UNIVERSITY OF SOUTHAMPTON

THYROID HORMONES AND THE DEVELOPMENT OF ADRENAL FUNCTION IN THE RAT

by

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ABSTRACT

FACULTY OF SCIENCE

Doctor of Philosophy

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by Frances Mary Fullerton

The development of glucocorticoid function has been studied in the rat from birth to weaning. It has been shown that both the total plasma corticosterone (B) concentration and the relative adrenal weight are high at birth, decrease during the first week post-partum, remain low during the second week and increase during the third week. The response of the adrenal axis to stimulation at the hypothalamic, pituitary and adrenal levels has been assessed by measuring the changes in total plasma B concentration after acute challenges of insulin, ADH and ACTH. The responses to all three agents are similar and follow the same pattern as the resting plasma B concentration. The binding of B has also been measured over this period and found to decrease over the first week post-partum and increase in the third. It is suggested that the changes in plasma binding may partly account for the changes in adrenal function seen at this time.

Thyroid hormones are known to be regulators of corticosterone binding globulin (CBG) synthesis in the adult rat and their concentrations increase from day 7 post-partum. Administration of thyroxine (T_4) has been shown to produce an increase in plasma binding of B in the 9 day old rat, and also to produce an increase in total plasma B concentration, relative adrenal weight and an increased response to stimulation. The effect of different doses and schedules of treatment has been investigated and the results are consistent with the suggestion that the change in binding is the primary and causative change. In spite of increased total plasma B concentration and increased response to stimulation the corresponding free hormone concentrations are approximately constant after T_4 treatment.

Treatment with propylthiouracil, an inhibitor of thyroid function, partially prevented the increase in binding normally seen by day 21 and also diminished the increase in plasma B concentration and adrenal responsiveness normally seen by this age thus providing further evidence for thyroid hormones being the physiological stimulus for the binding changes seen after day 9. The system has also been used to assess the activity of the thyroid hormones tri-iodothyronine (T_3) and reverse tri-iodothyronine (rT_3). T_3 was shown to be 10 times more potent than T_4. rT_3 had no action alone at any age, was able to significantly decrease the effect of T_4 at day 9 but had no effect on the response to T_3 at this age. This provides *in vivo* evidence of rT_3 acting as a β-deiodinase inhibitor under physiological conditions.

These observations are discussed in the context of the role of the thyroid and adrenal hormones in the development of organ function in the young rat.

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADH	antidiuretic hormone, vasopressin
ADP	adenosine diphosphate
ATP	adenosine triphosphate
B	corticosterone (Kendall's Compound B)
BSA	bovine serum albumin
BMR	basal metabolic rate
°C	degrees centigrade
^{14}C	carbon-14
CBG	corticosteroid binding globulin
CBP	competitive protein binding (assay)
CNS	central nervous system
cpm	counts per minute
CRF	corticotrophin releasing factor
cyclic AMP	adenosine 3,5 monophosphate
DNA	DEOXYRIBONUCLEIC ACID
eg	for example
EGF	epidermal growth factor
F	cortisol (Kendall's compound F)
g	gramme
^{3}H	tritium
^{125}I	iodine-125
ie	that is
ip	intra peritoneal
iv	intra venous
mg	milligramme
MW	molecular weight
ng	nanogramme
NGF	nerve growth factor
PTU	propylthiouracil
RIA	radioimmunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
rT_3	reverse tri-iodothyronine
Sb	bound steroid
sc	subcutaneous

SNR	stress non-responsive
SNS	sympathetic nervous system
Su	unbound steroid
T_4	thyroxine
T_3	tri-iodothyronine
TSH	thyroid stimulating hormone
TRH	thyrotrophic releasing hormone
u	micron
uCi	microcurie
ul	microlitre

INTRODUCTION

THE MAMMALIAN ADRENAL AXIS

The Adrenal Glands

The mammalian adrenal glands are small paired organs lying on either side of the mid-line of the abdominal cavity above the kidneys. The glands consist of an outer cortex and an inner medulla which have different embryological origins and thus form two separate functional and morphological structures within a single capsule. The cortex develops from coelomic epithelium and is of mesodermal origin, whilst the medulla is derived from sympathogonia which migrate from the neural crest and is therefore ectodermal in origin. The cortical tissue is the first to appear and can be recognised between the fourth and sixth week of gestation in the human (Melby, 1979) and about day 13 in the rat (Josimovich, Ladman and Deane, 1954), (gestation is 40 weeks in the human and 21-22 days in the rat). Jost suggested that the first appearance of adrenal tissue and the biochemical capacity to synthesise hormone were independent of a functioning pituitary but that secretion of hormones into the blood required an intact pituitary (see Jost and Picon, 1970). The growth of the adrenal cortex in the fetus is dependent upon an intact fetal pituitary. Lack of an intact fetal pituitary leads to a reduction in adrenal size and atrophy of the cortical tissue in the following species:- human, (Brewer, 1957); sheep, (Liggins and Kennedy, 1968); rat, (Wells, 1947) and mouse, (Eguchi, 1961). The adrenal medulla develops later than the cortex. The first cells to invade the fetal adrenal cortex are seen from around the 7th week in the human and on day 16 in the rat (Pankratz, 1931). In the adult secretion by the adrenal medulla is controlled by pre-ganglionic sympathetic nerve fibres which release the neurotransmitter acetyl choline.

Adrenaline is the predominant catecholamine secreted by the adrenal medulla. It is released directly into the circulation when the sympathetic nervous system (SNS) is activated. The release of adrenaline gives a different spectrum of response from the rest of the SNS where noradrenaline is released locally. Adrenaline is predominantly a β agonist whilst noradrenaline is an α agonist.

α agonists cause general vasocontriction and relaxation of the gut, compared with β agonists which cause relaxation of the gut, vasodilation in skeletal muscle, an increase in heart rate and contractility and have important actions on lipolysis and glycogenolysis. These effects are particularly important in the flight or fight response. About 35% of noradrenaline is removed in a single passage through the pulmonary circulation while the pulmonary removal of adrenaline is negligible (Gryglewski, 1980). The functioning of the adrenal medulla is not considered essential for life.

In contrast, the adrenal cortex must function if life is to be maintained in a normal environment. Lack of adrenocortical function leads to loss of weight, inability to withstand stress, loss of carbohydrate reserves and lowering of blood glucose concentration, loss of sodium, loss of vasomotor TONE, an increased sensitivity to insulin and an increase in non-protein nitrogen (Balfour, 1962). Rats are able to survive adrenalectomy if maintained in a protected environment and given saline to drink, (Balfour, 1962). The adrenal cortex comprises 80% of the weight and volume of the adult human adrenal and is divided into three layers. These three layers differ in their light microscopic and electron microscopic appearance, enzyme content and hence steroid composition as well as their response to regulatory peptides.

When examined by light microscopy, the outer glomerulosa is a narrow zone which lies adjacent to the adrenal capsule consisting of small epithelial cells. The structure of the mitochondria, under electron microscopy, is different from those of the other layers. In the zona glomerulosa the mitochondria are elongated and possess lamella cristae. The glomerulosa secretes the mineralocorticoid aldosterone, which acts on epithelia to conserve sodium and therefore plays an important part in salt and water balance.

The middle layer, the fasiculata, is the largest layer of the adrenal and its columnar cells are continuous with the cells of the zona glomerulosa. The cells of the fasiculata are arranged in cords radiating from the glomerulosa and contain both large numbers of lipid droplets and cytoplasm which is highly vacuolated. The mitochondria vary in size and are larger than those of the zona glomerulosa and the inner zone, the zona reticularis. They contain

cristae which are short, tubular vesicular invaginations of the inner mitochondrial membrane or free lying vesicles in the mitochondrial matrix. The zona fasiculata secretes the glucocorticoids which control aspects of intermediary metabolism. In the human the major glucocorticoid is cortisol (Kendall's compound F) and in the rat it is corticosterone (Kendall's compound B).

The zona reticularis is adjacent to the medulla and is composed of a network of interconnecting cells which vary in size, shape and density. There are fewer lipid droplets in the cells of the reticularis than the cells of the other two zones. The mitochondria are similar to those of the zona fasiculata although they tend to be more elongated and contain flattened cristae. These cells produce the C-19 steroids (the adrenal androgens) and C-18 steroids (the adrenal oestrogens). The C-19 androst-5-ene structure steroids are produced predominantly. These hormones cause the development of secondary sexual characteristics. Their role is thought to be of limited importance except in disease states.

The adrenals have a high blood flow for their size and oxygen consumption and thus the adrenal venous blood is only slightly desaturated with oxygen. The medulla receives some of the arterial blood directly but most comes from the capillaries of the adrenal cortex, and, apart from the adrenal cortex itself, the cells of the medulla are exposed to higher levels of corticosteroids than any other tissue in the body. The enzyme phenylethanolamine-N-methyltransferase (PNMT) which converts noradrenaline to adrenaline in the medulla is dependent on a high glucocorticoid concentration for its activity (Wurtman and Axelrod, 1966). It has also been suggested that the full functioning of the cortex itself is dependent on a high arterial blood supply. L'Age, Gonzales-Luque and Yates (1970) suggest that the adrenal cortical response to a given ACTH injection is greater when the blood flow is higher.

The adrenal steroids are synthesised from the parent compound cholesterol. The adrenal cortex is able to synthesise cholesterol from acetyl Co A via melavalonate and squalene (Samuels and Nelson, 1975) but it is also able to use cholesterol directly from the blood. Blood cholesterol can be derived either from the diet or from liver biosynthesis. The relative contribution from the diet or from adrenal

synthesis is variable, species dependent and may be related to the extent of the cortical stimulation (Ichii and Kobayashi, 1966). Cholesterol is stored in the cortex in a free or an esterified form. The main sequence of reactions which leads to the adrenal steroids is shown in Fig. I.1. The steroid production from each zone depends on the individual enzymes present. The anterior pituitary hormone, adrenocorticotrophic hormone (ACTH) is required both to maintain adrenal steroidogenesis and adrenal size. After hypophysectomy the adrenal atrophies and loses its responsiveness to ACTH. Kiruma (1969) demonstrated that the activity of the side-chain cleavage enzyme, the rate-limiting step in steroid synthesis, was reduced after hypophysectomy, so the adrenal becomes less responsive to exogenous ACTH as it can only produce limited amounts of steroids. ACTH administration is able to restore the activity of this enzyme. This effect is less marked in the zona glomerulosa where ACTH is only required to maintain a basal secretion. The Renin-Angiotensin system and plasma potassium concentration regulate the secretion of aldosterone above the basal secretion promoted by ACTH in the human. The Renin-Angiotensin system is less important in the rat (Edmonds, 1978) and other factors such as plasma potassium concentration may be more significant in the regulation of aldosterone secretion in this species.

Control of Adrenocortical Function

ACTH is a single chain polypeptide consisting of 39 amino acid residues (M.W. = 4500) of which the N 1-24 fragment is necessary for biological activity. This 1-24 fragment is considered to be common to many mammals (Imura, Sparks, Grodsky and Forsham, 1965) whereas the 24-39 fragment shows species variation and may play a role in transport or protection from enzymatic degradation (Landon, James, Cryer, Wynn, Franklan, 1964). ACTH is the smallest pituitary trophic hormone and is present at very low plasma concentrations. Its secretion is thought to be stimulated by corticotrophin releasing factor (CRF). ACTH is secreted from the corticotrophs, a type of basophil cell in the anterior pituitary. CRF is released from the hypothalamus and reaches the anterior pituitary via the portal system of the

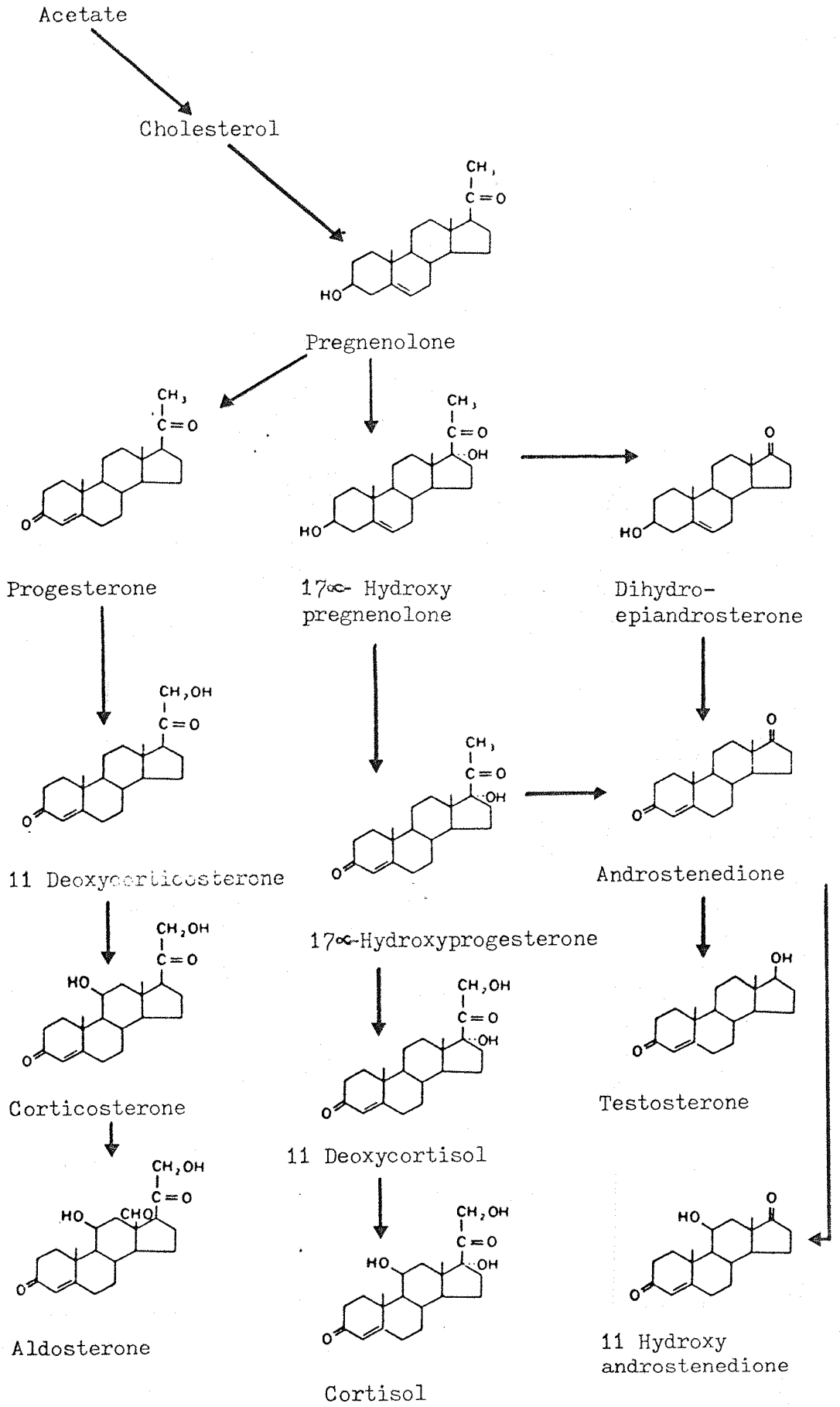


Fig. I.1. Steroid synthesis in the adrenal cortex.

hypothalamo-hypophyseal tract. Harris (1948) has demonstrated the existence of CRF but its exact chemical nature still remains unclear. The posterior pituitary peptide vasopressin (ADH) has been suggested as a possible candidate for CRF.

ADH is able to cause ACTH secretion both *in vitro* (Fleischer and Vale, 1968) and *in vivo* (Rivier, Vale and Guilleman, 1973). However ADH may not be the sole component of CRF activity as large doses are used for *in vitro* experiments, and *in vivo* ACTH secretion is not always associated with an increase in peripheral ADH concentration. Furthermore, some CRF activity is still present in the Brattleboro rat which is not able to produce ADH (Buckingham and Leach, 1980). Walkins, Schwabedal and Bock (1974)proposed the presence of a CRF-associated neurophysin in the external zone of the median eminence and Stillman, Recht, Rosario, Seif, Robinson and Zimmerman (1977) demonstrated a direct zonal pathway to the hypophyseal portal system containing ADH and a neurophysin which was controlled by the adrenal cortex. Gillies and Lowry (1979) chromatographed stalk-median eminence extract and found a large peak with CRF activity which was associated with ADH and a smaller peak with CRF activity in the void volume. They assayed for CRF activity using a dispersed pituitary column preparation. They showed that these two components caused a three-fold potentiation of CRF activity when added to the pituitary column. They suggested that ADH and at least one other factor was involved in CRF activity. More recently Vale, Spiess, Rivier and Rivier (1981) have isolated and characterised a 41 amino acid peptide which has CRF activity. Gillies, Linton and Lowry (1982) reported that this peptide was more potent than ADH at releasing ACTH from their pituitary columns. They also reported that the new peptide and ADH produced a four-fold potentiation of CRF activity. However, as this new peptide also potentiated the activity of ADH-free hypothalamic extracts they suggested that this new CRF peptide did not account for all the non-ADH portion of the CRF complex. Thus, it still remains for the full complexity of CRF to be finally elucidated. Higher centres in the brain modulate ACTH release through CRF. These include the amygdala, the hippocampus and the limbic system. It is possible to modify ACTH secretion by local administration of small amounts of glucocorticoids to these areas of the CNS (Donovan, 1970).

