3	1
	$10^{-8}M$	+ (2) − (5)	↓ ↓ (7)	2	0
	$10^{-6}M$	− (6) + (1)	↑ (4) ↑ (3)	1	3

of operation performed for the primary tumour included segmental excision, simple mastectomy, and Patey and radical mastectomies. Tumour tissue for culture was obtained at operation or from biopsy specimens in the advanced cases.

Results

Biopsy tissues from seven malignant cases maintained in T_8 medium containing $10^{-5}M$ 17β-œstradiol showed high dehydrogenase activity and an improved histology when compared with their uncultured tissue. Five of these tissues did not survive in Trowell's T_8 medium when no œstrogen was added, and two survived poorly. Histological examination showed few cells surviving, and pentose-shunt studies revealed no dehydrogenase activity in five and very little activity in two of these cases. Tissues maintained in T_8 medium containing $10^{-6}M$ 17β-œstradiol had less biochemical activity than those maintained at the higher œstrogen dose in all seven cases, but showed improvement over the results obtained with T_8 medium alone (control). Tissues maintained at $10^{-6}M$ œstradiol showed either a higher or about the same activity as their initial controls (uncultured tissue). These results show that breast tissue from patients 1–7 required œstrogens for growth and maintenance in vitro. Tissues from the other seven malignant cases showed a striking decrease in dehydrogenase activity when maintained in T_8 medium containing $10^{-6}M$ 17β-œstradiol and compared with their initial controls. However, although four of these specimens showed less activity when maintained in T_8 medium containing $10^{-6}M$ 17β-œstradiol, tissues from the remaining three cases showed higher dehydrogenase activity than their initial controls. The results in the steroid-dependent and steroid-independent cases are summarised in table I. Preliminary semi-quantitative results of a dependent and

TABLE II—SEMIQUANTITATIVE RESULTS

Conditions of culture	% formazan/picture-point		% matrix/picture-point	
	Clearly independent	Dependent	Clearly independent	Dependent
Uncultured	8	10	92	90
T_8 medium only	1	1	99	99
$10^{-5}M$ 17β-œstradiol	0·8	54	99·2	46
$10^{-6}M$ 17β-œstradiol	5	3	96	97

TABLE III—SUMMARY OF TREATMENT AND PROGRESS: ŒSTROGEN-DEPENDENT CASES

Case	Age	Treatment before T.C. (and stage of tumour)	Histological type of tumour	Menstruating at time of T.C.	Clinical state at time of T.C.	Treatment after T.C.	Clinical progress
1	60	Radiotherapy, simple mastectomy (stage III)	Scirrhous*	No	Lumbar and cervical spine deposits	Stilbœstrol, ethinyl œstradiol, nandrolone, I.C.I. 46,474, bilateral adrenalectomy and oophorectomy; radiotherapy to vertebral deposits	Worse on œstrogens, slight improvement on nandrolone; died
2	41	Radiotherapy, radiation menopause (stage III)	† No report	No	Cervical nodes, no recurrence in other breast	Nandrolone	Excellent remission until nodes recurred
3	49	Simple mastectomy and radiotherapy	No report	Yes	Widespread dissemination	Radiation menopause, dexamethasone	No remission; died
4	46	Radical mastectomy, bilateral oophorectomy, norethisterone	Cellular (medullary)	No	Secondary deposits, pleural effusions	Norethisterone; chemotherapy	Good general health, nodes static, pleural effusion minimal
5	70	None	No report	No	Ulceration of breast, lymphœdema, pain	I.C.I. 46,474	Excellent remission, ulceration healed, pain gone, lymphœdema resolving
6	55	Extended simple mastectomy (stage III)	Scirrhous	No	No dissemination	None	Well
7	42	Radical mastectomy	Scirrhous	Yes	No dissemination	None	Well

* Later histology on mastectomy specimen showed no viable cancer cells.
† Cervical-node biopsy confirmed secondary carcinoma.

TABLE IV—SUMMARY OF TREATMENT AND PROGRESS: OESTROGEN-INDEPENDENT CASES

Case	Age	Treatment before T.C. (and stage of tumour)	Histological type of tumour	Menstruating at time of T.C.	Clinical state at time of T.C.	Treatment after T.C.	Clinical progress
8	43	Patey mastectomy (stage I)	Scirrhous	Yes	No dissemination	None	Well
9	54	Extended simple mastectomy, radiotherapy, nandrolone	Scirrhous	No	Hepatic and vertebral metastases	Bilateral adrenalectomy and oophorectomy, chemotherapy	Died (? brainstem haemorrhage)
10	49	Segmental excision	Mucoid	No	No dissemination	Radiotherapy postoperatively	Well
11	57	Radical mastectomy	Scirrhous	No	No dissemination	Radiotherapy to node fields only	Well
12	75	Radiotherapy (stage IV)	No report	No	Fungating, infected breast; lung metastases	Chemotherapy	No remission; died
13	57	Radiotherapy, non-ethisterone, previous hysterectomy (? ovaries removed)	Scirrhous	No	Skin nodules, pleural effusion	I.C.I. 46,474, non-ethisterone with-drawn	Regression of nodules and effusion on I.C.I. 46,474; well, no metastases
14	63	Simple mastectomy, bilateral oophorectomy, radiotherapy to axilla, stilbœstrol, nandrolone	No report*	No	Recurrent axillary nodules	Excision of axillary nodule	Oophorectomy and stilbœstrol did not control axillary recurrences; nandrolone caused improvement but not regression; free of recurrence

* Axillary-node biopsy confirmed secondary carcinoma.

a clearly independent case are shown in table II. The apparatus used for this purpose was quantimet 720. A matrix, resulting from division of the image by digital control, had precise and reproducibly positioned picture points. A scan line of 100 picture-points was chosen. Formazan and matrix within this area were measured at ten random positions in each section. The average in each treatment was calculated, and % formazan/picture-point and % matrix/picture-point was calculated from these results. The clinical follow-up of all malignant breast-tumour patients is summarised in tables III and IV. All the tissues obtained from benign cases survived well in T_8 medium containing no œstrogen. Some showed a little improvement on addition of œstrogens and all the uncultured biopsy specimens from these patients had no dehydrogenase activity.

Discussion

In seven of the fourteen malignant cases tumour tissue required œstrogens for maintenance and growth in vitro. Dehydrogenase activity of these tissues decreased when they were maintained at lower concentrations of 17β-œstradiol, and their histological appearances showed less improvement compared with those at the higher steroid dose. Five of the seven œstrogen-requiring tissues did not survive in Trowell's T_8 medium alone.

Only a few cases have been investigated so far, but there seems to be good correlation between in-vitro steroid dependence and clinical progress on antiœstrogenic therapy (e.g., cases 2, 4, and 5). In case 1, where the test suggested œstrogen dependence but stilbœstrol and subsequently ethinylœstradiol were prescribed, the patient got worse if anything: antiœstrogenic therapy was tried at a late stage, with some symptomatic improvement, but the patient already had widespread dissemination and no remission could be obtained.

The other seven malignant cases, showing variable results when maintained at $10^{-8}M$ 17β-œstradiol but an unmistakable decrease in dehydrogenase activity at the higher dose of œstrogen, are classed as breast-cancer tissues not requiring œstrogens. However, it may be that survival of three of these tissues by biochemical criteria, and one by histological criteria, at low levels of œstrogen (table I), further subdivides this group, suggesting that more than two classes of breast tumours may exist, at least in respect

of steroid dependence. We are now routinely culturing tumour tissues with varying concentrations of prolactin and testosterone, and this may throw some light on this subject. In the non-œstrogen-requiring group there is some correlation with the in-vitro tests—e.g., in the complete failure to obtain remission with antiœstrogenic therapy in cases 9 and 14. In case 13, antiœstrogenic treatment (norethisterone), given despite in-vitro œstrogen independence, did not control recurrences, yet complete regression and control were subsequently obtained with I.C.I. 46,474 (1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenyl-1-ene), a new non-steroid preparation which has varying œstrogenic and non-œstrogenic effects in laboratory animals, but which, at the recommended dose of 10 mg. twice a day, is said to be antiœstrogenic in man. Its mode of action is not certain, and we cannot explain its efficacy in case 13 when norethisterone treatment had failed.