ACTH acts on the adrenal cortex, via the second messenger - cyclic AMP, to cause an increase in the synthesis and secretion of glucocorticoids. ACTH stimulates the synthesis of glucocorticoid by increasing the activity of the side chain cleavage enzyme, which converts cholesterol to pregnenolone. ACTH also causes a dramatic increase in adrenal blood flow (Edwards, Hardy, Malinowska, 1974), depletion of adrenal cholesterol and ascorbate and cortical growth mainly of the fasiculata and reticularis. The time course of these responses is different. The increase in glucocorticoid secretion and the increase in blood flow occur within minutes, adrenal ascorbic acid and cholesterol depletion within a few hours and adrenal growth after a few days. In addition to its actions on the adrenal cortex ACTH acts directly on adipose tissue to cause increased lipolysis (Desbal, Desbal and Agid, 1970). However, the physiological significance of this is not clear.

ACTH secretion from the pituitary maintains a plasma glucocorticoid concentration which follows a diurnal rhythm, peak concentrations occurring early in the morning in the human (Nichols and Tyler, 1967) but late in the day in the rat which is a nocturnal animal (Guilleman, Dear and Liebett, 1959). ACTH secretion from the pituitary is not constant but occurs in discrete pulses (Yates and Maran, 1975). This pulsatile secretion of a trophic hormone is thought to be essential to maintain full responsiveness of the target tissue (Bergquist, Nillius and Wide,1979). ACTH also stimulates a rise in plasma glucocorticoids when the animal is exposed to a variety of noxious stimuli, e.g. injury, hypoglycaemia, cold, heat or infection. ACTH secretion can be inhibited by high plasma glucocorticoid concentration. It is still unclear whether glucocorticoids feedback directly to the pituitary, via the hypothalamus, or a combination of both. Feedback directly to the pituitary may affect ACTH secretion only whilst feedback to the hypothalamus may affect both synthesis and secretion (Krieger, 1979). As CRF has not been fully characterised it has not been demonstrated conclusively that changes in ACTH secretion are accompanied by changes in portal CRF activity. CRF stimulates a rapid rise in plasma ACTH concentration very quickly leading to an increase in adrenal steroidogenesis within minutes. Little is known of the time course of CRF suppression.

Generally it is considered that there are two types of negative feedback by glucocorticoids on ACTH secretion. One is a rate-sensitive mechanism and the other is a level sensitive mechanism. The rate-sensitive mechanism responds if plasma glucocorticoid concentration increases rapidly. In male rats this increase must be greater than 13 ug/min/litre (Jones, Brush and Neame, 1972). This mechanism is of short duration and when the plasma glucocorticoid concentration reach a plateau this inhibition disappears (Jones, Gillham, Mahmoud and Holmes, 1979). This mechanism is unusual as it is an action of glucocorticoids which does not require protein synthesis. The level-sensitive mechanism has a delayed action and operates if glucocorticoids are elevated for over 2 hours. It is possible that the delayed-feedback mechanism serves to reduce the activity of the adrenal axis and that the rate-mechanism carries out the finer control. The delay in the onset of the level sensitive mechanism allowed glucocorticoids to continue to increase if the stimulus to secretion is prolonged and not acute. There are other sites of feedback within the adrenal axis as, already mentioned application of glucocorticoids to specific areas of the brain modifies ACTH secretion (Donovan, 1970). It has been suggested that glucocorticoids feedback directly on the adrenal cortex (Loose, Do, Chen and Feldman, 1980) and that there is another short feedback loop between the pituitary and the hypothalamus (Upton, Corbin and Mabry, 1973). Some workers suggest there is a tissue CRF which modifies ACTH secretion (Lymangrover and Brodish, 1973).

The release of ACTH from the pituitary occurs within 2 minutes of exposure to stressful stimuli and the peak increase is seen about 10 minutes later. Plasma glucocorticoid concentrations increase more gradually, peak 30 minutes after the stress and remain elevated for 1-1½ hours. The difference in the time course of these two hormones plasma concentrations is caused, in part, by their different half-lives. ACTH is broken down very rapidly and has a half-life of about 10 minutes compared with glucocorticoids which have a half life of between 30 minutes and 1 hour. The fate of ACTH is poorly understood, and although it is taken up by the kidneys as well as the adrenals none appears in the urine. Glucocorticoids are mainly metabolised in the liver. They are reduced and conjugated with either glucuronate or sulphate which makes them more water soluble, before being excreted in the bile or the urine.

Transport of Hormones

Both albumin and a specific globulin, corticosteroid binding globulin (CBG) bind glucocorticoids in the plasma, with differing affinities. Albumin has an affinity constant in the region of 10^3 litres/mole at 37°C compared with CBG which has an affinity constant of 10^7 to 10^8 litres/mole. Thus at normal plasma glucocorticoid concentration most of the glucocorticoids are bound to CBG. When glucocorticoids are protein bound they are protected from degradation and renal filtration thus buffering the total plasma hormone concentration. The local plasma hormone concentration is also buffered by plasma binding of a hormone this means that the tissue hormone supply is independent of blood flow provided the tissue uptake is slower than the rate of dissociation of the steroid from the binding protein (Hillier, 1971). Protein binding also increases the amount of glucocorticoids which can be carried in the plasma as they are not very water soluble. Thus, glucocorticoids exist in the plasma in a bound and free form (Keller, Sendelbeck, Richardson, Moore and Yates, 1966) and it is the free form which is considered to be physiologically active in tissues of low protein permeability. Keller, Richardson and Yates (1969) have demonstrated that the enzyme alanine transferase was inducible by dexamethasone in the pancreas and liver in the male rat. When CBG concentration and thus the total plasma B concentration were increased by prolonged oestrogen treatment only the hepatic enzyme increased in activity. Since the free hormone concentration did not change this suggested that the liver responded to the total plasma glucocorticoid concentration whilst the pancreas, being less protein permeable, responded to the free hormone concentration. Keller et al. (1969) also showed that B which is bound to a greater degree than dexamethasone in plasma, induced both the pancreatic and liver enzyme in the male but only the liver enzyme in the female rat. This finding was interpreted as a sex-dependent difference in binding activity. Thus, when considering the active plasma concentration it is necessary to consider changes in hormone binding as well as changes in total plasma hormone concentration.

THE MAMMALIAN THYROID AXIS

The Thyroid Gland

The adult thyroid gland consists of two lobes joined by an isthmus which crosses the trachea in front of the second to fourth cartilage rings, the highly vascular nature of the thyroid gives it a brownish red appearance. The gland is enclosed by connective tissue which is contiguous with the pretracheal fascia. This outer capsule is loosely connected to a deeper layer of connective tissue which forms the inner capsule. The space between the two capsules contains the recurrent laryngeal nerves and, generally, the parathyroid glands. Septa extend from this inner capsule into the gland dividing it into lobules of varying size and shape, which are composed of follicles. Each follicle is a spherical structure of about 50 u in diameter. In the rat the superficial follicles are larger than the deeper ones, but this pattern is not observed in the human. A follicle consists of a single layer of epithelial cells enclosing a cavity, the follicular lumen, which is filled with a viscous, proteinaceous fluid, the colloid. Follicles are surrounded by a thin basement membrane and have a rich capillary plexus. The blood vessels within the follicles have a rich sympathetic innervation. The thyroid receives a parasympathetic nerve supply but its distribution is not well documented. The shape of the follicular cells varies among species and among follicles within an individual but the cell height is uniform within individual follicles. Generally if the cell height is low the cells are resting and if the cell height is increased the cells are active.

The thyroid develops from the primitive gut and is formed by an invagination of the pharyngeal epithelium. There are three stages of histological development in the thyroid:- the precolloid stage, the beginning of the colloid formation and finally follicular growth. In the human these occur at 42-72 days, 73-80 days and beyond 80 days respectively. In the rat the lobes and the isthmus of the fetal thyroid are clearly distinguishable but no capsule is present by day 14 of gestation. The first colloid droplets are seen by day 17 and the follicles are present by day 18 (Carpenter and Rondon-

Tarchetti, 1957). The early development is independent of a fetal pituitary, however the later development and full maturation of the ability to trap iodine and hormone synthesis are dependent on an intact pituitary (Jost and Picon, 1970).

The follicle is the smallest functional unit of the thyroid. It is here that the iodine containing thyroid hormones are synthesised from the large glycoprotein thyroglobulin. In addition to the two major thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) calcitonin is synthesised in the parafollicular or 'C' cells which are found in the follicular walls or the interfollicular spaces. Calcitonin is concerned with the regulation of calcium homeostasis and will not be considered further here.

Hormone Synthesis and Secretion

T_4 and T_3 are derivatives of iodinated amino acids of the L configuration. The following processes are involved in their synthesis and secretion:-

1. synthesis of thyroglobulin and its secretion into the follicular lumen,

2. iodination of thyroglobulin and coupling of the tyrosine residues,

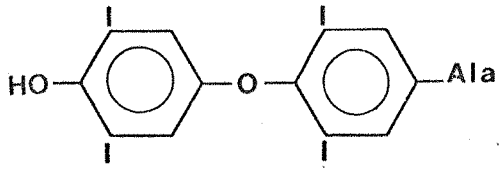
3. storage of the thyroglobulin in the follicular lumen,

4. resorption of this material and its degradation to form T_4 and T_3 with the other constituents being recycled within the gland.

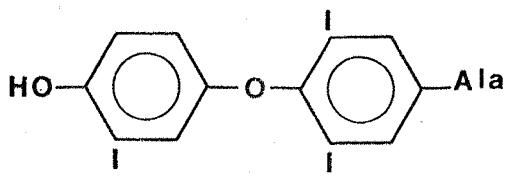
Thyroglobulin is a very large glycoprotein (M.W. 660 000). A considerable part of the dry weight of the tissue consists of thyroglobin. It is mainly synthesised in the rough endoplasmic reticulum of the follicle cells. Various carbohydrate moieties are added in the Golgi apparatus as the protein migrates from the basal to the apical portion of the cell. The thyroglobulin molecule is then transferred from the Golgi apparatus to the apical cell

surface enclosed by vesicles, and following fusion with the cell surface they empty their contents into the follicular lumen. The iodide from the diet has to be converted to either free iodine or the very reactive $^{\bullet}I^{+}$ radical before it can be incorporated into the thyroglobulin molecule. This conversion is carried out by an enzyme peroxidase system. The site of iodination of thyroglobulin is not certain with both a cellular and a luminal site having been suggested. Electronmicroscopical studies in rats using ^{125}I indicate that this is concentrated in a narrow zone on the periphery of the lumen close to the apical membrane 30 seconds after its administration (Elkholm and Wollman, 1975) and not in the cell. The site of iodination will be closely related to the localisation of thyroperoxide the enzyme considered responsible for the iodination of the tyrosyl residues and for the coupling reaction which forms the iodothyronines. Histological evidence indicates that the peroxidase reaction product is found in association with the apical membrane (Tice and Wollman, 1974) which suggests that the site of iodination is on the apical membrane surface and that the thyroglobulin is then released into the lumen of the follicle. Such a mechanism has the advantage that it produces minimal iodination of intracellular proteins and thyroglobulin is stored in the colloid in the lumen of the follicle. This very large store of preformed hormone is unique among endocrine glands as is the mechanism of hormone release with endocytosis followed by proteolysis. Having been taken up into the follicular cell the colloid vesicles are disrupted by lysosomal enzymes and the thyroid hormones are released and diffuse into the blood. As well as the two major thyroid hormones other iodoamino acids are released. These include monoiodotyrosines (T_1) and diiodotyrosines (T_2). The structures of these iodo compounds are shown in Fig. I.2. The inactive T_1 and T_2 are broken down within the cell and the iodide is recirculated into thyroglobulin.

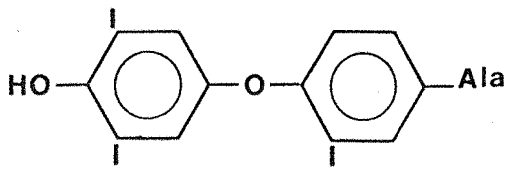
The thyroid needs an adequate supply of dietary iodide for normal hormone production. Iodide is present in the diet in trace amounts and this iodide is concentrated within the gland by a powerful iodide pump. (The iodide pump generates a thyroid to serum ratio of 25:1). The total iodide content of the thyroglobulin reflects the iodide status of the animal and varies between 0.2 and 1% of the protein.



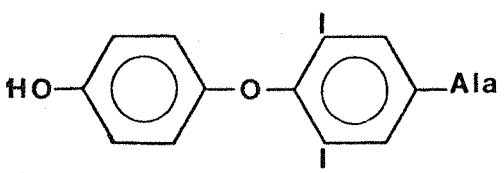
Thyroxine (T_4)



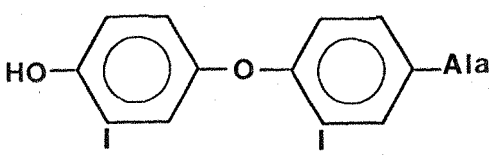
3,3',5-Triiodothyronine (T_3)



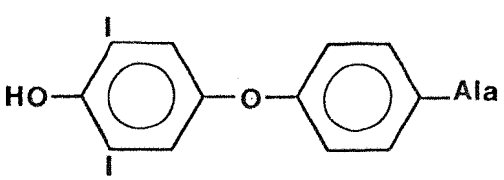
3,3',5'-Triiodothyronine (rT_3)



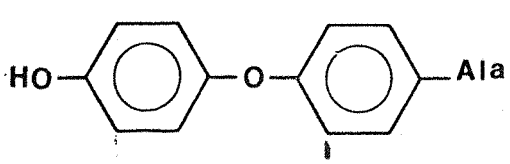
3,5-Diiodothyronine (3,5-T_2)



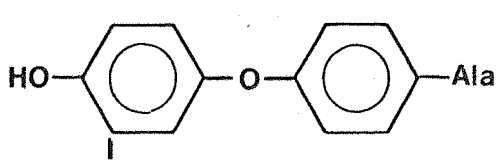
3,3'-Diiodothyronine (3,3'-T_2)



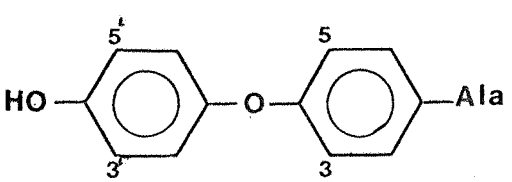
3',5'-Diiodothyronine (3',5'-T_2)



3 -iodothyronine (3-T_1)



3'-iodothyronine (3'-T_1)



β ring α ring

Fig. I.2 The iodothyronines.

Control of Thyroid Hormone Secretion and Metabolism

Thyroid stimulating hormone (TSH), secreted by the thyrotrophs of the anterior pituitary, stimulates synthesis and secretion of the thyroid hormones. TSH is a two chain polypeptide hormone having a β subunit in common with LH and FSH. It acts through the second messenger cyclic AMP. TSH causes an increase in iodide trapping from the plasma, increased synthesis and iodination of thyroglobulin and an increased rate of degradation of thyroglobulin. The resulting increase in the secretion of T_4 and T_3 is accompanied by an increased blood flow through the gland. TSH secretion from the anterior pituitary is stimulated by the hypothalamic releasing hormone, TRH. TRH is a tripeptide consisting of pyroglutamyl histidyl proline amide. Sympathetic stimulation increases thyroid secretion but it is normally of limited importance because if it is inappropriate, TSH secretion will decrease thus the set point wil not be altered.

T_4 is the major hormone secreted by the thyroid gland. It exists in the plasma mainly in a bound form. It is bound to thyroxine binding globulin (TBG), a glycoprotein (Mol. Wt. 55 000), to albumin and to a pre-albumin protein (TBPA). In humans about 99.95% of the plasma T_4 is protein bound. The same proteins also bind plasma T_3 but with a lower affinity such that 99.5% of the T_3 is bound. The normal total plasma T_4 concentration is about 80 ng/ml whilst the normal total plasma T_3 concentration is about 1.5 ng/ml. Part of this difference in total plasma hormone concentrations is made up for by the fact that the proportion of T_3 which is free is about 10 times the proportion of T_4 which is free, 42% of the whole body turnover of T_3 in the human derives not from thyroidal T_3 secretion but from β deiodination of T_4 in the tissues (Singer and Nicoloff, 1972). T_3 is more potent than T_4 and this is considered to be the major route through which T_4 exerts its hormonal effects. Thus the control of thyroid hormone activity is not only exercised by regulating the secretion of hormone from the gland but also by regulating the rate of conversion of T_4 to T_3 (β deiodination) in the tissues and hence the tissue T_3 concentration.