Semiquantitative determinations show conclusively that the total dehydrogenase activity in the two groups is very different. There seems to be about a tenfold increase in activity with œstrogen-dependent tumours and a tenfold decrease with œstrogen-independent tumours, when maintained in a concentration of $10^{-5}M$ 17β-œstradiol.

The advantage of this type of cellular and biochemical approach is that each breast-cancer patient's tumour tissue can be studied individually, and the response determined nearer to physiological conditions, because the method does not involve the use of cell suspensions or homogenisation techniques. In addition, the period of incubation is kept down to 24 hours to try to avoid serious changes in the biology of the tissues. The total dehydrogenase activity has been very easy to assess and is a more reliable indicator of survival than is histology alone. Armed with the knowledge of the steroid-dependence of each patient's own tumour, at least in vitro, the clinician may be able to make a more rational and effective decision as to hormonal therapy in advanced breast cancer.

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Requests for reprints should be addressed to J. R. H.

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PROLACTIN DEPENDENCE IN HUMAN BREAST CANCERS

H. SALIH H. FLAX
W. BRANDER J. R. HOBBS

Tumour Biology Group,
Westminster Hospital and Medical School,
London S.W.1

Summary Prolactin dependence has been directly demonstrated in 32% of fifty human breast cancers. The pentose-shunt activity of the fresh-frozen tumour is compared to that of slices kept only 24 hours in culture media with and without added hormones (sheep prolactin, 17β-œstradiol, testosterone). Results may have predictive value for the individual patient.

Introduction

IN laboratory animals, prolactin is a factor for the growth of some mammary carcinomas. Dimethylbenzanthracene-induced mammary carcinoma in Sprague-Dawley rats is a prolactin-dependent tumour.[1,2] After the hypophysectomy-induced remission œstradiol benzoate injections did not reactivate tumour growth, whereas sheep prolactin did. Boot [3] considered prolactin to be the hormone most directly responsible for mammary tumour formation in mice. Cassell et al.[4] found that ergot alkaloids inhibited mammary-tumour growth in rats, both directly and by increasing prolactin-inhibitory factor (P.I.F.) to decrease pituitary prolactin secretion.

In man, Salih et al.[5] have used an in-vitro method for determining œstrogen sensitivity of breast cancer under conditions close to its freshly excised state. This method has the following advantages:

(1) Clearcut results can usually be obtained within 24 hours for the individual patient.

(2) The tumour is reasonably similar to its natural condition. It has not been exposed to trypsin, nor has there

been prior selection of those single cells surviving in tissue culture.

(3) Histochemical localisation enables assessment of the carcinoma cells alone. Assays in relation to a given mass of whole tumour cannot allow for the varying content of stroma.

(4) The pentose-shunt activity accords well with the condition and growth of the tumour.

Follow-up of those patients studied indicates a reasonable correlation between in-vitro findings and response to therapy. The method was extended to see whether prolactin affected the growth of human breast cancer, and we report here our preliminary results.

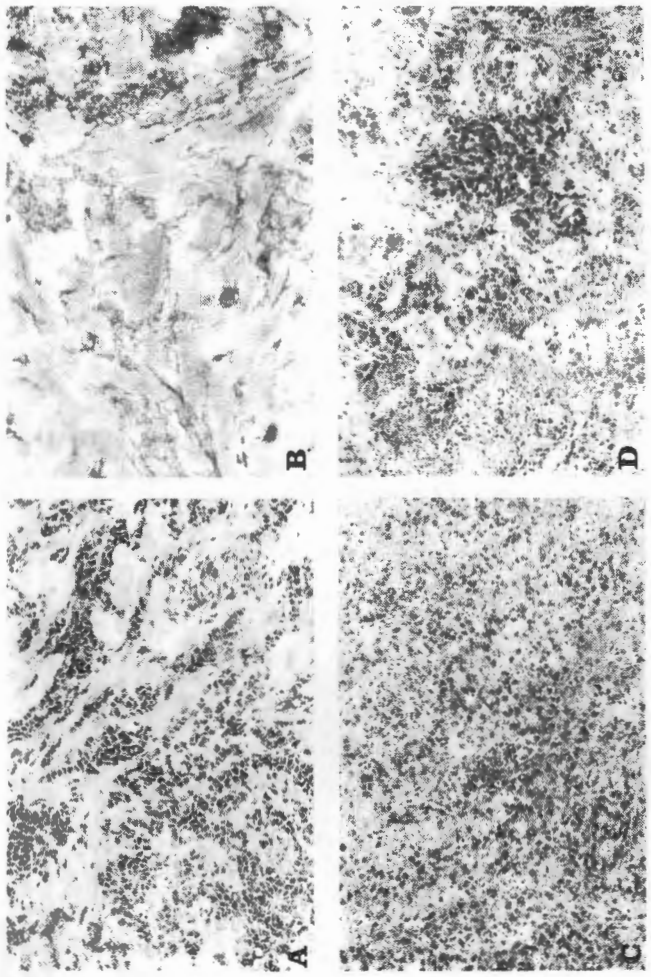
Materials and Methods

Biopsy tissues were obtained from fifty breast-cancer patients under aseptic conditions and placed in Trowell's T_8 medium. A piece from the biopsy material was frozen immediately in liquid nitrogen, and the other thin slices were maintained for 24 hours in Trowell's T_8 medium alone and also Trowell's T_8 medium containing various concentrations of hormones. Sheep prolactin was kindly supplied by the Medical Research Council Division of Biological Standards, in ampoules containing 22 I.U. per mg., and was the same material as that described by Bangham et al.[6]

Sheep prolactin is closely related to human prolactin,[7] and we estimated that in non-pregnant women serum levels would be equivalent to about 200 mI.U. sheep prolactin per ml. Since there is no evidence of prolactin binding to serum-proteins we chose for convenience 220 mI.U. per ml. as one concentration of free sheep prolactin added to the protein-free Trowell's T_8 culture medium. Preliminary dose-response curves on breast cancers showing dependence indicated no potentiation of tumour growth at 2·2 mI.U. per ml., some potentiation at 22 mI.U. per ml., and actual toxic suppression of growth at 2200 mI.U. per ml. We therefore used 22 and 220 mI.U. per ml. for the initial screening of breast cancers. The maintenance culture techniques have already been described.[5] After the maintenance period the tissues were transferred to liquid nitrogen. Using a cryostat, 8 μ sections were made of the initial uncultured frozen controls and of the cultured specimens. The total dehydrogenase activity of the pentose shunt was determined in duplicate by a histochemical method. At first the histochemical results were carefully measured using 'Quantimet 720', but with experience reliable comparisons can easily be made by eye.

From the same frozen specimens, sections adjacent to those used for histochemistry were stained by hæmatoxylin and eosin (H.&E.). The blocks were then processed through to paraffin, so that even better sections could be prepared for H.&E. staining.

The diagnosis of carcinoma of the breast was confirmed



Cryostat sections of human secondary breast-cancer tissue taken from a skin nodule and stained with H.&E. (reduced to 5/12 of × 120).

(A) Appearance of tissue taken at operation.
(B) Same biopsy material after maintenance in T_8 medium for 24 hours without prolactin. The tissue has died.
(C) Same specimen after 24 hours: T_8 medium containing 220 mI.U. prolactin per ml. The tissue showed survival and thus a requirement for prolactin.
(D) Same specimen after 24 hours' incubation with 22 mI.U. prolactin per ml. The tissue showed surviving cells.

Pentose-monophosphate-shunt studies of the same breast-cancer tissue as in A–D.

Exposed sections show the total dehydrogenase activity of this pathway deposited as formazan after reduction of neotetrazolium chloride. Phenazine methosulphate was used as the first hydrogen acceptor (reduced to 5/12 of × 120).

(E) Formazan deposits in the tissue frozen at operation.

(F) No formazan deposited after the tissue was maintained in T_s medium alone.

(G) After maintenance in T_s medium containing 220 mI.U. per ml. for 24 hours. There was very high activity of the pentose-shunt pathway, essential for the survival of cancer tissue.

(H) Same tissue after 24 hours' incubation with 22 mI.U. prolactin per ml. Cells still showed activity.

in all patients by histological examination. Following the W.H.O. classification [8] 74% of the tumours were C II infiltrating carcinomas and 26% were C III (a, medullary; b, papillary; c, cribriform; or d, mucous).

Results

Clearcut results for one of the breast-carcinoma biopsies are shown in the figure. While paraffin-embedded blocks produced technically better sections, for the purposes of recognising deterioration compared to the control (e.g., pyknotic nuclei and cell debris) such paraffin sections were not superior to the cryostat sections. The cryostat sections also had the advantage that adjacent sections had been studied both by H.&E. and histochemistry. The H.&E. results were reviewed by W. B. independently of the histochemical assessments. In 148 of the 150 comparisons there was agreement between the older familiar ground of H.&E. results and the newer (but quicker and easier) histochemical results. We all preferred the histochemical assessment, which yielded much more clearcut results.

THOSE HUMAN BREAST CANCERS SHOWING ENHANCED TOTAL DEHYDROGENASE ACTIVITY WHEN COMPARED TO THEIR FRESH FROZEN CONTROL

—	No.	Cultured control	Cultures with added sheep prolactin	
			220 mI.U./ml.	22 mI.U./ml.
Enhanced by prolactin	16	1*	16	4
Not requiring prolactin	34	0	0	0

* The one cultured control with improved activity showed even more activity with prolactin.

The results for the fifty patients were all quite definite with regard to prolactin (see table). Cancer tissues from sixteen patients showed higher dehydrogenase activity after culture with prolactin added at 220 mI.U. per ml. than in the uncultured control and even more so when compared to that cultured with no added hormone.

Four of these cancers also showed clear enhancement even when prolactin was added at 22 mI.U. per ml. (about one-tenth the normal serum level). The other twelve were maintained somewhat better than the cultured controls.

The histological classification of this prolactin-dependent group showed that 81% were C II, not

differing significantly from 74% C II of the series of fifty.

Of the remaining 34 breast cancers 12% showed only œstrogen dependence and 2% only androgen dependence. In 54% we could detect no enhancement in culture, although the tumours did survive with some remaining pentose-shunt activity. The few failed cultures were excluded from the series.

Discussion

Prolactin improved in-vitro maintenance and growth in sixteen (32%) of the fifty malignant breast cancers studied. For 20% the only requirement discovered was for prolactin. For 12% enhancement also occurred with 17β-œstradiol as well as with prolactin, and in one cancer androgen and prolactin could each enhance tumour growth.

If these in-vitro enhancements at physiological levels of the hormones (as far as is known) also apply in vivo it can readily be understood how oophorectomy and adrenalectomy could fail to benefit patients whose tumours are promoted by prolactin. In that prolactin secretion from the anterior pituitary is held in check by hypothalamic prolactin inhibitory hormone (P.I.H.), it can also be seen how pituitary stalk section can sometimes fail to induce a remission of progressive breast cancer. Furthermore, when any of these operations are done in the patients whose breast cancers show no requirement for prolactin, œstrogen, or androgen it could be anticipated little good would follow.

Several of our original patients whose primary breast cancers showed œstrogen dependence have developed evidence of disseminated tumour and then shown a good clinical response to antiœstrogen measures. In two patients who were unknowingly given stilbœstrol frank efflorescence of their tumours occurred. Because of these results we have started a trial of management of metastatic tumour based on the in-vitro findings.

Where only prolactin dependence is detected we plan to avoid staged operations and either proceed direct to pituitary ablation or to treat with bromergocryptine, which has been successful [9] in reducing high prolactin levels in patients with galactorrhœa.

With either of these treatments it will be essential to see how low the serum-prolactin level is reduced, and 5 A.M. would seem a suitable time of day for blood-

sampling.[10] In one of our patients, hypophysectomy reduced a preoperative serum-prolactin level of 19 milliampoules per ml. to 2 milliampoules per ml., but this then rose again within 8 days to 5 milliampoules per ml. (These are units for human prolactin, kindly measured by Dr. A. R. Boyns, of the Tenovus Institute, Cardiff.) 1 milliampoule per ml. seems roughly equivalent to 20 mI.U. sheep prolactin per ml.—a level at which our studies predicted prolactin enhancement, so that the patient's tumour would (and did) persist. It is therefore essential with either treatment to know how low the serum-level can be reduced, and also to know what level will still enhance the tumour, for three others of the breast cancers studied were still enhanced at only 22 mI.U. per ml. in a serum-free medium. Such tumours provide quite a challenge to present methods of hypophysectomy.

Phenothiazines can suppress P.I.H. and increase prolactin secretion,[11] so tranquillisers such as chlorpromazine or sulpiride may be contraindicated in patients whose breast cancers show prolactin dependence.

Where œstrogen or testosterone enhancement is also present with that shown for prolactin, appropriate treatment will also be tried.

We have yet to confirm that our tissue survival results with sheep prolactin apply for human prolactin, but we hope that the above approach may make less of a gamble the choice of treatment for the individual patient with recurrent breast cancer.

We thank our clinical colleagues in the Westminster Hospital Group, especially Mr. G. Westbury and Dr. K. Newton, for their encouragement; Prof. D. H. Mackenzie and Dr. J. Earle for their cooperation over biopsy material; and the Dame Barbara Hepworth, Fane, and Lawson Trusts for their generous support and interest.

Requests for reprints should be addressed to J. R. H.

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ARE SOME WOMEN'S BREAST CANCERS ANDROGEN DEPENDENT ?

H. FLAX H. SALIH
K. A. NEWTON J. R. HOBBS

Tumour Biology Group, Westminster Hospital and Medical School, London SW1

Summary In-vitro studies of breast-cancer tissues from a hundred and thirty patients demonstrated testosterone dependence in fourteen women, of whom thirteen were postmenopausal. Six could have been conditioned by prior androgen therapy. Anti-androgen measures were successful in six of eight patients, and adrenalectomy may be the best treatment of testosterone-dependent breast cancer. In two other patients œstrogen treatment failed (as predicted in vitro) because œstradiol and testosterone are not always antagonistic.

Introduction

THE endocrine status of breast-cancer patients remains difficult to define precisely, and the growing belief that prolactin also plays an important role adds a new dimension to the problem. In particular, urinary-steroid-excretion patterns in these patients have differed widely in various reports and conflicting results have been reported after ablative surgery.[1] In the case of bilateral adrenalectomy, the conventional view has been that when it produces a remission in a postmenopausal patient it does so by removing adrenocortical œstrogen production. However, this hypothesis cannot explain the behaviour of many breast cancers, only some of which respond to ablative surgery, nor does it explain the common

clinical experience that some of these tumours respond to œstrogen administration and others to androgens.

Breast cancers can metabolise testosterone actively,[2] and Jenkins and Ash [3] demonstrated that the formation of 5α-dihydrotestosterone was greatest in the most undifferentiated carcinomas but did not occur in normal breast tissue. The Shionogi androgen-dependent rat breast tumour, described by Minesita and Yamaguchi,[4] was produced by passaging a mammary adenocarcinoma, originally from a female rat, from one male to another. It was completely suppressed by œstrogens and its androgen dependence was attributed to adaptation of the cell in parallel with modification of the hormonal milieu intérieur. Minesita and Yamaguchi suggested that some cases of recurrence in man might arise by the same mechanism. In 1942 Farrow and Adair [5] demonstrated that carcinoma of the human male breast was an androgen-dependent tumour, but as far as we know this concept has never been investigated in the case of breast cancers in women. Farrow and Woodard [6] claimed exacerbation of bony metastases in breast-cancer patients by administration of large doses of testosterone propionate (on the basis of development of hypercalcæmia), but it is now known that œstrogen as well as androgen administration can produce hypercalcæmia in these patients,[7] and Jessiman et al.[8] have demonstrated that it may arise spontaneously in the course of the disease without administration of any steroids. Thus, development of hypercalcæmia cannot be taken as evidence that actual tumour growth has been stimulated.