In the adult human T_4 has a plasma half life of 7 days and T_3 has a half life of 2 days. In the rat T_4 has a half life of 14 hours and T_3 has a half life of 7 hours (Schwartz, Surks and Oppenheimer, 1971).

There are a number of important feedback mechanisms in the thyroid axis. T_4 has been shown to have a negative feedback effect on the pituitary secretion of TSH (Yamada and Greer, 1959) but more recently Reichlin, Martin, Mitnick, Boshans, Grimm, Bollinger, Gordon and Malacara (1972) have suggested that T_4 has a positive feedback upon hypothalamic TRH production. It is generally considered that TSH secretion is a balance between the positive effects of TRH from the hypothalamus determining the 'set point' of the system and the negative feedback effects of the plasma T_4 concentration. The mechanism of the β deiodinase enzyme in the pituitary is such that most of the available T_4 is converted to T_3. Balfour (1969) demonstrated that pituitary T_3 content inhibited TSH secretion to a greater degree than T_4 pituitary content. Thus, it is really pituitary T_3 receptor occupancy which suppresses TSH secretion but this in turn depends on the plasma T_4 concentration. There is much evidence to suggest that in addition to the control through TSH the thyroid itself has an ability to regulate its hormone production in response to changes in iodide availability. Hypophysectomised rats respond to iodide deficiency with a slight elevation of iodide transport activity (Halmi and Spirtos, 1955) and administration of T_4 is reported to reduce the thyroid:serum ratio for iodide to a lower level than hypophysectomy (Halmi, Granner, Albert and Doughman, 1959). More recent evidence indicates that thyroid hormones directly inhibit the thyroid adenyl cyclase and thyroid response to cyclic AMP (Friedman, Lang and Burke, 1977). During iodine deficiency the gland also secretes a higher proportion of T_3 compared with T_4 thus producing the hormone with the greater metabolic activity in larger quantities (Greer, Grimm and Studer, 1968).

The peripheral conversion of T_4 to T_3 is another, much more recently recognised site for regulation of the thyroid axis. It is important because it directly regulates the amount of active hormone present in the tissue. Increased sympathetic activity is known to enhance T_4 to T_3 conversion. Wiersinga, Modderman and Touber (1980) showed that α and β agonists increased the *in vitro* T_4 to T_3 conversion and that α and β antagonists decreased the *in vitro* T_4 to T_3 conversion. Starvation (Vagenakis, Burger, Portnay, Rudoph, O'Brian, Azizi, Arky, Nicod, Ingbar and Braverman, 1975) and glucocorticoid treatment (Chopra, Williams, Orgazzi

and Solomon, 1975a) are known to reduce T_4 to T_3 conversion. Recently the thyroid compound rT_3 has been suggested as an important inhibitor of T_4 to T_3 conversion. This may have important physiological consequences as during starvation and glucocorticoid therapy when plasma T_3 concentrations are reduced, plasma rT_3 concentrations are increased (Chopra et al., 1975a). However much of this work has been carried out *in vitro* and the results require careful interpretation since Kaminski, Kohrle, Kodding and Hesch (1981) showed that the *in vitro* actions of rT_3 are pH-dependent. It still remains to be shown whether rT_3 has an active role to play in the regulation of T_4 to T_3 conversion or whether T_4 is converted to a less metabolically active compound when thyroid hormone activity requires to be low.

The actions of thyroid hormones on metabolism require that T_4 and T_3 concentrations are kept tightly regulated with generally no rapid fluctuations in the hormone concentrations resulting in most negative feedback mechanisms being generated over a period of days or weeks. However if T_4 and T_3 concentrations need to be increased rapidly this can be achieved if plasma free fatty acids are increased. This results in the displacement of T_4 and T_3 from the plasma binding proteins (principally albumin) so increasing the active free concentration (Hollander, Scott, Burgess, Rabinowitz, Merimee, Oppenheimer 1967). This may occur physiologically in cold exposure and in the newborn (Comline and Silver, 1972).

Plasma rT_3 concentrations are generated in the peripheral tissues from T_4 (Chopra, 1976). Thus, about 80% of T_4 is shunted into either T_3 or rT_3. The division is approximately 40% to each in humans whilst only 17% is converted to T_3 in rats (Schwartz, Surks and Oppenheimer, 1971).

The fate of thyroid hormones in the body is progressive deiodination with modification of the iodothyronine molecule. The most important modifications are the phenolic conjugations with β glucuronate and sulphate and oxidative deamination or transamination of the alanine side chain. The breakdown products are then excreted in either the bile or urine, with the former route predominating. Small quantities of both T_4 and T_3 are excreted unchanged. Some of the biliary T_4 is deconjugated in the intestine

and reabsorbed but the extent to which this occurs depends to some extent on the bulk of the intestinal contents (Van Middlesworth, 1957).

THE ACTIONS OF THYROID HORMONES AND GLUCOCORTICOIDS IN THE ADULT

Thyroid hormones and glucocorticoids act through induction or repression of rate-limiting enzymes. Most of their actions can be blocked by inhibitors of protein synthesis or DNA synthesis. As they act through increasing the amount of enzyme present within the cell there is a time lag between the increase in hormone concentration in the blood and an observed change in cellular metabolism.

Lack of these hormones has marked effects. In humans hypothyroidism in the adult causes the person to become lethargic, cold and generally unresponsive to environmental changes and lack of adrenal function causes an 'Addisonian crisis', characterised by loss of carbohydrate reserves, decreased blood pressure caused hypovolaemia and hyponatraemia and a general inability to withstand stress.

Calorigenesis is one of the best known actions of the thyroid hormones. This calorigenic action is important in cold exposure and thyroid hormones are essential for an animal to tolerate prolonged exposure to cold. Thyroid hormone secretion is increased during cold exposure and the rate of conversion of T_4 to the more metabolically active T_3 is enhanced. Whole animal studies and *in vitro* individual tissue studies indicate that thyroid hormones act to increase oxygen consumption and heat production. Most tissues in the body respond in this way with the exceptions of the brain (excluding the anterior pituitary), the gonads, the lymph nodes, the spleen, the thymus and the dermis (Barker and Klitgaard, 1952).

Thyroid hormones cause an increase in basal metabolic rate causing a high cellular respiration rate which is independent of the availability of ADP (Hoch, 1974). Several theories have been proposed to explain the thyroid hormone induced increase in basal metabolic rate (BMR). Thyroid hormones are known to act at the mitochondrial level but may also increase the rate of oxidation by inducing key enzymes involved in metabolic regulation. They may also potentiate the calor genic actions of the other hormones such as catecholamines, insulin and glucagon.

Thyroid hormones cause increased incorporation of amino acids into protein (Buchanan, Primack and Tapley, 1971) and modifications in the turnover of mitochondrial DNA and protein. T_4 has been shown to induce a soluble protein fraction which increased state 4 respiration (mitochondrial respiration in the resting state, with oxygen and oxidizable substrate but with all ADP converted to ATP) when added in vitro to mitochondria from hypothyroid rats (Herd, Kaplay and Sanadi, 1974). These authors suggested that T_4 induced a cytoplasmic protein which acts at the mitochondrial level. There is a mitochondrial protein , MW 54 000, which can be induced by T_3 (Baudry, Clot, Bouhnik, Michel and Michel, 1975). These observations, together with the demonstration of high affinity binding sites for T_3 in the mitochondria (Sterling and Milch, 1975) suggest possible physiological action of thyroid hormones at the mitochondrial level.

It has been suggested that thyroid hormones act by increasing the activity of the futile shunts leading to heat production, e.g. the α glycerophosphate shunt. Thyroid hormones are known to enhance the synthesis of this enzyme (Tarentino, Richet and Westerfeld, 1966) and its cofactor (Wolf and Rilvin, 1970). However, the overall contribution of this enzyme to total oxygen consumption is small and in animals such as the guinea pig no change in the amount of this enzyme can be seen despite thyroid hormone induced increases in BMR (Lee, Liu and Hsu, 1970).

Another possible mechanism of thyroid hormone induced calorigenesis has been suggested from the observation that ouabain inhibits the T_4 or T_3 induced increase in oxygen consumption by rat liver slices (Edelman and Ismail-Beigi, 1974). As ouabain is an inhibitor of the Na^+/K^+ dependent ATPase, or the sodium pump, it has been inferred that thyroid hormones exert their effects through an increase in the activity of this enzyme possibly by making the cell membrane more permeable to sodium. Administration of T_3 increases the activity of this enzyme in parallel with the rise in oxygen consumption (Bernal and Refetoff, 1977). This action may be important as in mammalian tissues 20 to 40% of the resting respiratory rate is dependent on the membrane transport of sodium. The ADP produced by the hydrolysis of ATP would in turn stimulate the mitochondria.

In addition to calorigenesis thyroid hormones have actions on lipid, protein and carbohydrate metabolism. Thyroid hormones stimulate the synthesis, mobilisation and particularly the degradation of lipids. They decrease lipid stores and their plasma concentration. Thyroid hormones decrease serum cholesterol by increasing the faecal loss and the conversion to bile salts (Miettinen, 1968). They also increase the turnover of the low density lipoproteins which bind plasma cholesterol (Walton, Scott, Dykes and Davies, 1965). Thyroid hormones enhance the action of other lipolytic agents (Bressler and Wittels, 1966).

Thyroid hormones modulate carbohydrate metabolism by affecting the actions of other hormones such as insulin and catecholamines. The effect on glycogen metabolism is biphasic, administration of small amounts of thyroid hormones causes increased glycogen synthesis, whilst larger amounts induce glycogenolysis. Thyroid hormones enhance absorption of glucose which exceeds deposition, insulin release is delayed and its turnover may be enhanced (Elgee and Williams, 1955). These actions of thyroid hormones can explain the paradoxical responses of the thyrotoxic patient who has glycosuria and postprandial hyperglycaemia but a normal or accelerated response to an iv glucose administration (Lamberg, 1965).

The initial action of thyroid hormones is to cause protein synthesis. However the subsequent increase in BMR causes obligatory protein wastage. Thyroid hormones influence the turnover of many proteins, e.g. the half life of albumin is prolonged in hypothyroidism and shortened in thyrotoxicosis (Kekki, 1964).

Thyroid hormones are also required for full maintenance of nervous function. Although a lack of them leads to an impairment of brain function and decreased responsiveness of the brain, the exact mechanism of action is unclear.

Glucocorticoids are required for the regulation of carbohydrate metabolism. Their action on glycogen deposition in the adrenalectomised rat has been utilised in some of the early bioassays (Steele, 1975). Glucocorticoids are generally considered to oppose the actions of insulin. They protect blood glucose concentration through increasing protein degradation in the skeletal muscles and amino acid metabolism in the liver. Glucocorticoids thus cause a net transfer of protein from peripheral tissues to the liver. The rate of liver transamination

and deamination of amino acids is stimulated. This provides more substrate for gluconeogenesis which is enhanced by glucocorticoids. This leads to an increase in blood glucose concentration. In most tissues in the body with the important exceptions of the brain and the liver, glucocorticoids inhibit glucose uptake, thus reducing peripheral glucose utilisation.

The effects of glucocorticoids on fat deposition depend on the nutritional and insulin status of the animal. In the diabetic animal glucocorticoids increase lipid mobilisation and cause ketosis. However, in the starved animal with an intact pancreas, glucocorticoids actually suppress ketosis. This may be due to the glucocorticoid elevation of blood glucose concentration which stimulates insulin secretion. Insulin, in turn blocks the stimulatory effects of glucocorticoids on lipolysis and peripheral glucose utilisation. The effect of glucocorticoids depends on the extent that insulin antagonises their actions.

Glucocorticoids depress the activity of the lymphoid tissue. They depress the growth of both the thymus and bone marrow and this depresses both the T and B cell components of the immune system. They inhibit growth through inhibition of glucose and amino acid uptake and depress the activity of RNA polymerase so the protein production of the cells is reduced. Glucocorticoids may have a role in the preventing of adverse effects of overactivity of the immune system. This anti-inflammatory action is used clinically in organ transplant operations to reduce tissue rejection.

The potency of catecholamines is increased by glucocorticoids. The action may be due to an inhibition of catecholamine uptake, an inhibition of the enzyme catechol-o-methyl transferase which catalyses the breakdown of adrenaline and noradrenaline or a combination of both (Foster, Goldie and Paterson, 1983). This action is one of the few that does not require a protein synthesis step and is therefore a fast action of glucocorticoids. Thus glucocorticoids are very important for the functioning of the adrenal medulla, increased conversion of noradrenaline to adrenaline(discussed earlier) and increased duration of action of the catecholamines. Glucocorticoids are also required to maintain vasomotor tone. The exact mechanism is unclear but it may be through their action on increasing the

half-life of adrenaline and noradrenaline which contribute to resting vasomotor tone.

Glucocorticoids are released quickly in response to stress. An adrenalectomised animal is unable to tolerate severe stress (e.g. cold exposure or starvation). The ability to withstand stress is restored if glucocorticoid replacement is given. The response of the adrenal axis to stress is rapid and the feedback mechanisms ensure that the plasma B activity is not elevated for long. It is difficult to see precisely how hormones which act on protein synthesis with a considerable time lag between their release and their onset of action could be advantageous during the acute stage of a stress, although their presence at such times is known to be crucial. The extra adrenal actions of ACTH on (e.g.) lipolysis may be advantageous and the permissive role played in the actions of adrenaline and noradrenaline may also be important. During stress the SNS and the adrenal medulla are activated and the rapid actions of the glucocorticoids may be expressed here. Barton and Little (1978) showed that, if glucocorticoid synthesis was blocked, thus reducing the increase in plasma B concentration in response to injury in the rat, the lethality of the injury was increased and the hyperglycaemia associated with injury was prevented. B administration prevented the lethal effects of the injury and restored the hyperglycaemia associated with injury. Thus, they concluded that the glucocorticoids were involved in the early circulatory and metabolic responses to injury in contrast to their reported permissive role in the later catabolic phase.

There is some interdependence in the adult animal between the thyroid and adrenal axes. In the rat thyroid hormones are known to stimulate CBG synthesis, (Labrie, Raynaud, Ducommun and Fortier, 1964) thus influencing the proportion of the hormone which is free and active in the plasma. Thyroid hormones decrease tissue levels of ACTH in the hypothalamus and in the corpus striatum and thyroid hormone deprivation reduces tissue levels of ACTH in the pituitary and the frontal lobe (Gambert, Garthwaite, Pontzer and Hagen, 1980). Glucocorticoids are known to reduce TSH secretion (Wilber and Utiger, 1969) and have been shown to inhibit peripheral conversion of T_4 to T_3. Plasma T_3 concentrations are depressed in patients receiving

glucocorticoid therapy (Chopra et al., 1975a). This occurs physiologically in situations of maintained stress when plasma glucocorticoid concentrations are elevated such as during starvation and non-thyroidal illness (Chopra, Chopra, Smith, Reza and Solomon, 1975b).

ACTIONS OF GLUCOCORTICOIDS AND THYROID HORMONES DURING DEVELOPMENT

A functioning adrenal is essential for the development of liver function in mammals. At birth there is a switch of function from the mother and placenta to the neonatal liver. Some of the earliest fetal endocrine studies by Jost and Hatey (1949) showed that the litters of rabbit fetuses decapitated in utero contained 12 times less glycogen than normal litter mates at term. Later, they demonstrated that if glucocorticoids were administered to decapitated fetuses from day 26 of gestation (term in the rabbit is 32 days) the liver glycogen content was normal. Small quantities of prolactin were required if glucocorticoid treatment was started before day 26 for liver glycogen to be normal at term (Jost and Picon, 1970). The effect of fetal hypophysectomy in the rat is less marked unless accompanied by maternal adrenalectomy as the rat placenta is much more permeable to glucocorticoids (Jost and Picon, 1970). In the rat Leakey and Dutton (1975) have shown the enzyme glycogen synthetase UDP-glucose to be induced prematurely by glucocorticoid treatment. The action of glucocorticoids in large animals is similar. Barnes, Comline and Silver (1978) demonstrated that fetal hypophysectomy or adrenalectomy in the sheep decreased liver glycogen at term and that infusion of cortisol restored liver glycogen content. This is the best known action of glucocorticoids on liver development, however there are many examples of glucocorticoid dependent maturation of liver enzymes. Much work has been carried out on rat liver enzyme systems. Glucocorticoids are found to be required for the increase in tyrosine transaminase activity seen within a few hours of birth (Sereni, Kenney and Kretchmer, 1959). If glucocorticoids are not present hormones such as glucagon are unable to increase the activity of this enzyme and the nutritional status of the animal has no effect (Wicks, 1968). Other liver enzymes which require glucocorticoids for their development include the arginine synthetase system (Raiha and Suihkonen, 1966), the ornithine aminotransferase (Hertzfeld and Greenard, 1969), tryptophan oxygenase (Greenard and Dewey, 1971) and glucokinase (Jambar and Greenard, 1970). Thus gluco-

corticoids are required for the induction of enzymes involved in both protein and carbohydrate metabolism.