We have described a method [9] for determining the hormone dependence of human breast cancers in vitro and have found that a certain number of them appear to be testosterone dependent. There is some evidence in the clinical progress of these patients to support this hypothesis, which may explain the behaviour and response to treatment of these tumours.

Methods

A hundred and thirty patients have now had biopsies of their primary or metastatic tumours tested in vitro for hormone dependence. The overall results are shown in table I. Of these patients, fourteen demonstrated in-vitro dependence on testosterone alone and a further eight on testosterone and prolactin. The method has been described in detail previously.[9] A tumour is classed as " dependent " only when the total dehydrogenase activity of the pentose

TABLE I—IN-VITRO HORMONE DEPENDENCE OF 130 BREAST CANCERS

—	No. patients	%
Independent	62	48
Dependent	68	52
Prolactin	19	15
Œstradiol	14	11
Testosterone	14	11
Prolactin and œstradiol	13	10
Prolactin and testosterone	8	6

shunt pathway is greater than in both the fresh-frozen uncultured biopsy specimen and the biopsy specimen cultured in medium alone without added hormones. The problem of what concentrations to use in an in-vitro system is a difficult one, particularly since there is no guide as to the degree of selective trapping and concentration of the hormone that might occur in the vicinity of the tumour. After a series of trials with concentrations of testosterone of 10^{-5} to $10^{-9}M$, we decided to use 10^{-5} and $10^{-6}M$, which we found bracketed all our testosterone-dependent cases. Although the plasma-testosterone levels are probably of the order of 10^{-7}–$10^{-8}M$, we have found 10^{-5} and $10^{-6}M$ to be the most effective in vitro and to relate to the clinical situation. In our system the tissue is not immersed in the medium but relies on absorption up a lens paper, covering the grid on which it rests. The concentrations we use, in fact (equivalent to 2·884 and 0·2884 μg. per ml.), are less than those used by Burstein et al.[10] and Mioduszewska et al.[11] (both 5 μg. per ml.), Heuson and Legros [12] (11 and 33 μg. per ml.), and Tchao et al.[13] (50 μg. per ml.).

Table II shows the distribution of the testosterone-dependent tumours in relation to the menstrual status of the patients. We propose to discuss only those patients whose tumours showed pure testosterone dependence in our system. All patients were treated quite freely and independently by the clinicians concerned, with no attempt to conform to any trial, although the results and recommendations of the hormone-dependence studies were available for appli-

TABLE II—IN-VITRO TESTOSTERONE DEPENDENCE IN RELATION TO MENOPAUSAL STATUS

Status	No. patients	No. dependent on testosterone only	No. dependent on testosterone and prolactin
Premenopausal	21	1	0
< 5 yr. postmenopausal	29	1	2
> 5 yr. postmenopausal	78	12	6

cation. All tumours were histologically confirmed and none of these patients had any overt clinical features of masculinisation. The age and menstrual status in each case refer to the time the biopsy was taken, and the final comment on the clinical progress is valid at the time of writing (early April, 1973).

Case-reports

Case 1.—Age 62. Menopause at 48. Excision biopsy of a left-breast lump in October, 1972, showed testosterone dependence. No further treatment. No signs of disease at present.

Case 2.—Age 60. Over 5 years postmenopausal. Simple mastectomy for right-breast carcinoma February, 1972, followed by radiotherapy. No sign of recurrence.

Case 3.—Age 62. Left radical mastectomy May, 1965. Recurrent nodule removed from scar October, 1965. Well until presenting with terminal stage-IV carcinoma in January, 1973, with widespread visceral and soft-tissue metastases. Biopsy of a soft-tissue nodule taken a few days before death showed testosterone dependence.

Case 4.—Age 64. Simple mastectomy for right-breast carcinoma in 1955. A right axillary recurrence was excised in 1963 and oophorectomy performed, with radiotherapy to the axilla. In 1970 several lumps appeared in the right arm and pectoral region. Stilbœstrol stimulated growth of the lumps, so nandrolone phenylpropionate was given with some improvement. In March, 1972, a large axillary excision with skinflap cover was performed for advancing disease. Nandrolone was stopped, since testing of the tumour showed testosterone dependence. In August, 1972, several small nodules appeared on the right arm, but these have remained constant in size for many months and she is on no treatment.

Case 5.—Age 58. Over 5 years postmenopausal. Right simple mastectomy in March, 1973, followed by radiotherapy. Testing showed testosterone dependence. Remains well on no treatment at present.

Case 6.—Age 62. Postmenopausal. Presented in May, 1972, with a stage-III left-breast carcinoma. Biopsy showed testosterone dependence with very poor survival in œstradiol. Ethinylœstradiol (1 mg. daily) started as anti-androgenic treatment with excellent objective remission and complete disappearance of the lump and axillary nodes. Remains well.

Case 7.—Age 76. Menopause at 40. Right simple mastectomy and postoperative radiotherapy in September, 1970. Nodules appeared in the scar in October, 1972, and one was biopsied. This showed testosterone dependence and complete death of the tumour in œstradiol. Ethinylœstradiol (1 mg. daily) was started as anti-androgenic

treatment and an excellent objective remission was obtained. She remains well and free of disease.

Case 8.—Age 62. Menopause at 50. Simple mastectomy and postoperative radiotherapy in August, 1969, for a left-breast carcinoma. In October, 1971, a mass appeared in the left supraclavicular fossa but regressed completely with the anti-œstrogen ICI 46474. In July, 1972, the mass recurred together with a left axillary mass. The latter was biopsied and showed testosterone dependence. Adrenalectomy was advised and carried out as anti-androgenic treatment, bringing about a good remission with pronounced shrinkage of the supraclavicular mass (though as yet not complete disappearance), and disappearance of the axillary mass. She remains well with no other signs of disease.

Case 9.—Age 68. Stage-III carcinoma of the left breast treated by radiotherapy in 1964. Left pleural effusion and local disease reappeared in September, 1965. This progressed despite stilbœstrol therapy. In October, 1968, with extensive local disease present, a radiation castration was performed and norethisterone acetate administered. This produced 6 months of remission, but extensive local disease recurred in October, 1969, and a trans-sphenoidal hypophysectomy was carried out with an excellent result. After 2½ years of remission the tumour recurred locally in July, 1972, and biopsy showed it to be testosterone dependent with poor survival in œstradiol. Radiotherapy in July, 1972, produced some shrinkage in the lump but not disappearance. She was put on to ethinylœstradiol as anti-androgenic treatment, with some further shrinkage, and at present the lump remains static with no signs of activity on œstradiol treatment. She is otherwise well and no other metastases have appeared.

Case 10.—Age 58. Over 5 years post-menopausal. Presented with an ulcerated stage-III carcinoma of the left breast in February, 1966. Treated with radiotherapy and stilbœstrol without remission, and 5-fluorouracil and cyclophosphamide also failed to control the disease. In December, 1969, drostanolone propionate was started and produced some remission. In March, 1970, nodules had recurred on the chest wall and axillary nodes reappeared. She was then given cytotoxic chemotherapy until September, 1971, and after that she was given ICI 46474 until August, 1972, when the disease again began advancing locally. Biopsy showed testosterone dependence and she was treated with norethisterone acetate and prednisone, which have resulted in a good objective remission of the local disease which has so far been maintained.

Case 11.—Age 62. Menopause at 50. Presented with a stage-III carcinoma of the left breast in February, 1972, and treated with radiotherapy. The local disease failed to resolve completely and in October, 1972, biopsy showed testosterone dependence but—significantly—also moderate survival in œstradiol. Although adrenalectomy was suggested, it was decided to try prednisone first. This

brought no remission and ethinylœstradiol was started in November, 1972, which brought some subjective improvement but no objective remission. We had anticipated this in view of the tumour's in-vitro survival in œstradiol. The patient went home to discuss the question of adrenalectomy with her family, but having decided in favour, she died suddenly at home on Dec. 31, 1972.