The action of glucocorticoids seems to be in two distinct phases. One is at the time of birth, the time of transition from the mother and placenta to the neonatal liver and the second phase is at the time of weaning when there is a considerable change in the constituents of the diet. In the rat, for example, the induction of tyrosine transaminase is in the first phase and the induction of tryptophan oxygenase is seen around the time of weaning. This corresponds with high total plasma corticosterone concentrations at birth and after day 14. The actions of glucocorticoids on individual enzyme systems are dependent on the tissue being at the right stage of development to be capable of responding to the signal. For example, it is not possible to induce tryptophan oxygenase prematurely by administering glucocorticoid before day 6 in the rat (Fiegelson and Killewich, 1979). This combination of tissue development and hormone signals ensures the enzyme activity increases at the appropriate time.

In addition to these actions on liver enzymes, glucocorticoids are essential for the growth of the liver and the postnatal rise in liver RNA seen in the rat (Sereni and Barnabei, 1967). The fetal liver synthesises red blood cells but this function is transferred to the long bones after birth. The disappearance of liver haematopoietic tissue is glucocorticoid dependent (Jacquot, 1971).

Fetal glucocorticoids are important for maturational changes in organs associated with the gut. One such example is the maturation of the insulin secreting capacity of the β cells of the pancreas. Glucose is transported across the placenta by a facilitated diffusion mechanism (Widdas, 1952) with the result that the fetal plasma glucose concentration is lower than that of the mother but shows similar changes in concentration throughout the day. The newborn animal, however, needs to be capable of regulating its own blood glucose concentration once independent life is established. Jack and Milner (1975) have shown that glucocorticoids influence the development of insulin secretion in the fetal rabbit. The fetal lamb is able to respond to a glucose challenge with only a poor secretion of insulin but this increases at term or after ACTH

administration (Liggins, 1976). Rall, Pictet, Githens and Rutte, 1977 suggest that the glucocorticoids are important for the development of the exocrine pancreas in the fetal rat. They showed that these caused an increase in the enzyme levels of various pancreatic enzymes including amylase, chymotrypsinogen and the procarboxy peptidases A and B as well as increasing the numbers of zymogen granules present in the rough endoplasmic reticulum.

The gradual rise in fetal plasma glucocorticoid concentration seen towards the end of gestation is responsible for the induction of phenylethanolamine N-methyl-transferase, which converts noradrenaline to adrenaline in the adrenal medulla, in the rat (Roffi, 1971) providing an increase in the amount of β catecholamine activity in the newborn. In larger animals the increase in plasma glucocorticoid concentration seen before term has also been shown to alter the peripheral metabolism of thyroid hormones so that a greater proportion of the plasma T_4 is converted to T_3 in preparation for the increase in metabolic rate required by the newborn (Thomas, Krane and Nathanielsz, 1978; Osathanondh, Chopra and Tulchinsky, 1978).

There is a marked difference between VARIOUS SPECIES in the role of glucocorticoids in the timing of parturition. Fetal glucocorticoids do not have a precise role to play in the timing of parturition in small animals although their presence is necessary for degenerative changes in the placenta (Wellman and Volk, 1972). In contrast, the increase in fetal plasma glucocorticoid secretion seen in some large animals such as the sheep or goat appears to initiate the mechanisms of parturition. Cortisol or ACTH infusion to the fetus is able to cause premature delivery and ACTH replacement after fetal hypophysectomy is able to initiate parturition in an animal whose gestation would otherwise be prolonged for up to 30 days beyond normal term. The slow rise in plasma cortisol concentration seen before delivery is necessary for the placenta to switch from progesterone to oestrogen biosynthesis and for the degenerative changes to take place in preparation for placental separation at birth (Liggins, Fairclough, Grieves, Kendall and Knox, 1973).

Some glucocorticoid dependent changes also rely on a functioning thyroid axis. It is important that the lungs are mature at birth

so that the newborn animal is able to establish adequate ventilation. Glucocorticoids are important in this development in both large and small animals. Buckingham, McNary, Sommers and Rothschild (1968) first suggested that rabbit lung maturation was under glucocorticoid control. Liggins (1969), observed that lambs born prematurely after fetal glucocorticoid administration were viable and not suffering from respiratory distress. Administration of thyroid hormones stimulates lung development in rabbits (Kikkawa, Orzalesi, Motoyama, Kaibara, Zigas and Cook, 1973) and in rats (Morishige, Joun and Guernsey, 1982). Both glucocorticoids and thyroid hormones cause the development of the more mature type II epithelial cells and accelerate the appearance of pulmonary surfactant and increased activity of alkaline phosphatase. This improves the stability of the lungs after inflation hence reducing the amount of effort required to keep them inflated. The recent evidence of Morishige,(1982)indicated that T_4 is able to increase the number of glucocorticoid receptors in the lung in the newborn rat. Thus T_4 may exert its effects by increasing the lung responsiveness to glucocorticoids rather than having a direct action of its own.

In small animals such as the rat and the rabbit much development takes place postnatally. Thyroid hormones and glucocorticoids are required for many of these changes. Some of the most important changes occur in the gut. The rough endoplasmic reticulum and the granules of the chief cells in the stomach are increased by glucocorticoids in the rat (Tatematsu, Takahashi, Tsuda, Hirose, Furihata and Sugimura, 1975) as well as the induction of pepsinogen (Furihata, Kawachi and Sugimura, 1972). The maturation of the intestinal enzymes and the development of the glands and the villi are dependent on a functioning adrenal. Intestinal enzymes induced by glucocorticoids include intestinal invertase (Doell and Kretchmer, 1964), sucrase (Moog, 1971) and maltase (Moog, 1979). Salivary and pancreatic amylase are dependent on both T_4 and B for their induction (Kumegawa, Maeda, Yajima, Takuma, Ikeda and Minamide, 1980; Kumegawa, Maeda, Yajima, Takuma, Ikeda and Hanai, 1980). In young rats the gut cells are 'open' and allows the passage of large macromolecules across it (Halliday, 1955). In this way immunity can be transferred passively from the mother to the neonate. At weaning, i.e. at about 21 days post-partum, the gut closes

and macromolecular uptake is no longer possible. This change can be induced prematurely with glucocorticoid administration (Daniels, Hardy, Malinowka and Nathanielsz, 1973) and with T_4 (Chan, Daniels and Thomas, 1973). Malinowka, Chan, Nathanielsz and Hardy (1974) suggest that this action of T_4 probably involves the adrenal cortex and may be another example of T_4 increasing tissue responsiveness to glucocorticoids.

Thyroid hormones themselves are essential for proper growth, brain maturation, bone ossification and the establishment of thermo-regulation. In an animal with a long gestation, e.g. the sheep, fetal thyroidectomy at day 81-96 leads to a 33% decrease in body weight at delivery (Hopkins and Thorburn, 1972). However, hypothyroid human babies usually have a normal body weight at term (Anderson, 1960). The effect of surgical thyroidectomy in small animals with a short gestation, e.g. the rat, is difficult to evaluate since there is not sufficient time between surgery and delivery for tissue hormone concentrations to fall substantially due to the long half-life of T_4 and its prolonged cellular actions. Lack of T_4 has a marked effect on bone maturation. In hypothyroid human fetuses dysgenesis of ossification centres could be observed as early as the 7th month of gestation (Wilkins, 1941). Ossification was shown to occur regularly and to proceed from multiple interspersed islets of cartilage rather than from a concentric centrally located zone of ossification. Similar patterns were observed in animals thyroidectomised *in utero*. Hopkins and Thorburn (1972) demonstrated a delay in bone maturation in lambs made hypothyroid. Hamburgh, Lynn and Weiss (1964) stated that rats made hypothyroid using thiouracil administered to the mother had normal ossification at birth. This may be because the degree of hypothyroidism achieved was insufficient. Geloso, Hemon, Legrand, Legrand and Jost (1968) suggested that thyroid hormones were important in fetal rat bone development since fetuses decapitated *in utero* showed marked retardation of bone development. Some of the effects of surgical thyroidectomy in larger animals may however result from the loss of parathyroid hormone or calcitonin.

Thyroid hormones have important actions on the development of the skin. Chapman, Hopkins and Thorburn (1974) showed that the earlier thyroidectomy was performed in the lamb fetus the

greater was the retardation of the development of the primary wool follicles, sebaceous glands and sweat glands. T_4 administration accelerated the development of all skin components. Infusions of epidermal growth factor (EGF) for 3 to 10 days into lamb fetuses at 115 days (term 147 days) caused an increase in skin wrinkling, hypertrophy of the cells in the sebaceous glands, follicular sheath and sweat gland ducts and a shedding of wool fibres (Thorburn, Waters, Young, Dolling, Buntine and Hopkins, 1981). EGF infusions also caused a fall in fetal plasma T_4 concentrations suggesting a link between the control of the secretion of thyroid hormones and EGF.

Thyroid hormones have a critical phase of influence on brain development in the rat (Eayrs, 1961). The effect of neonatal thyroidectomy becomes less marked as the animal gets older and if it is delayed until after day 25 there is no detectable difference in brain development (Eayrs, 1961). If T_3 replacement therapy is used it is totally successful if given from day 10 but has no effect if delayed until after day 25 (Eayrs, 1961). Hypothyroid neonatal rats show a decrease in axonal development and dendritic arborisation (Eayrs, 1955), a decrease in total brain protein synthesis (Geel, Valcana, and Timiras, 1967) and a decrease in average cell mass(Pasquini, Kaplun, Garcia-Argiz and Gomez, 1967). T_4 administration restored the levels of succinate dehydrogenase and GABA aminotransferase activity in the rat cerebral cortex after thyroidectomy (Gomez, 1971). Recently it has been suggested that the thyroid may exert some of its actions through increasing the levels of Nerve Growth Factor (NGF) and T_4 administration to adult mice increases NGF concentrations in the cerebral cortex, cerebellum and brain stem. There are problems with this hypothesis however since propylthiouracil (PTU) an inhibitor of thyroid hormone secretion and action, does not reduce the levels of NGF (Walker, Weichsel, Fisher, Guo and Fisher, 1979).

Thyroid hormones are important in the establishment of thermoregulation in the newborn mammal. In utero the fetus is in a protected environment and relies on the mother for the maintenance of a stable body temperature. Once independent life is established the newborn animal must be capable of maintaining its own body temperature. Thyroid hormones are important in establishing many

mechanisms in non-shivering thermogenesis. The increase in T_4 secretion after birth and the enhanced conversion to the more metabolically active T_3 which occurs at this time are important in the stimulation of basal metabolic rate. In the rat there is a period of thermoregulatory incompetence after birth when the young animal relies heavily on the mother for warmth. From day 8 there is an improvement and independent thermoregulation is fully established by day 21. T_4 treatment of the young rat can accelerate the establishment of adequate thermoregulation and treatment with PTU can delay it (Steele and Wekstein, 1972).

Thus, the development of the thyroid and adrenal axes are of interest because of their profound influence on the development of other organs. A functioning adrenal cortex is essential for the newborn to survive the first few days of life (Balfour, 1962). The rat adrenal in utero appears to be very active. The fetal rat adrenal is capable of maintaining maternal plasma B concentration after the mother has been adrenalectomised (Kamoun, 1970), and normally causes the rise in maternal plasma B concentration seen towards the end of gestation (Kamoun, 1970). The fetal rat adrenal is capable of responding to noxious stimuli with a decrease in adrenal ascorbate (Milkovic and Milkovic, 1961). In contrast, Jailer (1950) observed that 4 day old rats did not respond to convulsive doses of ADH and adrenaline. Schapiro, Geller and Eiduson, (1962) defined this period as the stress non-responsive period (SNR period) and postulated that the lack of response was a result of brain immaturity. When more sensitive assay techniques became available, Zarrow, Haltmeyer, Denenberg and Thatcher (1966) showed that 2 day old rats responded to stressful stimuli with an increase in total plasma B concentration whilst 9 day old rats did not. Haltmeyer, Denenberg, Thatcher and Zarrow,(1966) and Levine, Gluck and Nathane (1967) were able to demonstrate that rats from day 4 to day 11 post-partum were stress non-responsive.

Brain immaturity now seems an unlikely reason for the SNR period as the fetus and a 1 or 2 day old rat are able to respond to stressful stimuli. Thus, the aim of this work was to examine the development of glucocorticoid function in the neonatal rat,

and to investigate the responses to agents which stimulate the release of B, by acting at different levels of the hypothalamic-pituitary-adrenal axis.

Concentrations of hormones were measured using modern radio-immunoassay techniques which are more specific and more sensitive than techniques used previously; care was taken to validate the techniques used. The % free B (ie the proportion not bound to plasma protein) was measured using a modification of the method of Pearlman and Crepy (1967). This technique, like all others, overestimates the % free hormone, as described in detail on page 182. However, these measurements, together with the measurements of the total plasma B concentration were used to indicate that the circulating concentration of unbound, active hormone does not decrease during the period from day 1 to day 21 post-partum.

Thyroid hormones are known regulators of CBG synthesis in the adult rat and Schapiro and Norman (1967) have shown that T_4 treatment abolishes the SNR period in the young rat. For these reasons the effects of thyroid hormones and antithyroid drugs were investigated on the binding of B in the neonatal period. The assessment of the free active B concentration between birth and weaning in the young rat may lead to a better understanding of the physiological role and the development of the adrenal gland in this period.

METHODS

1. Care of the Animals

From day 14 of gestation pregnant rats were housed in individual cages. They were kept on a 16 hours light, 8 hours dark lighting regime. They were fed ad libitum on laboratory chow. The day of birth was noted (gestation 21-22 days) and this was designated as day 1 post-partum.

2. Materials

All solvents, acids and buffer reagents were of a high analytical grade. Other chemicals were obtained from the following sources:-

Amberlite Resin (IRA-400 Chloride Form 14-52 Mesh)	Hopkins and Williams Ltd.
2,2' azino-di(3-ethyl benz-thiazoline sulphonic acid) (ABTS)	Sigma
Bisbenzimide	Sigma
Bovine serum albumin (BSA)	Sigma
Calf Thymus DNA	Sigma
Charcoal Norit A	BDH Chemicals Ltd.
Charcoal Norit GSX	Hopkins and Williams Ltd.
Coomassie Brilliant Blue G-25	BDH Chemicals Ltd.
Corticosterone-21-acetate-3-CMO:BSA	Steraloids Ltd.
Dexamethasone(Decadron Shock Pack 20 mg/ml)	Merck, Sharp and Dohme Ltd.
Dextran T-70	Pharmacia Fine Chemicals Ltd.
Florisil (60-100 US Mesh)	BDH Chemicals Ltd.
Freund's Complete adjuvant	Difco Laboratories Ltd.
Gelatin (Swine skin, G-2500)	Sigma
Glucose	BDH Chemicals Ltd
Glucose oxidase	Sigma
Hydrocortisone 21-hemisuccinate	Sigma
Hydrocortisone Sodium Succinate (100 mg/Ampoule)	Organon
Perioxidase	Sigma
Propylthiouracil	Sigma

Reverse Tri-iodothyronine	Calbiochem.
Sephadex G-25 superfine G-100 superfine	Pharmacia Fine Chemicals Ltd.
Steroids	Sigma
^{3}H steroids	Amersham International
Thyroxine	Sigma
Tri-iodothyronine	Sigma
^{125}I Thyroxine	Amersham International

Scintillation Fluid

For all β counting, tritoscint was used as the scintillant. It was made to the following recipe:-

667 mls Xylene (puriss)	Koch-Light Labs.
4 g Diphenyloxizole (PPO) (scintillation grade)	Int. Enzymes
0.5 g Dimethyl bis phenyl oxazoyl benzene	Cambrian Chemicals
333 mls Synperonic NXP (scintillation grade)	ICI

Krebs Ringer Bicarbonate

Stock solutions of the following were prepared:-

sodium chloride	82.6 g/500 mls
calcium chloride	3.32 g/500 mls
potassium dihydrogen phosphate	1.94 g/500 mls
magnesium sulphate	7.16 g/500 mls
potassium chloride	4.22 g/500 mls

50 mls of each stock solution were taken and mixed together. The calcium chloride was added last slowly to avoid precipitation. Finally 2.0 g of sodium bicarbonate was added and the solution was made up to 1 litre. The solution was then gassed with 95% O_2:5% CO_2 until the pH was 7.4.

3. Radioimmunoassay for Total Plasma Corticosterone (B) Concentration in Rat Plasma

Total plasma B concentrations were measured using a specific radioimmunoassay. A first supply of antiserum to B was obtained from Dr G. Reed of the Tenovus Institute of the Welsh National School of Medicine and a second antibody was raised by the immunisation of rabbits in the laboratory. The basis of the assay was adapted from the method described by Fahmy, Reed and Hillier, (1975) for the assay of cortisol in human plasma.

Immunisation of Rabbits

The immunogen, corticosterone-21-acetate bound via a 3-O-carboxymethyloxime linkage to bovine serum albumin (B-21-acetate-3-CMO:BSA), was emulsified in a mixture of Freund's complete adjuvant and saline (3:1 v/v). 4 mg of the protein-steroid conjugate was injected subcutaneously into each of six New Zealand White rabbits at multiple sites along the back. Blood samples were taken from the marginal ear vein 50 days after primary immunisation and 14 days after subsequent immunisations.

For both antisera, the assessment and validation of the assay included:- determination of B antibody titres, an estimation of the cross-reactivity with related steroids, a demonstration of parallelism and the determination of the recovery of the assay. Each of these parameters was checked using the assay system described below.

Assay Buffer and Dextran/Charcoal Mix

The assay buffer was 0.01 M sodium phosphate and 0.15 M sodium chloride, pH 7.4 with gelatin (0.1% w/v). The dextran/charcoal suspension was prepared by dissolving 50 mg of dextran in 200 ml of assay buffer, 500 mg of charcoal (Norit GSX) was then added to the buffer and stirred continuously to form a fine suspension. This suspension was stirred, over ice, for at least one hour before it was used.

Standard B Solutions

A B standard solution of 100 ng/ml in ethanol was stored at 4°C until required. A solution of 10 uCi/ml of ^{3}HB in ethanol was prepared from the stock solution in toluene and ethanol and stored at 4°C. This solution was diluted with assay buffer before each assay to give a final concentration of approximately 20 000 cpm/100 ul.

Assay Procedure

Standards were prepared by diluting the 100 ng/ml solution of B in ethanol to give a series of Lp3 tubes (in duplicate), containing 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 ng of B per tube, each in 100 ul of ethanol. The same volume of ethanol was added to 2 further tubes to form a zero standard, thus measuring the total amount of antibody bound ^{3}HB. 2 tubes were used to determine the total amount of ^{3}HB added and 2 more tubes were prepared without antibody to act as non-specific binding tubes.

Plasma samples were extracted (in duplicate) in 3 times their volume of ethanol, vortexed for 30 seconds and the protein precipitated by centrifugation for 20 minutes at 2 500 rpm at 4°C. Half of the ethanolic supernatant was transferred into an assay tube. The standard solutions and the ethanolic extracts of plasma samples were evaporated to dryness under compressed air at 40°C. The optimum antiserum dilution was prepared, and 100 ul was added to each tube with 100 ul of buffer except for the total and non-specific tubes which each received 200 ul of buffer only. After vortexing briefly the tubes were allowed to stand in a water bath for 30 minutes at 30°C. 100 ul of ^{3}HB solution was then added to all tubes and after vortexing briefly again the tubes were returned to the water bath and incubated for a further hour at 30°C. At the end of the incubation the tubes were placed on ice for 15 minutes, then 0.5 mls of dextran/charcoal was added to all tubes except the totals. The tubes were vortexed briefly and allowed to stand on ice for a further 15 minutes before centrifugation at 4°C for 10 minutes at 2 500 rpm. The supernatant was decanted into a scintillation vial, 7 mls of tritoscint was added and the vials were counted in a Beckman LS 7000 liquid scintillation counter to within a 2% counting error and at a tritium efficiency of 56%.

Initially the standard curves were constructed by plotting the counts bound expressed as a % of the total counts added against the amount of cold B in the standard tubes. For later routine use a Hewlett Packard computer programme, involving a logit transformation, was used (Rodbard and Lewald, 1970).

Plasma samples containing 0.5 ng/ml of B were extracted and run through each assay to monitor the inter-assay variation and to act as a quality control for individual assays.

Validation

Fig. M.1 shows the antibody dilution curves at zero B concentration for each of the 6 rabbits bled 50 days after the primary immunisation. Rabbit D had produced the best antiserum and so was further immunised and bled 14 days later. Fig. M.2 shows 4 point standard curves for antiserum dilutions of 1:100, 1:500, 1:1000 and 1:10 000. This suggests that the optimum antiserum dilution for use in the assay is 1:500. Fig. M.3 shows 2 point standard curve for the Reed antiserum at dilutions 1:100, 1:500, 1:1000 and 1:10 000 suggesting a similar optimum dilution of 1:500 (representing a final antiserum dilution in the assay tube of 1:1500 before dextran/charcoal separation). Fig. M.4 shows the full standard curve for both antisera. The antiserum from rabbit D (antiserum 2) has a useful range of 0.1 to 10.0 ng per tube and the Reed antiserum (antiserum 1) has a useful range of 0.05 to 10.0 ng per tube under the assay conditions.

The cross reactions for each antiserum were determined for a variety of related steroids. The % cross reaction with B was assessed at concentrations of 1 ng and 10 ng per tube for each steroid. The cross reaction was low in each case (Table M.1).

This low cross reaction was further confirmed by demonstrating parallelism for plasma and extracted B with both antisera using the following procedure. 1 ml of rat plasma was extracted with 4 mls of ethanol. The ethanol extract was diluted 1:10 and the following volumes were aliquoted:- 100 ul, 200 ul and 300 ul. The standard curve for B and the standard curve generated by the plasma extracts are parallel (Figs. M.5 and 6) indicating that the antisera are measuring only B in the plasma extract.

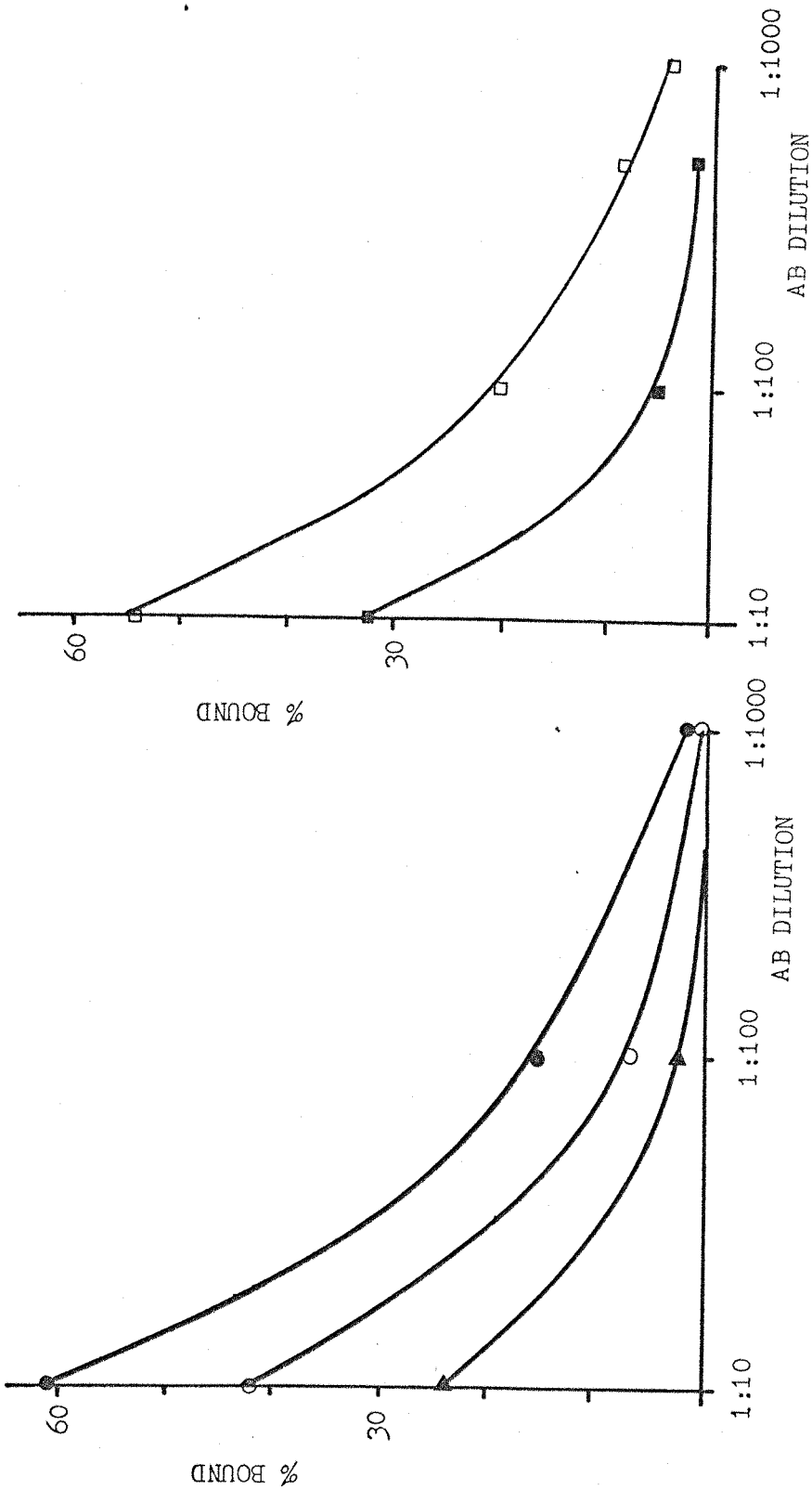


Fig. M.1. Ab dilution curves at zero B concentration. Rabbit A (○—○), Rabbit B (●—●), Rabbit D (□—□), Rabbit E (▲—▲) and Rabbit F (■—■).

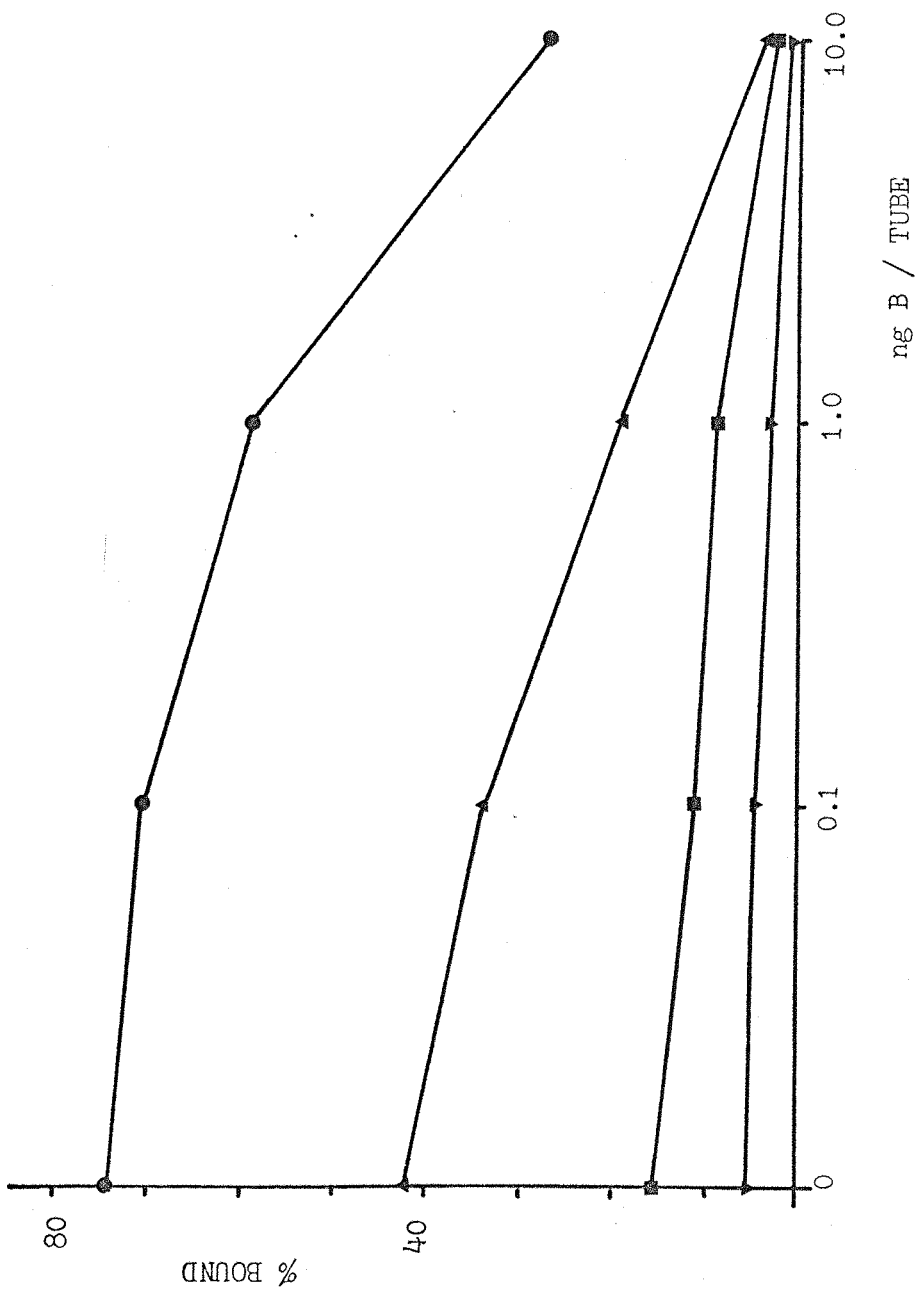


Fig. M.2. 4 point standard curves at Ab dilution 1:100 (●—●), 1:500 (▲—▲), 1:1000 (■—■) and 1:10 000 (▼—▼) for rabbit D antibody.

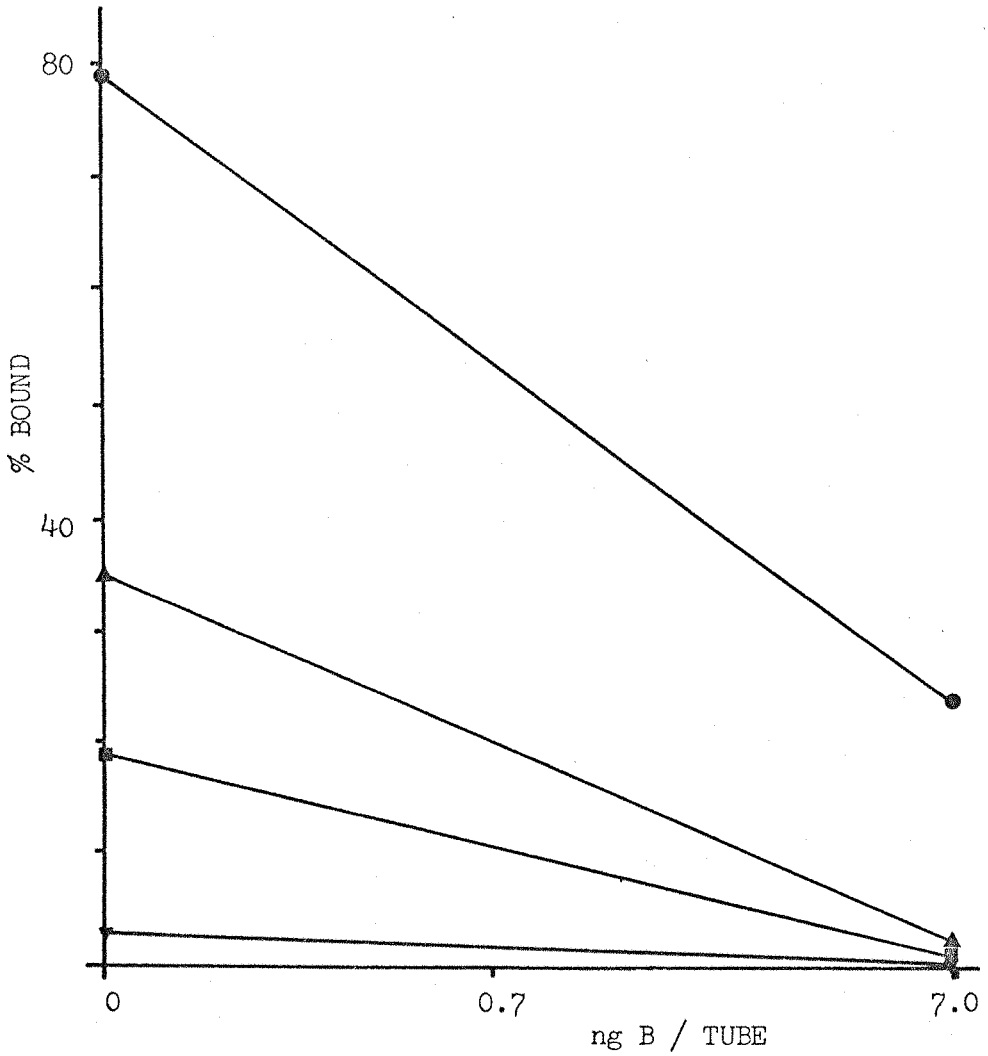


Fig. M.3. 2 point standard curves at Ab dilution 1:100 (●—●), 1:500 (▲—▲), 1:1000 (■—■) and 1:10 000 (▼—▼) for Reed antibody.