Case 12.—Age 46. Left simple mastectomy for carcinoma in December, 1970, followed by radiotherapy 5 months later because of appearance of supraclavicular nodes. The nodes did not regress completely and bilateral oophorectomy was performed in September, 1971. The nodes regressed but returned in March, 1972. Skeletal metastases developed and trials of norethisterone, ICI 46474, and dexamethasone all failed to produce a remission. Testing of an axillary nodule showed testosterone dependence and moderate survival in œstradiol. Ethinylœstradiol was administered for 2 weeks with no effect and then changed to cytotoxic chemotherapy. This failed to control the disease and she died in November, 1972.

Case 13.—Age 62. Menopause at 52. Simple mastectomy with postoperative radiotherapy in July, 1970, for a right-breast carcinoma. 2 years later a chest-wall recurrence developed. Biopsy showed testosterone dependence. The patient was treated with norethisterone. This has failed to produce a remission and to date the local disease is advancing.

Case 14.—Age 40. Premenopausal. Modified radical mastectomy abroad in October, 1970, for left-breast carcinoma, followed by radiotherapy. Pulmonary and supraclavicular metastases appeared in April, 1972. Nandrolone phenylpropionate and 5-fluorouracil were tried with no effect, and biopsy in June, 1972, showed testosterone dependence. Adrenalectomy was suggested but the patient was deemed too ill for this. Norethisterone, prednisone, and chemotherapy were all tried, but she continued to deteriorate and died in October, 1972.

Discussion

How Does Androgen Dependence Develop ?

Tumours from all the above patients showed testosterone dependence on in-vitro testing. Thirteen of the fourteen women were postmenopausal at the time of testing, while the fourteenth had been treated with an androgen for several months before testing. In addition to patient 14, patients 8, 9, 10, 12, and 4—i.e., six of the fourteen patients—had all either been treated with androgens or some form of anti-œstrogenic treatment (such as an artificial menopause or an anti-œstrogen drug), before testing their hormone dependence. We feel that this suggests that the tumours have adapted themselves to live in an altered hormonal

environment, much like the Shionogi rat-tumour model, and that at the time of testing they were in fact being promoted in vivo by androgens. The fact, however, that patients 1, 2, 3, 5, 6, and 7 had tumours which were testosterone dependent without any previous hormonal treatment may indicate that a certain proportion of breast cancers in postmenopausal women may be androgen dependent ab initio. The menopause itself, like the administration of exogenous steroids, may bring about precisely the sort of hormonal alteration we are discussing.

Response to Anti-androgenic Measures

The fact that various anti-androgenic measures proved effective in causing a remission in some of our patients tends to support the thesis that in-vitro androgen dependence may reflect the in-vivo situation. In patients 6 and 7, where testing had shown testosterone dependence and also inhibition by œstradiol, œstrogen therapy alone produced excellent objective remissions, as did adrenalectomy in patient 8. Patient 9's disease is under control with œstrogen therapy, despite recurrence after hypophysectomy, though admittedly she had radiotherapy to the lesion as well. In patient 10 it is impossible to be certain whether norethisterone or prednisone produced the remission. The mode of action of norethisterone in breast cancer is complex and uncertain; some of the metabolites of the 19-nortestosterone group of which it is a member have œstrogenic metabolites and many of the progestogens are potent anti-androgens.[14] In addition, it may have feedback effects via the adrenal and pituitary glands. Prednisone, however, might well have been the effective agent by suppressing corticotrophin production and hence adrenocortical androgen secretion.

There are good clinical grounds in several of these patients for suspecting that the tumour had adapted itself after having been originally œstrogen dependent. Patient 4's tumour was actually stimulated by stilbœstrol and controlled by nandrolone 2 years before being found to be testosterone dependent in vitro. Patient 8's tumour had shown an excellent regression on anti-œstrogen therapy 9 months before recurring and being shown to be testosterone dependent. Patients 9 and 12 also had good remissions from artificial menopauses 6 months before recurrences were found to be testosterone dependent.

It is interesting and we believe relevant that in our culture system the effects of œstradiol and testosterone were not necessarily diametrically opposed. Although a testosterone-dependent tumour was usually markedly inhibited by œstradiol, this was not always the case, and in patients 11 and 12 we anticipated that œstrogen administration would be unlikely to produce a remission, since the tumour had survived moderately well in œstradiol in vitro.

Most androgen production in the female is derived either directly or via precursors from the adrenal cortex, although in the case of breast-cancer patients there may be para-endocrine activity and androgen synthesis by the tumour itself. Although only one patient in this small group has undergone adrenalectomy with the deliberate intention of reducing androgen production (followed by a successful remission), we wonder whether in fact this may not be the important underlying mechanism of at least some successful adrenalectomies.

We feel, therefore, that it is likely that certain female breast cancers may be promoted by male hormones, and that it is unnecessary to postulate an absolute rise in androgen production, or persistently high androgen levels, to produce this state of affairs. An alteration in the hormonal environment, such as the menopause, may permit an androgen-dependent tumour to arise, or the effect of anti-œstrogenic or androgenic treatment, or the menopause during the natural course of the disease, may bring about this adaptation. This would help to explain the not uncommon clinical situation of a recurrence after a successful artificial menopause or androgenic treatment which is followed by a further remission on anti-androgenic treatment, as happened in several of the cases we describe. The action of androgens, like other hormones, is not simply a peripheral one, and intricate endocrine feedback pathways may be involved. Furthermore, testosterone may well be converted to some other final active metabolite for use by the cell. However, the results of our in-vitro testing strongly support an androgen-dependence hypothesis by showing that only testosterone and neither œstradiol nor prolactin have enhanced the activity of these tumours above their control slices, and this has agreed fairly closely with the clinical situation.

We thank our colleagues in the surgical division (especially Mr K. Robinson and Mr G. Westbury) and in the oncology and histopathology divisions (especially Prof. D. Mackenzie and Dr W. Brander) for their cooperation in studying their patients. This work was supported by the Dame Barbara Hepworth, Lawson, and Fane Trusts.

Requests for reprints should be addressed to J. R. H., Department of Chemical Pathology, Page Street Wing, Westminster Medical School, 17 Page Street, London SW1P 2AR.

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technique applied to the angiograms accentuated the appearance of the vasospasm and of the vasodilatation as a result of treatment (Rickham, 1964).

The experimental model permits long-term survival and repeated production of subarachnoid haemorrhage. The tolerance to drug modification of vascular tone suggests that this may have clinical application in the control of cerebral vasospasm, and in the prevention of ischaemic sequelae following subarachnoid haemorrhage.

RICKHAM, P. P. (1964), *Br. med. J.*, **2**, 173.

51. The Effect of Ovariectomy on Experimental Mammary Carcinoma in the Rat with Portacaval Shunt: J. F. O'GRADY, MARGOT GRUENSTEIN, and F. A. REICHLE (P. Fitzgerald), Department of Surgery, University College, Dublin, and Temple University Hospital, Philadelphia, United States.

End-to-side portacaval shunt (P.C.S.) significantly reduces the incidence of experimental mammary carcinoma in the rat (Reichle et al., 1969). The mechanism of this inhibition is unknown. The major circulatory, metabolic, and hormonal changes which occur after portasystemic by-pass may be related to this tumour inhibition (Reichle et al., 1972).

Ten mg. of 7,12-dimethylbenz-(α)-anthracene (DMBA) were given in a single intragastric instillation to 105 Holtzman rats (150 g.). After 2 weeks P.C.S. (77 rats) and sham operations (28 rats) were performed. When palpable mammary tumours had developed bilateral ovariectomy was performed. A total of 19 rats (10 with shunts, 9 having had sham operations) with breast tumours underwent bilateral ovariectomy and survived long enough for adequate follow-up. Average increase in tumour size at death or 1 year after DMBA was $2{\cdot}48 \pm 0{\cdot}57$ cm. in sham-operated rats and $0{\cdot}82 \pm 0{\cdot}39$ cm. in rats with shunts ($P < 0{\cdot}05$). The average time of death after ovariectomy was $85{\cdot}7 \pm 11{\cdot}8$ days in sham-operated rats and $92{\cdot}7 \pm 18{\cdot}2$ days in rats with shunts (not significant). Approximately equal weight-gain following the sham or shunt operation was observed in both groups.

Following bilateral ovariectomy the suppression of DMBA-produced carcinoma growth is significantly greater in animals with a portasystemic by-pass. This may be due either to an altered hormonal response of the tumours in the shunted animals or to an effect of the P.C.S. on the hormonal changes associated with ovariectomy.

REICHLE, F. A., GRUENSTEIN, M., MERANZE, D. R., ROSEMOND, G. P., and SHIMKIN, M. B. (1969), *J. surg. Res.*, **9**, 559.
— — — — REICHLE, R. M., MERANZE, D. R., ROSEMOND, G. P., and SHIMKIN, M. B. (1972), *Archs Surg., Chicago*, **104**, 206.

52. *In vivo* Binding of Tritiated Oestradiol in Benign and Malignant Lesions of the Human Breast: N. O'HIGGINS, N. DESHPANDE, and J. I. BURN, Breast Unit, Department of Surgery, Royal Postgraduate Medical School, London, and the Imperial Cancer Research Fund.

Previous workers have examined the uptake of tritium-labelled hormones by mammary cancers (Braunsberg et al., 1967). The capacity to bind tritiated oestradiol-17 *in vivo* was determined in 8 benign and 13 malignant mammary cancers. Uptake was measured in whole-tissue slices and nuclear preparations.

A loading dose of 9 μc. of tritiated oestradiol was given intravenously. This was followed by a continuous slow infusion of 64μc. of tritiated oestradiol, which was started at least 3 hours before operation and continued until the lesion had been removed. Serial venous blood samples were examined to confirm that a steady state of oestradiol had been achieved. Radioactivity levels in the excised specimens were measured in a well counter.

The uptake in the benign lesions ranged between 808 and 2530 disintegrations per minute (d.p.m.) per mg. of wet tissue (mean 1229 d.p.m.). Levels in the malignant cancers ranged between 1456 and 12,563 d.p.m. (mean 2196 d.p.m.). There was a complete separation between postmenopausal patients with benign and malignant disease.

BRAUNSBERG, H., IRVINE, W. T., and JAMES, V. H. T. (1967), *Br. J. Cancer*, **21**, 714.

53. Macrophage Activity and Hormonal Responsiveness in Mammary Cancer: W. D. GEORGE, WENDY PARTRIDGE, and J. I. BURN, Breast Unit, Department of Surgery, Royal Postgraduate Medical School, London.

Changes in reticulo-endothelial macrophage activity occur in patients with cancer and may reflect the host's resistance to the disease (Old et al., 1961). Hormone therapy in advanced breast cancer should theoretically stimulate macrophage activity. Hepatic macrophage activity was studied serially in 14 patients with advanced mammary cancers before and after endocrine therapy. Activity was measured by the rate of clearance of radio-iodine labelled micro-aggregates of albumin given intravenously (Ilio and Wagner, 1963).

Seven patients received oestrogen therapy. Four responded successfully to treatment and all showed an increase in macrophage activity as therapy continued. Of 3 patients who failed to respond to oestrogens, 2 showed a decrease in macrophage activity whilst the third showed no change.

Seven patients underwent major endocrine ablations. Two responded successfully and both showed increase in macrophage activity. Of the 5 non-responders, macrophage activity decreased in 4 and remained unchanged in the other patient.

These results suggest that in patients with advanced mammary cancer successful endocrine therapy is related to the stimulation of reticulo-endothelial macrophage activity.

ILIO, M., and WAGNER, H. N. (1963), *J. clin. Invest.*, **42**, 417.
OLD, L. J., BENACERRAF, B., CLARKE, D. A., CARSWELL, E. A., and STOCKERT, E. (1961), *Cancer Res.*, **21**, 1281.

54. A New Method of determining the Hormone Dependence of Human Breast Cancer: H. FLAX (H. Ellis), Tumour Biology Group, Westminster Hospital, London.

A new technique has been developed to determine the hormone dependence of individual breast cancers (Salih et al., 1972) involving the 24-hour maintenance at 37° C. of tumour slices in Trowell's T8 medium with two near-physiological concentrations each of 17-β-oestradiol, testosterone, and prolactin. The technique has been successful for soft-tissue metastases as well as for the primary tumour. Results are determined by a histochemical reaction demonstrating the total dehydrogenase activity of the pentose shunt (Chayen et al., 1970) as well as by conventional haematoxylin and eosin staining (which showed 96 per cent correlation on independent histopathological assessment). The tumour is classed as dependent if it appears more active with a particular hormone or hormones than both the initial untreated biopsy and the medium control.

In the 100 patients studied so far, dependences have been as follows: prolactin only, 14 per cent; prolactin and oestradiol, 10 per cent; testosterone only, 12 per cent; prolactin and testosterone, 8 per cent; oestradiol only, 8 per cent. Where appropriate, endocrine-ablative surgery

was advised for the dependent cases. In the 48 per cent of cases that were independent the results, by indicating whether any particular hormone was strikingly inhibitory to the tumour, were helpful for selecting hormones for administration. Correlation with the clinical progress of these patients (only at 9 months so far) has been encouraging.

CHAYEN, J., ALTMANN, F. P., BITENSKY, L., and DALY, J. R. (1970), *Lancet*, **I**, 868.
SALIH, H., FLAX, H., and HOBBS, J. R. (1972), *Ibid.*, **I**, 1198.

55. The Effect of Vagotomy on the Exocrine Pancreatic Secretory Response to Pentagastrin and to a 400-g. Meat Meal in Dogs: M. DAVIS, SAROJ GUPTA, and J. B. ELDER, University Department of Surgery, Royal Infirmary, Manchester.

It has been stated that the pancreas becomes more sensitive to humoral stimulation after truncal vagotomy but the evidence is controversial (Konturek et al., 1971; Moreland and Johnson, 1971). The effect of truncal vagotomy on the exocrine secretory response to a 400-g. meat meal and to intravenous pentagastrin infusion at a dose maximal for acid secretion before and after vagotomy in 2 dogs with chronic pancreatic and gastric fistula (G.F.) has been studied.

With the G.F. closed the mean bicarbonate output after pentagastrin (8 μg. per kg. per hour) increased from $22{\cdot}98 \pm 1{\cdot}1$ mEq. to $33{\cdot}1 \pm 4{\cdot}6$ mEq. ($P < 0{\cdot}05$) after vagotomy; protein output to pentagastrin increased from 1898 ± 422 mg. to 3520 ± 342 mg. ($P < 0{\cdot}01$) after vagotomy. Bicarbonate output to the meat meal decreased significantly ($P < 0{\cdot}001$) after vagotomy but protein output was not significantly altered ($P < 0{\cdot}4$).

These results may be explained on the basis of an increased sensitivity of the pancreas to hormonal stimulation after vagotomy.

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MORELAND, H. J., and JOHNSON, L. R. (1971), *Gastroenterology*, **60**, 425.

56. Does the Vagal Nerve-supply to the Pancreas matter in Man? R. B. SMITH, J. P. EDWARDS, and D. JOHNSTON, University Department of Surgery, The General Infirmary, Leeds.

Reports on the function of the pancreas after severance of its parasympathetic nerve-supply in man (as in truncal vagotomy (T.V.)) are conflicting. These nerves are preserved in highly selective vagotomy (H.S.V.). The pancreatic response to insulin (0·2 U. per kg.) has been studied in 9 preoperative patients with duodenal ulcer (D.U.), and in patients more than 1 year after T.V. (6 patients) or H.S.V. (12 patients). The three groups of patients were matched for age and weight. Gastric acid was aspirated continuously and the duodenal *p*H remained greater than 7.

Outputs of amylase were found to increase significantly after insulin in D.U. and H.S.V. patients (from a mean of 9·1 to 20·5 kilo units in D.U. patients and from 6·0 to 16·5 kilo units in H.S.V. patients), whereas no significant increase was found in patients after T.V. (3·8 to 4·5 kilo units). Similarly, outputs of trypsin increased significantly in D.U. patients (from 16·0 to 31·1 I.U.) and in patients after H.S.V. (from 14·1 to 27·6 I.U.), whereas no such increase was observed after truncal vagotomy.