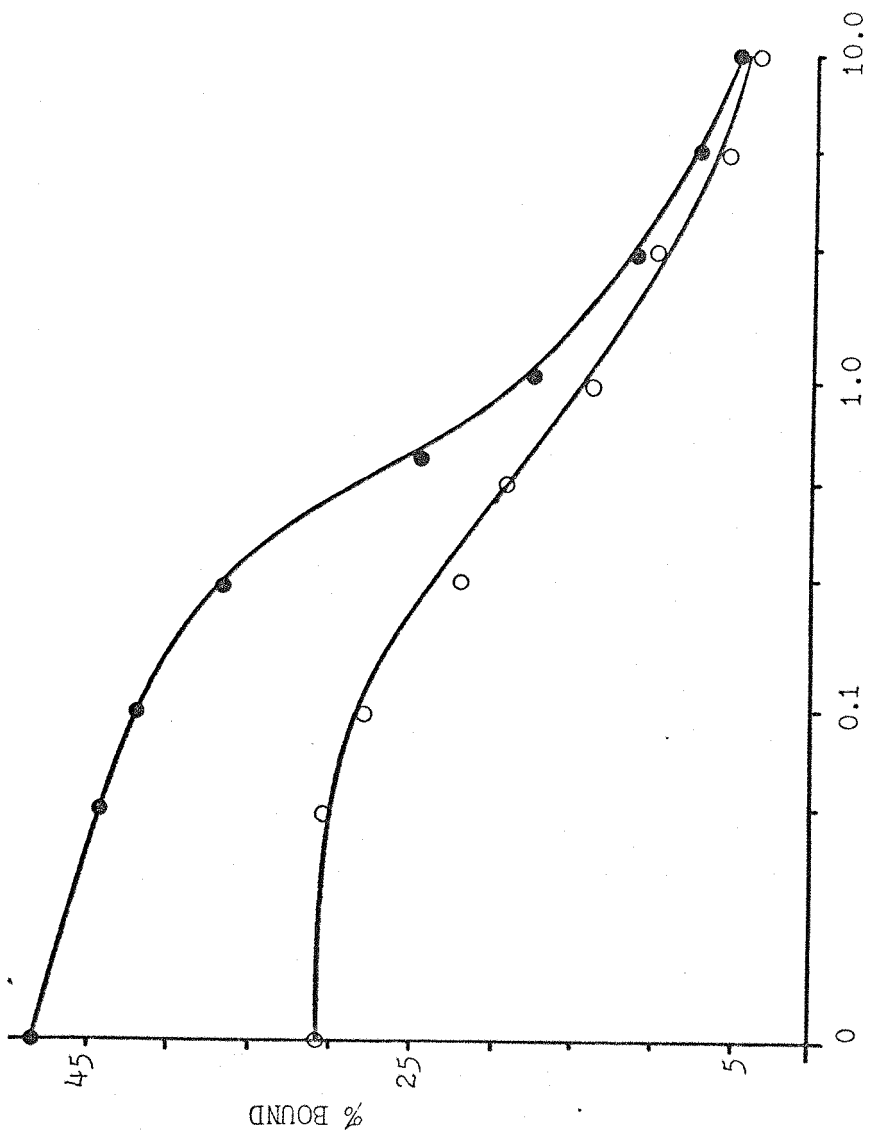


Fig. M.4. Full standard curves for the Reed antibody, Ab 1, (●—●) and for the antibody from rabbit D, Ab 2, (○—○).

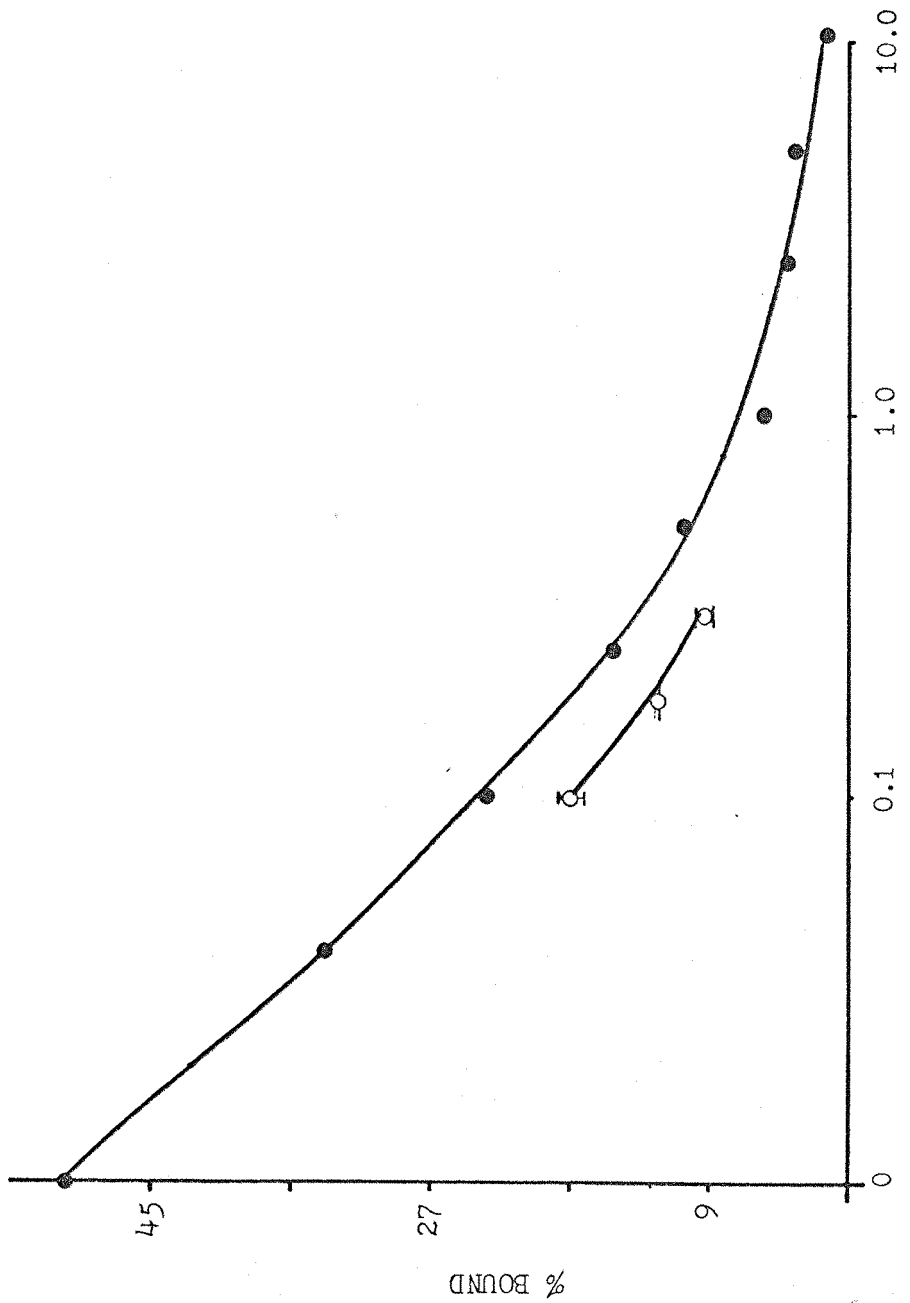


Fig. M.5. Parallelism between the standard curve of Ab 1 (●—●) and extracted plasma (○—○).

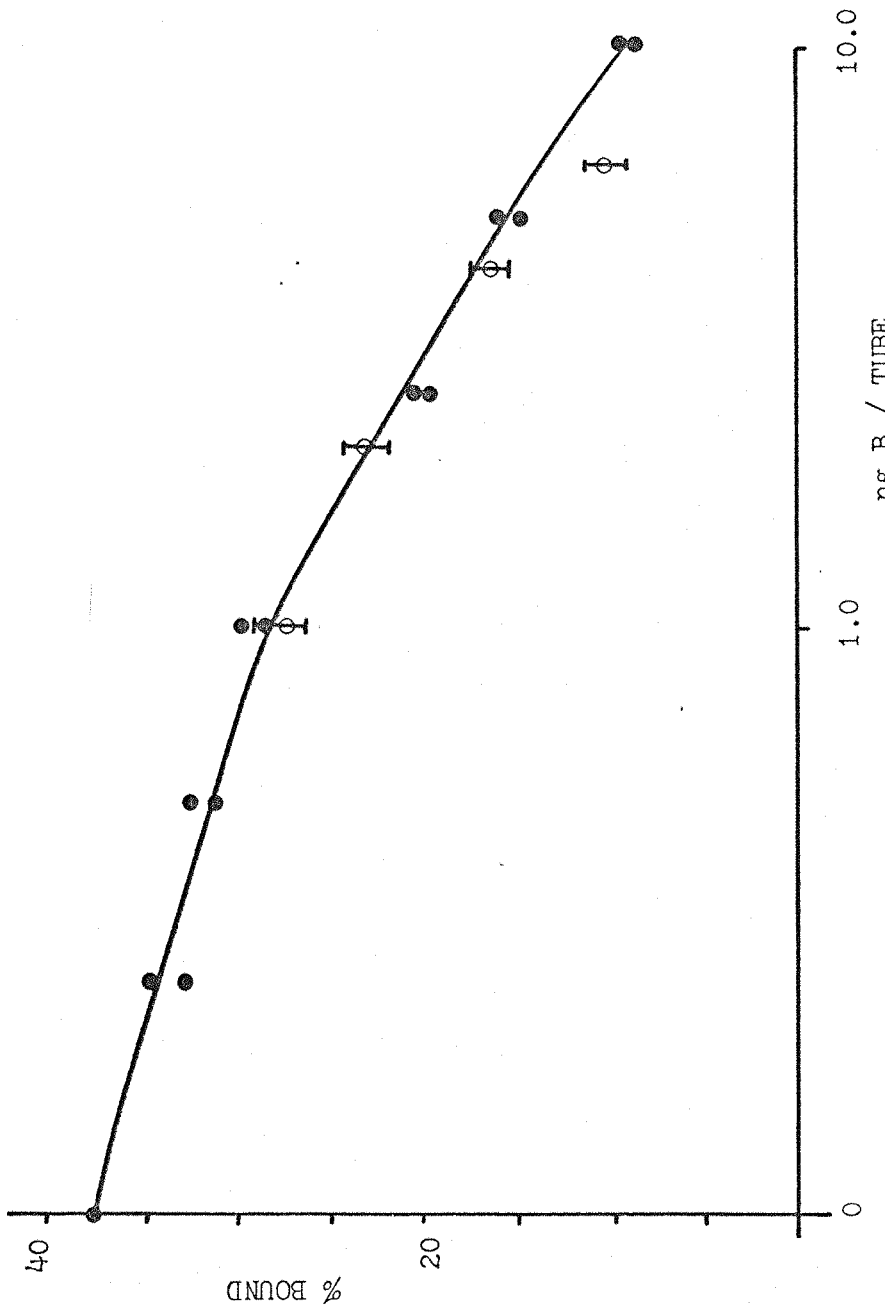


Fig. M.6. Parallelism between the standard curve of Ab 2 (●—●) and extracted plasma (○—○).

Table M.1: % Cross-Reaction

	Antiserum 1 ng steroid/tube		Antiserum 2 ng steroid/tube	
	10.0	1.0	10.0	1.0
Corticosterone	100	100	100	100
Cortisol	<0.5	< 0.1	< 1.0	< 0.1
Deoxy-corticosterone	3.3	< 0.1	5.6	< 0.1
Estradiol	< 0.1	< 0.1	< 0.1	< 0.1
Testosterone	< 0.1	< 0.1	< 0.5	< 0.5
Progesterone	2.0	< 0.1	< 1.0	< 0.5

The synthetic glucocorticoid, dexamethasone has a cross reaction of<0.2% at a concentration of 200 ug/tube with antiserum 1

The recovery of the assay was estimated by extracting and assaying plasma samples containing added B. The recovery was 84% for antiserum 1 and 103% for antiserum 2. The lines obtained from the 2 antisera were similar (Fig. M.7). The intra-assay coefficient of variation was determined for each antiserum using 10 determinations of a plasma sample which contained 0.5 ng per tube of B. The result for antiserum 1 was 14% and for antiserum 2 was 11%. From the intra-assay coefficients of variation it was possible to determine the sensitivity of the assay (i.e. the smallest amount of B it is possible to distinguish significantly from zero). The sensitivity will be equal to tS/N, where t = Student's t value at the level of probability of 0.05, S = standard deviation of the observation and N = number of degrees of freedom (Frankel, Cook, Graber and Nalbandov, 1967). For antiserium 1 the sensitivity is 0.05 ng/tube and for antiserum 2 the sensitivity is 0.07 ng/tube. The inter-assay coefficient of variation was determined from the quality control tubes from individual assays. This was 18% at 0.05 ng/tube for antiserum 1 and 17% for antiserum 2.

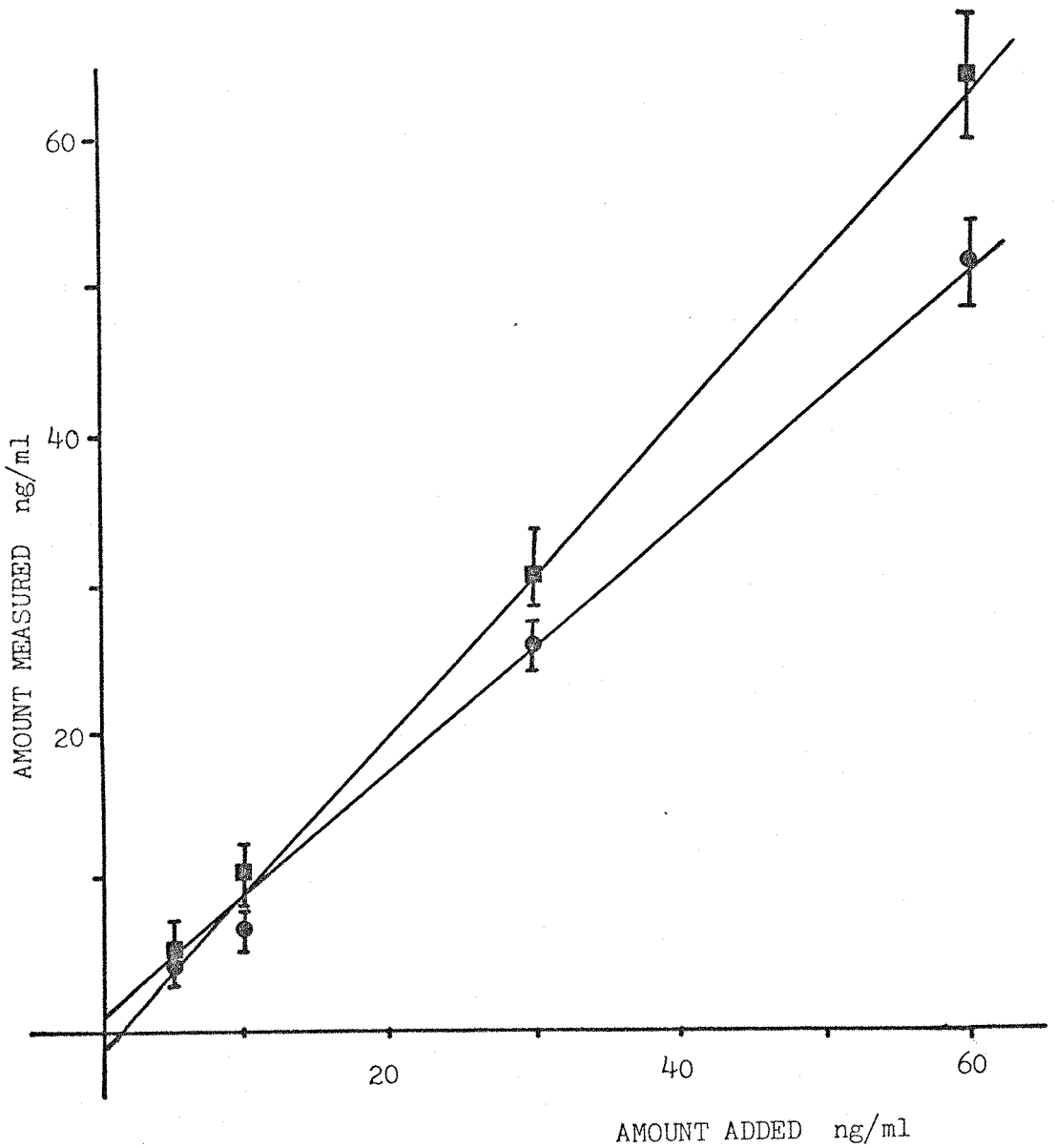
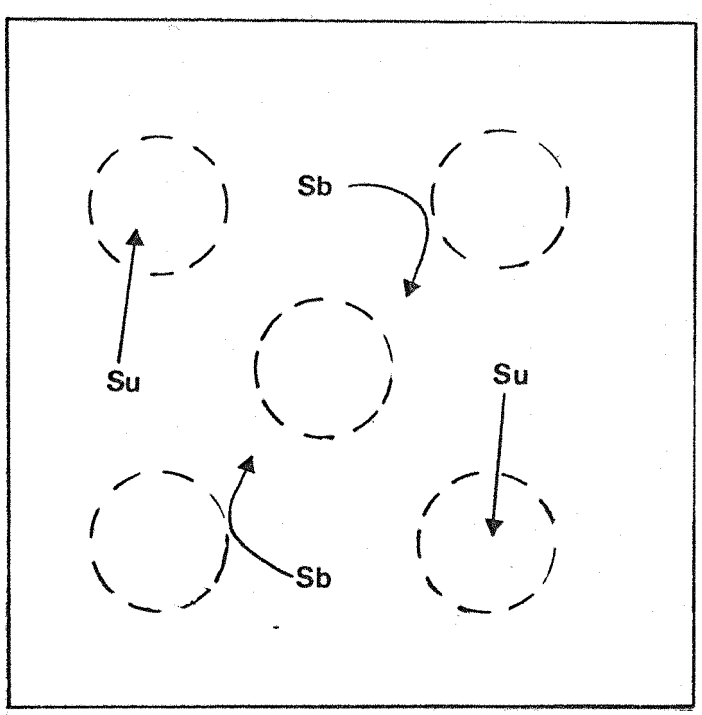
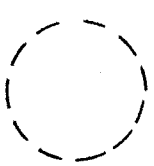


Fig. M.7. Recovery of the assay for each antibody.
Ab 1 (●—●); slope 0.84, intercept 0.25, R^2 0.98
Ab 2 (■—■); slope 1.03, intercept -1.5, R^2 0.96

4. Measurement of Plasma Binding of B

The binding of B to corticosterone binding globulin (CBG) was assessed using a modification of the method developed by Pearlman and Crepy, 1967. This method is based on the principle of equilibrium dialysis but uses the cross-linked dextran Sephadex G25 in place of the dialysis membrane as the means of separating the protein bound and free steroid. The measurement is made at equilibrium, making use of the major advantage of conventional dialysis methods but equilibrium is achieved very rapidly because of the large surface area provided by the Sephadex beads. The short equilibration time means that there is no bacterial growth, a common source of error in long dialyses. The basic principle of the method is illustrated in the diagram below.