These results are in agreement with previous reports of impaired (Pfeffer et al., 1952) or absent (Dreiling et al., 1952) pancreatic response to insulin after T.V. in man. After H.S.V. significant increases in enzyme output still occurred in response to vagal stimulation.

DREILING, D. A., DRUCKERMAN, L. J., and HOLLANDER, F. (1952), *Gastroenterology*, **20**, 578.
PFEFFER, R. B., STEPHENSON, H. E., and HINTON, J. W. (1952), *Ann. Surg.*, **136**, 585.

57. Disappearance of Acid from the Duodenum: N. J. DORRICOTT and W. SILEN (G. Slaney), Department of Surgery, Queen Elizabeth Hospital, Birmingham, and Harvard Medical School, Beth Israel Hospital, Boston, United States.

Experiments were carried out to investigate the poorly understood mechanism by which the duodenal mucosa contributes to the disappearance of hydrogen ions in the duodenum in the absence of pancreatic juice and bile.

In 7 standing awake dogs distal duodenal pouches were perfused by a constant-circulation technique with isotonic solutions ranging from 60 to 120 m*M* HCl (Dorricott and Silen, 1972). Fluxes of H^+, Na^+, K^+, and Cl^- were related to the initial concentration of H^+ and Na^+ and load of H^+ exposed to the duodenal mucosa per unit of time.

In all the experiments there was a loss of H^+ from the perfusate and a gain of Na^+, K^+, and Cl^-. A higher initial concentration of H^+ was associated with a greater rate of disappearance of H^+, e.g., 667 ± 70 μEq. per 30 minutes from 120 m*M* HCl and 312 ± 30 μEq. per 30 minutes from 60 m*M* HCl ($P < 0{\cdot}001$). Alteration of load of H^+ and initial concentration of Na^+ did not produce significant change in the rate of disappearance of H^+. An excess of cations over measurable anions, closely related to H^+ loss, entered the pouch, which can best be explained by a process of bicarbonate neutralization.

These experiments show a relationship between disposal of H^+ by the duodenal mucosa and intraluminal H^+ concentration, and not load of H^+ as found by Meyer et al. (1970).

DORRICOTT, N. J., and SILEN, W. (1972), *Surg. Forum*, **23**, 391.
MEYER, J. H., WONG, L. W., and GROSSMAN, M. I. (1970), *Am. J. Physiol.*, **219**, 971.

58. Human Duodenal Electrical Activity: W. WATERFALL, B. BROWN, and H. L. DUTHIE, University Department of Surgery, Royal Infirmary, Sheffield.

Stainless-steel electrodes (0·25 mm. in diameter) were mounted on a gastric tube and attached to the mucosa of the second part of the duodenum by suction in 16 subjects. Unipolar records of the electrical activity of the duodenum were obtained with the subjects in the resting state and also during intravenous infusion of pentagastrin (1·0 and 6·0 μg. per kg. per hour in 8 tests) and of secretin (0·25 and 2·0 U. per kg. per hour in 6 tests).

In the fasting subject a regular waveform of 11–12 cycles per minute was present all the time (pacesetter potential; basal electrical activity) with an amplitude of 0·2–1·6 mV. When motor waves were present (16 per cent of the time) they were accompanied by bursts of action potentials superimposed on the pacesetter potentials which then had their maximal amplitude. The frequency of the pacesetter potential was not affected by the infusion of pentagastrin or secretin. Action potentials and motor activity were increased significantly with pentagastrin and decreased with secretin. The contrasting actions of these hormones on the motor action of the duodenum have been confirmed and their effects on the electrical activity displayed.

59. *Ex vivo* Perfusion of Canine Jejunal Loop: Electrical and Mechanical Activities: C. MENDEL, D. JAECK, J. C. SCHANG, J. KACHELHOFFER, M. R. ELOY, and J. F. GRENIER (H. L. Duthie), Service d'Investigations Chirurgicales et Unité de Recherches de Biophysiopathologie Digestive de l'I.N.S.E.R.M. Pavillon Chirurgical B, Hôpital Civil de Strasbourg.

Letters to the Editor

HORMONE THERAPY IN MALIGNANT MELANOMA

SIR,—The evidence that some cases of malignant melanoma may be hormonally influenced has been reviewed by Bodenham,[1] who cites excellent regression in a small number of patients on hormonal therapy. 2 were treated by hypophysectomy, 1 by androgen administration, and 1 by œstrogen administration. He emphasised the need for a more widely applicable system to select these patients, in that the assessment in his cases depended on the use of an intralesional Geiger probe [2] which remained in situ for several days and involved a number of practical problems.

We have described [3] the use of a short-term maintenance culture system to study hormonal influences in human breast cancers and have found that the histochemical assessment of survival by measurement of the total dehydrogenase activity of the pentose-shunt pathway is a sensitive marker of tumour-cell activity. In a short project to test the same technique for malignant melanoma, biopsies from 11 patients with recurrent disease were treated in the way we have described for breast-cancer biopsies. After removal of the specimen, an uncultured fresh-frozen initial control slice was stored in liquid nitrogen, while a medium control (Trowell's T8 Medium) and cultures in 3 concentrations each (10^{-5}, 10^{-6}, and $10^{-7}M$) of testosterone and 17β-œstradiol were incubated for 24 hours in an oxygen and 5% carbon-dioxide mixture. The results are shown in the following table (comparisons between œstradiol and testosterone are in each case between identical concentrations—namely, those which demonstrated the difference in survival optimally):

Case	Sex	*Initial control*	*Medium control*	*Œstradiol*	*Testosterone*	
1	F	++++	++	+	+++	Relatively more
2	F	++++	++	+	++	active in testo-
3	M	++++	+	+	+++	sterone than in œstradiol
4	F	++	+++	+++	+++	No significant
5	F	+++	+	++	++	difference in activity
6	F	++++	+	+++	+++	between the two
7	F	+++	+	++	++	hormones
8	F	++++	+++	++++	+	Relatively more
9	F	++	+	+++	++	active in œstradiol
10	M	+++	++++	+++	+	than in testo-
11	F	+++	+	++	+	sterone

In only 1 (case 9) of the 11 cases was the enzyme activity in one of the hormones actually *higher* than that of *both* controls.

Four of these tumours were also cultured in 3 concentrations of corticotrophin (0·75, 0·075, and 0·0075 mI.U. per ml.), and they all survived well in these, although in no case better than both controls.

The technique is simple, and melanoma is easier to work with than breast cancers, in that it is more cellular and homogeneous. It may well be that modification of the concentrations or the time scale of the experiment may accentuate the differences in survival. The method also offers an opportunity of studying other hormones in relation to melanoma—for example, corticotrophin and β-M.S.H. Although we would agree that only a small proportion of patients may benefit from hormonal therapy in this disease, this in-vitro approach may merit further exploration.

Tumour Biology Group,
Westminster Hospital,
London SW1.

H. FLAX
H. SALIH
G. WESTBURY.

1. Bodenham, D. C. *in* Endocrine Therapy in Malignant Disease (edited by B. A. Stoll); p. 377. London, 1972.
2. Hale, B. T. *Lancet*, 1961, ii, 345.
3. Salih, H., Flax, H., Hobbs, J. R. *ibid.* 1972, i, 1198.

HORMONE THERAPY FOR MALIGNANT MYELOMA

SIR,—Because they reduce the negative calcium balance [1] and stimulate erythropoiesis,[2] androgens are useful in the management of the hypercalcæmia and anæmia of multiple myeloma. We have used androgens to treat patients with multiple myeloma.

34 patients with multiple myeloma were initially and adequately treated with alkylating agents (cyclophosphamide or melphalan) and prednisone. 18 of the 34 patients did not receive androgenic hormones, and 16 had adjuvant androgen therapy for at least one month in the form of either fluoxymesterone (10 mg. twice a day orally) or testosterone œnanthate (400 mg. intramuscularly twice a week for two to four weeks, then once fortnightly or monthly).