= Sephadex bead , 20-50 um

inside bead = internal volume
outside bead = external volume

Su = unbound steroid

Sb = bound steroid

The assay was assessed and validated using the method described below:-

Assay Buffer, Dextran/Charcoal, ^{3}H B Standard Solution

The assay buffer was 0.15 M sodium phosphate pH 7.4. The dextran/charcoal suspension was prepared as described previously except the suspension was stirred at room temperature. A 10 uCi/ml solution of ^{3}H B in ethanol was prepared from the stock solution in toluene and ethanol and diluted with assay buffer when required to give a final concentration of 50 000 cpm/100 ul.

Assay Procedure

200 mg of Sephadex was placed in a 6 ml capped plastic sample tube, using a calibrated spoon, with 1 ml of assay buffer. The tubes were vortexed briefly and the Sephadex was allowed to swell overnight. 200 mg of Sephadex G25 superfine absorbs 0.5 ml of buffer thus giving an internal volume of 0.5 ml and an external volume of 0.5 ml. The following day the external volume was made up to 1.5 ml by the addition of buffer, ^{3}H B and plasma which had been stripped of endogenous steroid. 2 tubes were used, containing no Sephadex to determine the total number of counts added and 4 tubes, containing no plasma were used to assess the distribution of ^{3}H B between the internal and external volume of the Sephadex in the absence of protein (K'). After the tubes had been recapped they were shaken for 5 mins at room temperature before centrifugation at 1000 rpm for 5 mins. The Sephadex was then allowed to settle for a further hour before 250 ul of the external volume was removed and transferred to a scintillation vial containing 10 ml of tritoscint and 750 ul of water. The samples were then counted in a Beckman LS7000 liquid scintillation counter.

Calculation

The distribution factor K' in the absence of protein equals:-

$$K' = \frac{x}{a - x} \qquad \text{Equation 1}$$

where a = total radioactivity present

x = radioactivity present in the external volume

In the absence of protein x equals the unbound steroid, Su, in the external phase

$$Su = K'(a - x) \qquad \text{Equation 2}$$

But, in the presence of protein, x equals Su + Sb(bound steroid).

Therefore

$$Sb = x - Su \qquad \text{Equation 3}$$

Substitute for Su (equation 2)

$$Sb = x - K'(a - x) \qquad \text{Equation 4}$$

From equations 2 and 4

$$\frac{Su}{Sb} = \frac{K'(a - x)}{x - K'(a - x)} \qquad \text{Equation 5}$$

and % free steroid:-

$$\frac{\frac{Su}{Sb}}{1 + \frac{Su}{Sb}} \times 100 \qquad \text{Equation 6}$$

All results were calculated using a PET computer programme.

Assessment and Validation of Assay

The assessment and validation of the assay included an estimation of the internal and external volumes of the Sephadex under assay conditions, a determination of the optimum shaking time, the effect of different concentrations of cold B on K', the optimum time for removal of endogenous steroid by dextran/charcoal, the effect of different volumes of plasma on Su/Sb, the effect of different label concentrations on Su/Sb and, finally a comparison of the method with conventional equilibrium dialysis.

The internal and external volumes of Sephadex were calculated using blue dextran and ^{3}H inulin. Both these reagents gave a value of 0.5 ml for the internal volume and 1.5 ml for the external volume when 200 mg of Sephadex was swelled in 2.0 ml of buffer. The equilibrium shaking time was found to be 5 mins (Fig. M.8). The value of K' was found to vary with the amount of unlabelled B present in the system (Fig. M.9) and as a result the B present in the plasma sample was removed using dextran/charcoal before assay. The minimum time required to remove the steroid from the plasma was determined using ^{3}H B and ^{3}H progesterone. All of the ^{3}H B added to a plasma sample was removed by incubating with dextran/charcoal for 20 mins at 37°C (Fig. M.10), and all of added ^{3}H progesterone (which is also bound to CBG) is removed within 60 mins (Fig. M.11). All samples were therefore incubated with the dextran/charcoal mixture for one hour before assay.

The value of Su/Sb measured decreased with increasing plasma volume (Fig. M.12). A volume of 60 ul of plasma was chosen for routine assay use, and a ^{3}H B concentration of 0.1 uCi per tube was also chosen (Fig. M.13). As a result of these preliminary experiments the optimum conditions for the assay were determined and are shown in Fig. M.14. The intra-assay coefficient of variation was determined from 10 duplicate determinations in a sample of human plasma and was found to be 5%. The inter-assay coefficient of variation was calculated from the 2 quality controls in all the assays and was 21%. Finally this method was compared with conventional equilibrium dialysis, using 18/32 visking dialysis tubing at 37°C, for a number of samples of rat plasma and was shown to be in close agreement, (Fig. M.15).

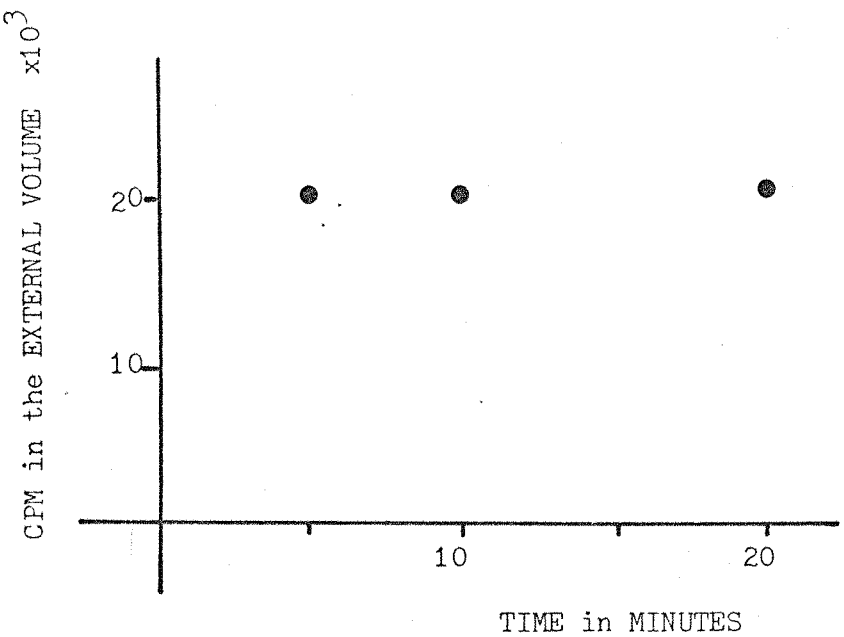


Fig. M.8. Counts per minute in external volume with shaking time.

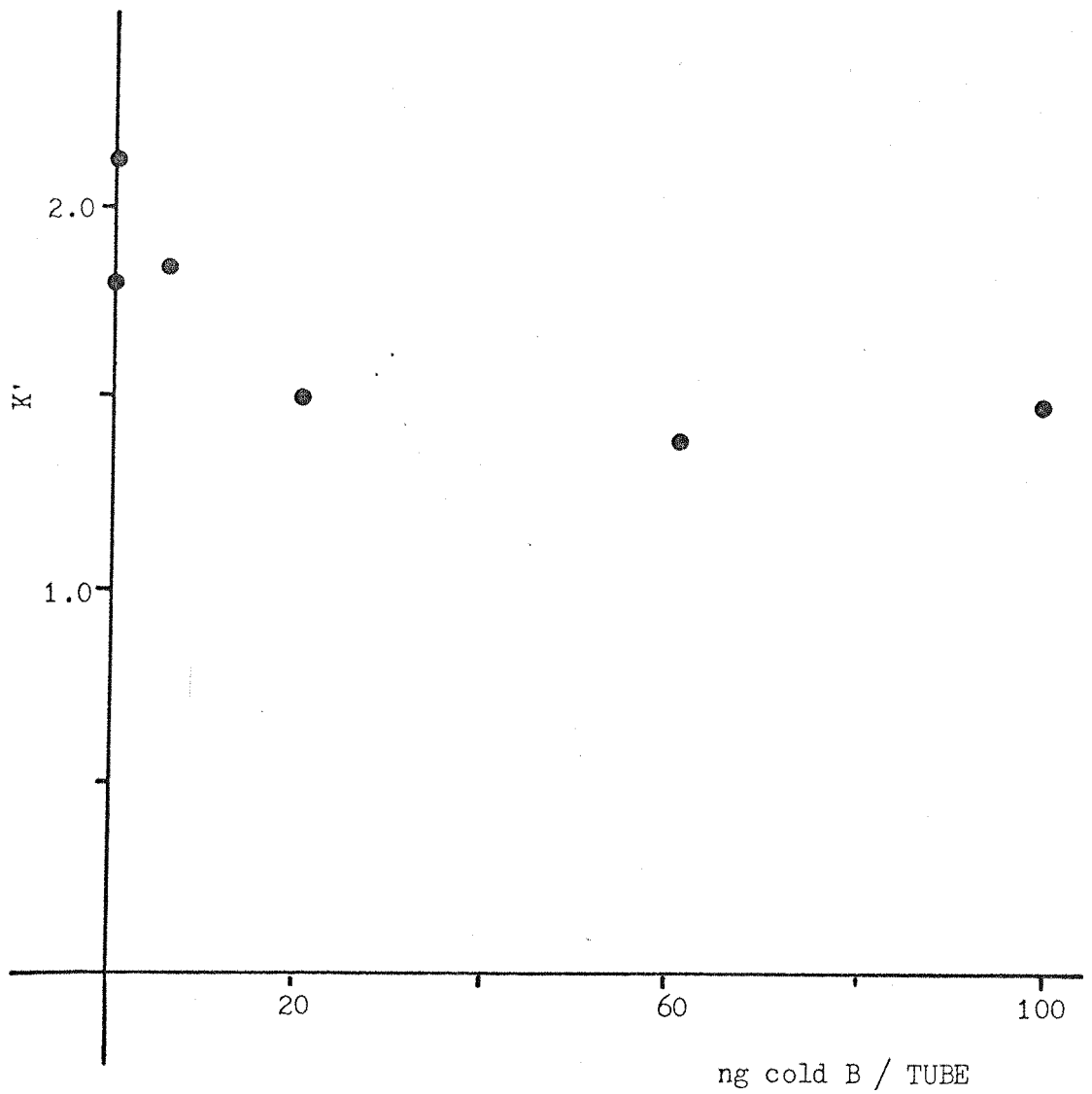


Fig. M.9. The effect of cold B added to the assay system on K', the partition factor.

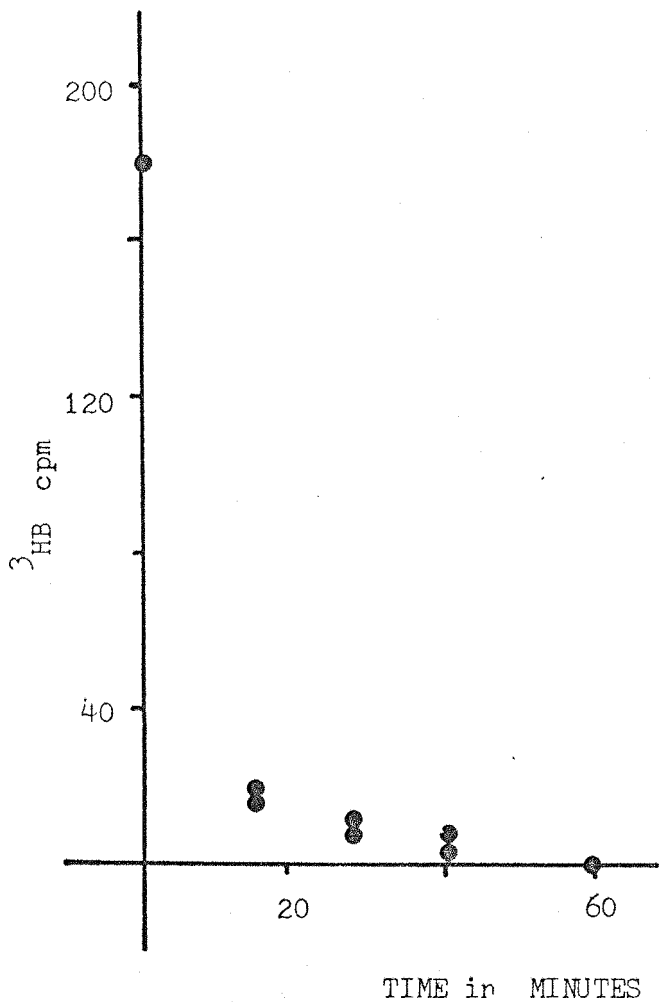


Fig. M.10. The removal of ^{3}HB from plasma by dextran/charcoal incubation. (Total counts added = 3500 cpm.).

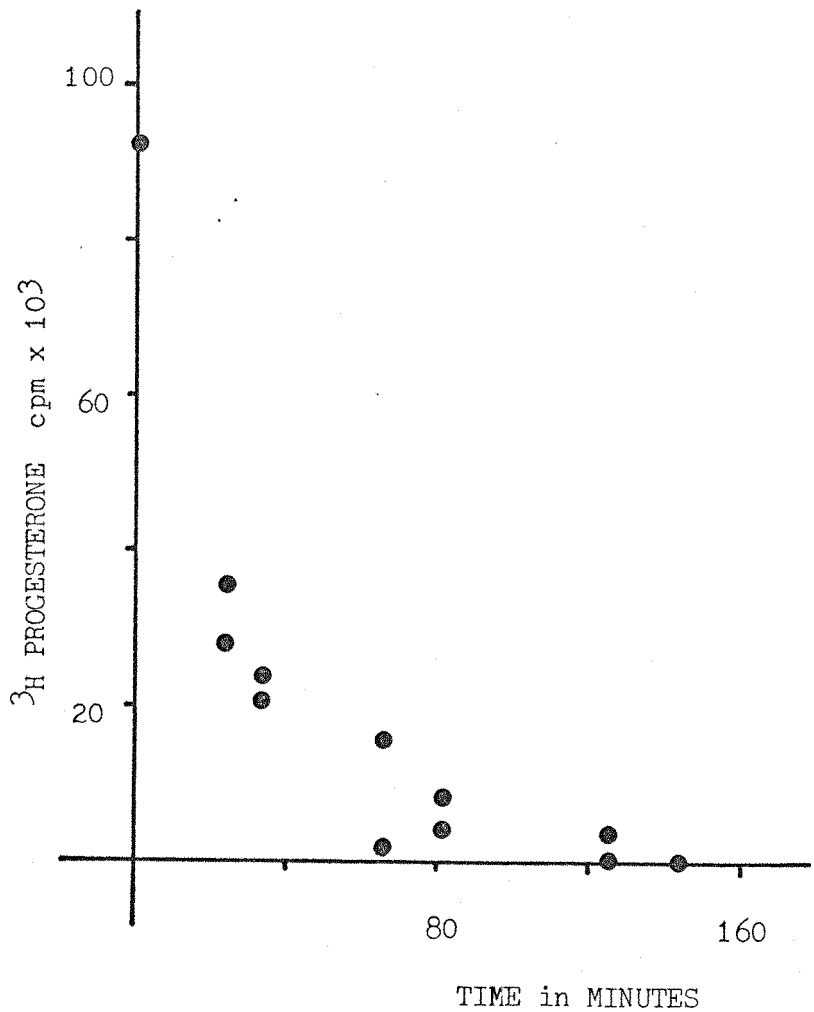


Fig. M.11. The removal of ^{3}H progesterone from plasma by dextran/charcoal incubation. (Total counts added = 1460 cpm.).