TABLE I—EFFECTS OF ANDROGENS IN MULTIPLE MYELOMA DURING CHEMOTHERAPY

—	Increase of Hb ≥2 g./100 ml.	Decrease of M-protein ≥50%	Correction of hypercalcæmia	Correction of azotæmia	Tumour response
Androgens	9/16 (*56*)	10/12	6/6	4/8	8/16 (*50*)
Non-androgens	4/18 (*22*)	5/12	3/5	2/6	2/18 (*11*)
P value	< 0·05	< 0·05	< 0·05	N.S.	< 0·01

N.S. = Not significant.
Percentages are shown in parentheses.

TABLE II—DURATION AND DOSAGE OF CYCLOPHOSPHAMIDE IN PATIENTS WITH OR WITHOUT ANDROGEN

Group	No. of patients *	Initial W.B.C. † ($\times 10^3$)	Cyclophosphamide †		
			Total dose (g.)	Duration (days)	mg./day
Androgen	12	6 (6·8)	10·7 (31·7)	450 (453)	75 (70)
Non-androgen	11	7 (6·5)	6·0 (10·4)	44 (159)	60 (65)

* Remaining patients received melphalan.
† Median (mean).
W.B.C. = White-blood-cell count.

The pretreatment mean hæmoglobin level (10 g. per 100 ml.) was identical in both groups. There was an increase in hæmoglobin of 2 g. per 100 ml. or more in 56% of the patients in the androgen group and in 22% of the non-androgen group ($P < 0·05$) (table I). More patients in the androgen group had their M-protein reduced and/or hypercalcæmia corrected during therapy than in the non-androgen group (both $P < 0·05$). The tumour-response rate, as judged by subjective and objective measurements other than hæmoglobin,[3] was 50% in the androgen group and 11% in the non-androgen group ($P < 0·01$). In the non-androgen group, of the 3 patients who did not respond, 2 achieved a good tumour response when androgen was subsequently added.

The total dose and the duration of cyclophosphamide therapy were greater in the androgen-treated group (table II). The higher dose and the longer duration of chemotherapy probably contributed to the better response-rate

1. Lafferty, F. W., Spencer, G. E., Jr., Pearson, O. H. *Am. J. Med.* 1964, **36**, 514.
2. Kennedy, B. J., Gilbertsen, A. S. *New Engl. J. Med.* 1957, **256**, 719.
3. Committee of Chronic Leukemia/Myeloma Task Force. *Cancer Chemother. Rep.* 1968, **1**, 13.

in the androgen group. Adjuvant androgen therapy seems to enhance the patient's tolerance of chemotherapy and thus improves the therapeutic potential of cytotoxic agents. The report [4] that androgens stimulate the proliferation of granulocyte precursor in mouse bone-marrow accords with our clinical observations.

Department of Medicine,
University of Minnesota
School of Medicine,
Minneapolis,
Minnesota 55455,
U.S.A.

DAVID T. KIANG
A. THEOLOGIDES
I. FORTUNY
B. J. KENNEDY.

B.C.G. PLUS PROTEASE I IN MALIGNANT MELANOMA

SIR,—The findings of Professor Thornes and his colleagues (June 16, p. 1386) corroborate work I have been doing with transfer factor combined with B.C.G. vaccine. In my technique, transfer factor replaces brinase with its toxic effects.

In 2 severely immunosuppressed patients with seronegative late-stage systemic lupus, B.C.G. vaccine was given in an attempt to improve cellular immunity. In both cases all 4 screening skin tests were negative. A series of six to eight B.C.G. injections was poorly tolerated and produced virtual abscess formation. The purified-protein-derivative skin tests remained negative. These patients then received transfer factor from an ABO compatible donor. Neither of the donors had a positive tuberculin skin test, although both had skin tests positive to either one or all of *Candida albicans*, trichophyton, and mumps. Four days after receiving transfer factor the recipients were retested. Both had strongly positive tuberculin reactions with vesiculation. One was strongly positive seven weeks after transfer factor. The other remained so up to five weeks after transfer factor. Follow-up was lost.

The third patient with stage-III malignant melanoma was totally anergic. This patient had received a series of B.C.G. vaccine, both intradermally and by the grid-scratch method of Mathé. Intratumour injections were given weekly. The patient evidenced no inflammatory reaction at any B.C.G. site. This was carried out over a period of three months. Repeated skin tests showed total anergy to the four antigens. The patient then received transfer factor from his mother, who carried a negative tuberculin skin test, a 3 cm. flare, and induration to mumps, although negative to monilia and trichophyton.

Four days later this patient developed positive P.P.D. skin tests with 12 mm. induration. Seven weeks later it was still positive. The mumps skin test also became positive. B.C.G. vaccinations now produced, for the first time in three months, active inflammatory reactions. The palm-sized group of chronic B.C.G. abscesses in the S.L.E. patient was 75% healed in two weeks.

From my observations, B.C.G. vaccinations at weekly intervals for at least one month, followed by transfer factor, will result in positive P.P.D. cellular immune response. The toxicity of brinase is avoided.

Rockwood Clinic,
312 West 8th Avenue,
Spokane, Washington 99204,
U.S.A.

S. K. MCILVANIE.

POPULATION AND THE N.H.S. BILL

SIR,—How interesting to see in your issue of July 7 an editorial (p. 29) on population problems and an account (p. 37) of the fate of the Lords' amendment to the N.H.S. Reorganisation Bill.

The restraints on population growth in the world are the ultimately limited amounts of food and natural resources which can be made available. Since Britain already consumes much more of these than Bangladesh, and Britain's population continues to grow, it ill becomes us to urge population control there, whilst taking no action at home.

UNROD recommends a Ministry of Population Affairs: a similar recommendation has been made for a Minister in Britain, but no action is forthcoming from the Government. One can only feel dismay that the Commons have finally succeeded in overcoming their more enlightened colleagues in the Lords and rejected a free family-planning service.

Royal Infirmary,
Edinburgh EH3 9YW.

D. M. PARKIN.

FLUOROCARBON PROPELLANTS IN AEROSOLS

SIR,—Dr Silverglade (June 9, p. 1325) mentions some 18 articles on the subject of the cardiotoxicity of fluorocarbon compounds used as aerosol propellants. He concluded that the weight of evidence attests to both the safety and efficacy of the pressurised bronchodilator aerosols when properly used.

Some of the articles, however, emphasise that the aerosol propellant compounds such as dibromotetrafluoroethane or trichlorofluoromethane are closely related in structure to halogenated hydrocarbons used in anæsthesia, such as halothane or chloroform. A degree of concern [1] has been expressed about the inhalation of trace levels of halogenated anæsthetics by theatre workers, as this chronic exposure might be implicated in explaining the various abnormalities revealed in statistical surveys. If the halothane which is absorbed by theatre workers to blood-levels [2,3] of around 100 μg. per 100 ml. can be suspected of producing spontaneous abortions [4] or liver damage, surely aerosol propellants with a very similar chemical structure and absorbed to equal or higher blood-levels [5,6] must also be suspect ?

Department of Clinical Physics
and Bio-engineering,
Western Regional Hospital
Board,
11 West Graham Street,
Glasgow G4 9LF.

IAN B. MONK.

DISPOSABLE SYRINGES FOR DIABETICS

SIR,—Regarding this correspondence (May 19, p. 1130; July 7, p. 41; and June 16, p. 1381) I should like to point out that, although disposable syringes are not generally available to diabetics on the National Health Service, in exceptional cases, where the general practitioner considers that disposable syringes and needles are needed on medical grounds, they can be obtained through hospitals, with the agreement of the consultant concerned. This applies chiefly to diabetic children, who often find the disposable syringes easier to manipulate.

The British Diabetic Association has made several pleas to the Department of Health and Social Security for the provision of disposable syringes for diabetics generally on the National Health Service, but the decision of the Department remains that the expenditure involved cannot be justified.

In answer to Professor Dundee (June 16, p. 1381), I should like to point out that a plastic spirit-proof container for the glass insulin syringe is now available on the National Health Service, which obviates the need for frequent

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5. Dollery, C. T., Draffan, G. H., Davies, D. S., Williams, F. M., Conally, M. E. *ibid.* 1970, ii, 1164.
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