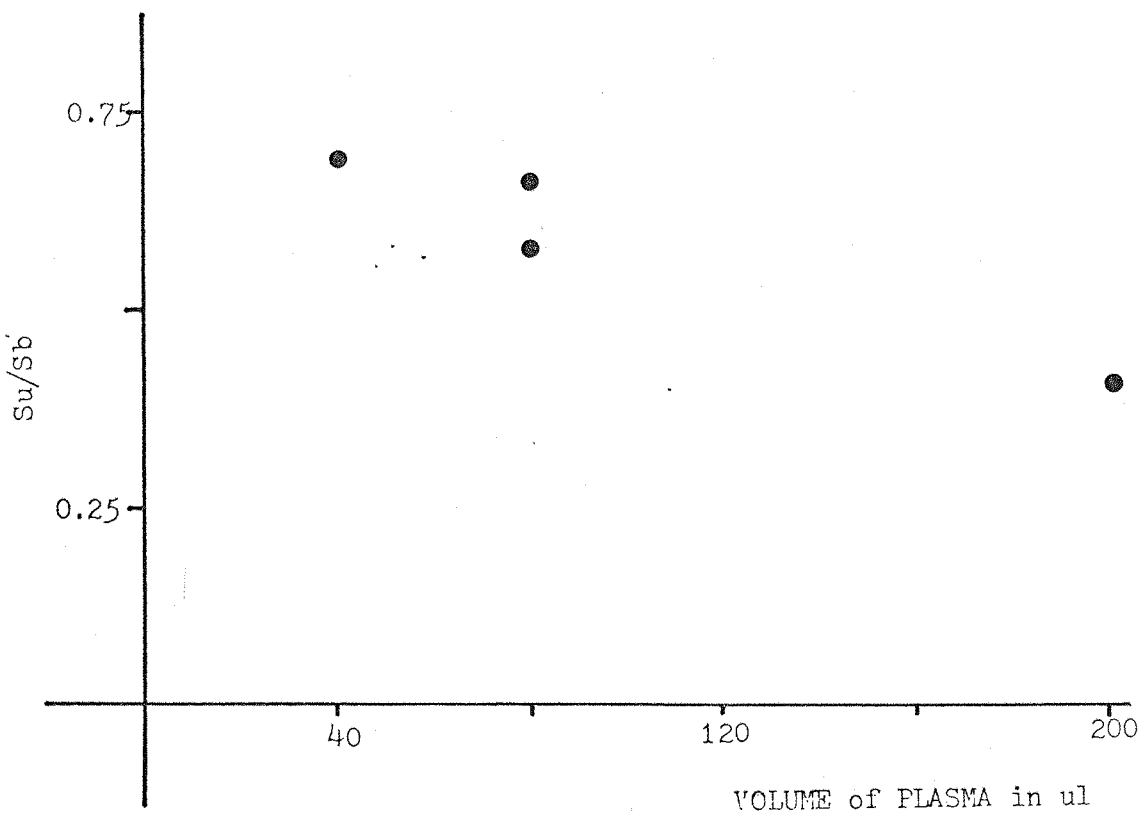


Fig. M.12. The effect of different plasma volumes on Su/Sb with constant label concentration.

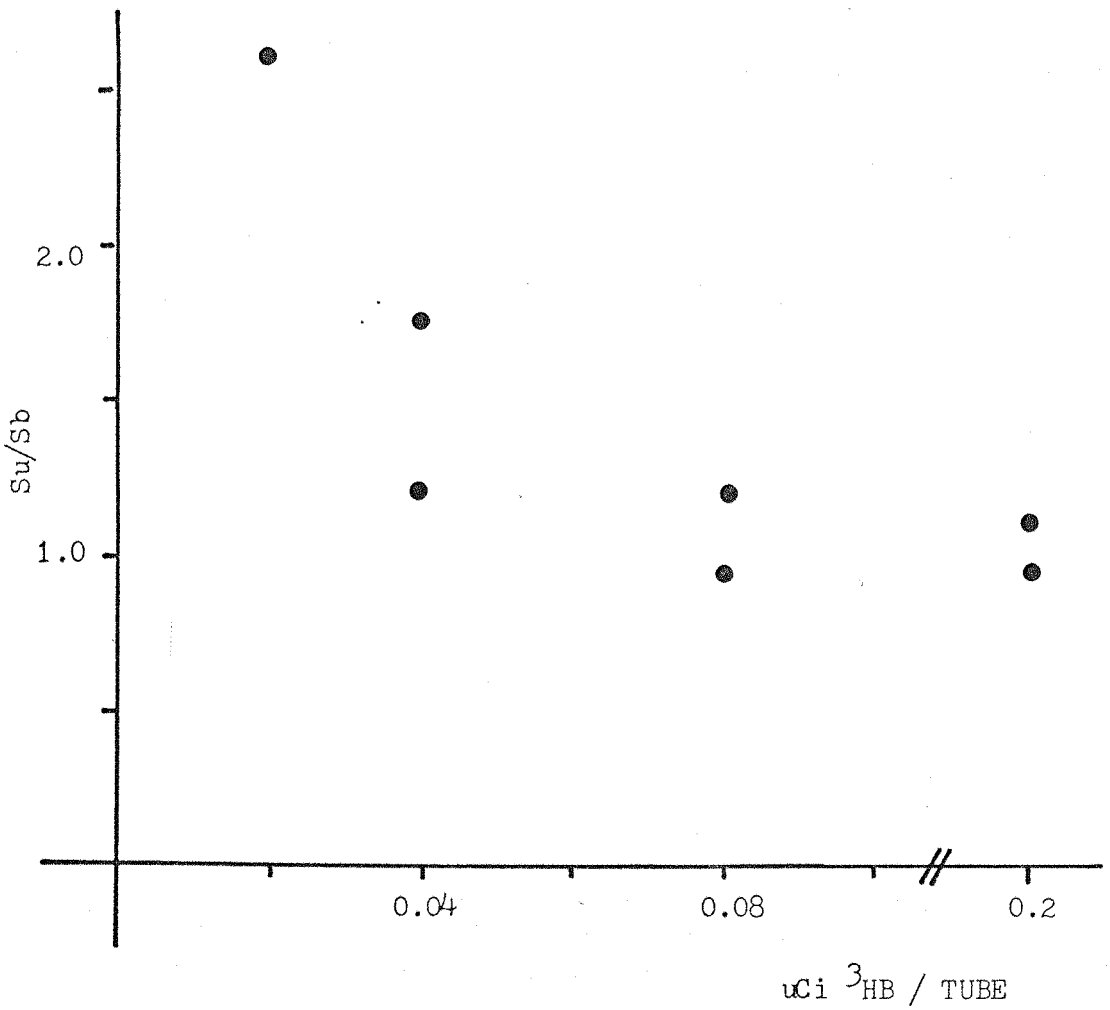


Fig. M. 13. The effect of different ^{3}HB concentration on Su/Sb with a constant plasma volume.

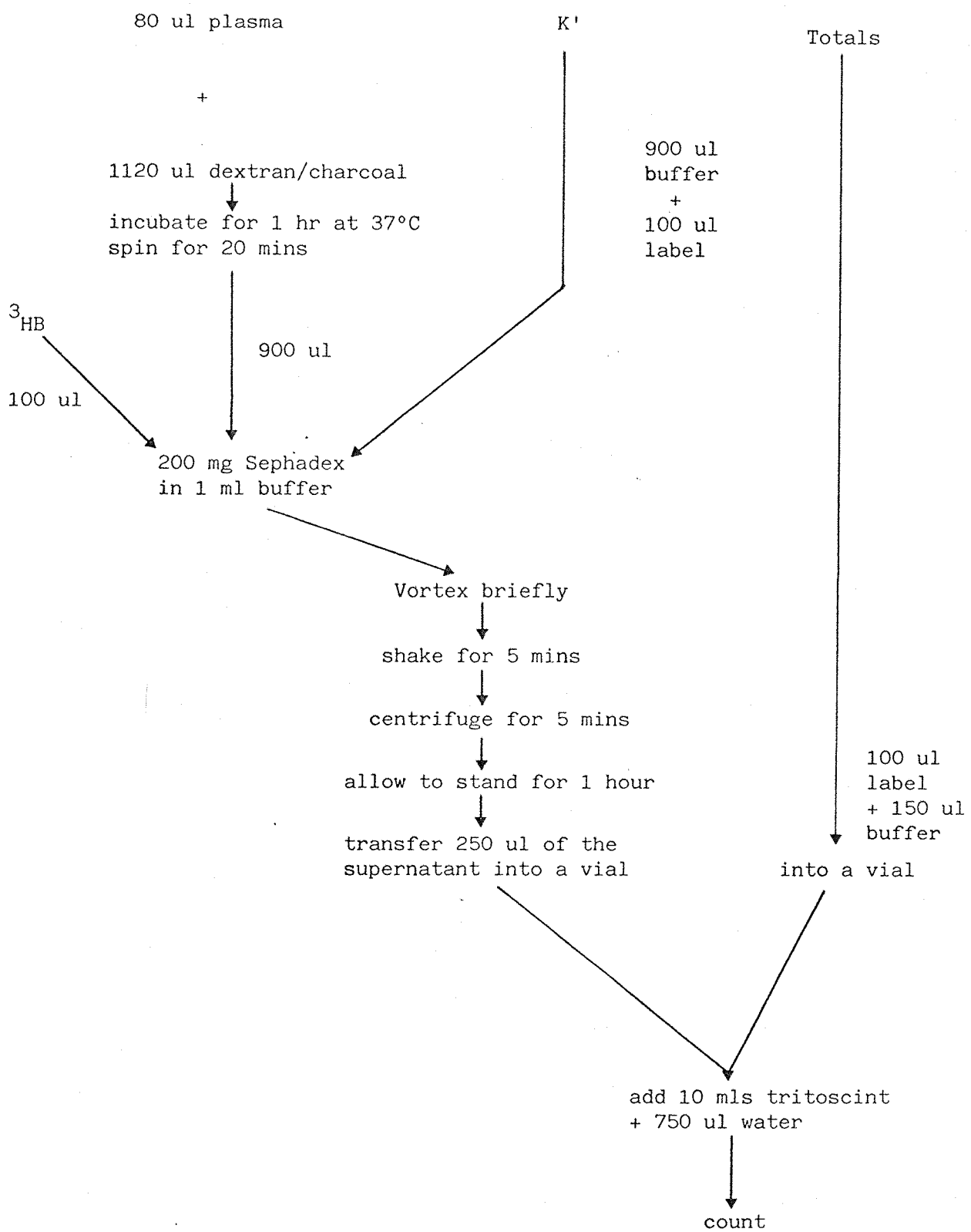


Fig. M.14: Diagram of Full Assay Method

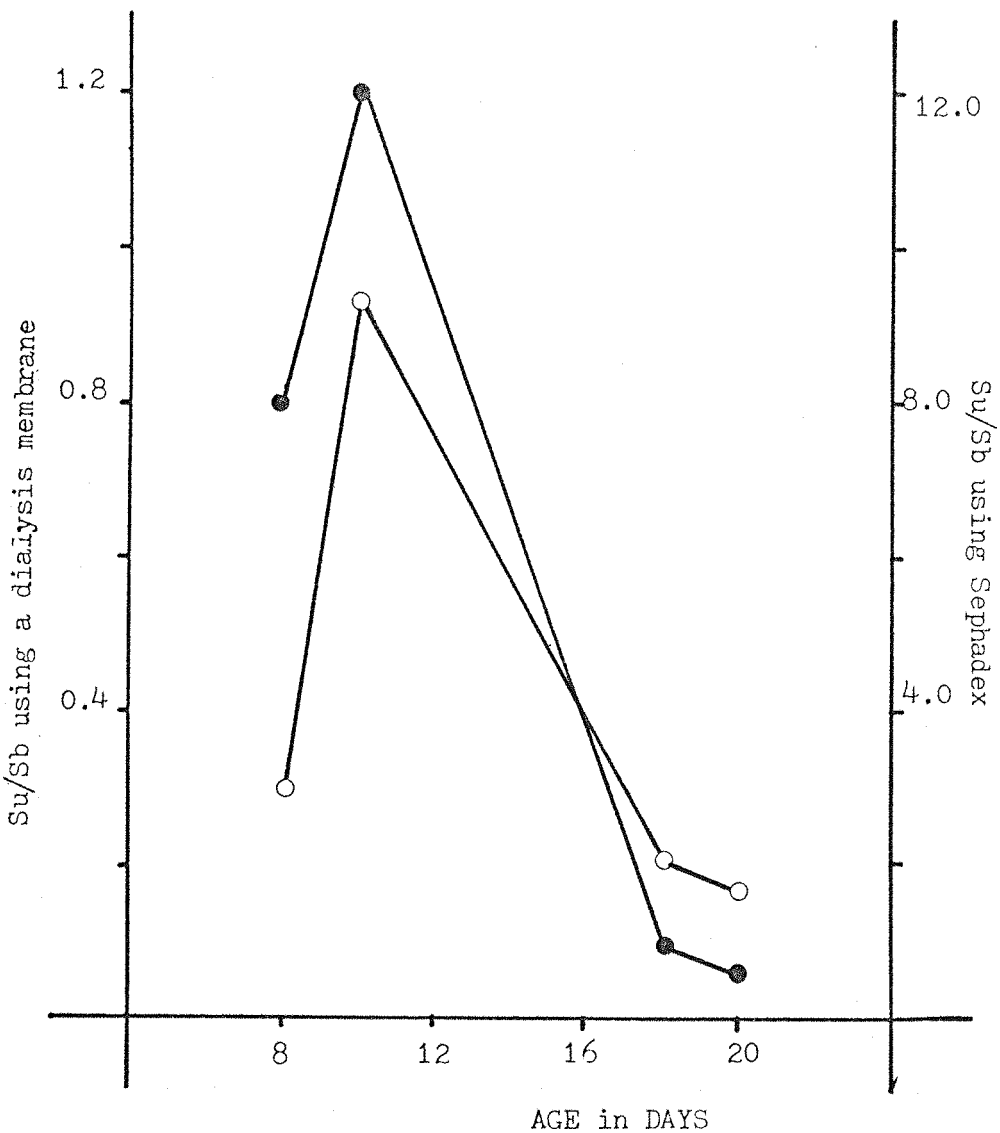


Fig. M.15. Comparison of Su/Sb measured by conventional equilibrium dialysis at 37°C (●—●) and Sephadex at room temperature (o—o) in young rat plasma.

5. Radioimmunoassay of Total Plasma Cortisol (F) Concentration in Rat Plasma

Total plasma F concentration was measured by specific radioimmunoassay using the same procedure used to measure total plasma B concentration except that ^{3}HF and F were used in place of ^{3}HB and B. The antibody was donated by Dr G. Reed of the Tenovus Institute of the Welsh National School of Medicine.

Validation

Fig. M.16 shows a standard curve at antibody dilution of 1:500 (the optimum dilution from Fahmy et al., 1975). The useful range of the standard curve is 0.1 to 5.0 ng/tube. The curve of extracted rat plasma is parallel with the standard curve indicating that the antibody is measuring only F in the plasma extract (Fig. M.17). The recovery of the assay was determined by extracting and assaying plasma samples containing added F. The recovery was 95%. The intra-assay coefficient of variation was calculated using 8 determinations of a plasma sample containing 1.0 ng/tube of F. This was 13%. The sensitivity of the assay was calculated from the intra-assay coefficient of variation (see total plasma B validation). This was 0.2 ng/tube.

6. Radioimmunoassay for Total Plasma ACTH Concentration in Rat Plasma

Plasma ACTH concentration was measured using an Immunoassay Kit for ACTH (Amersham International). The principle of the assay is that the ACTH in the plasma is concentrated by absorbing it on to glass beads. The ACTH is then desorbed using dilute acid and then acetone/water (50:50). The acetone/water extract is evaporated to dryness and the ACTH is measured by RIA with an antibody raised to the 1-24 fragment of human ACTH. The 1-24 fragment is also used as the standard.

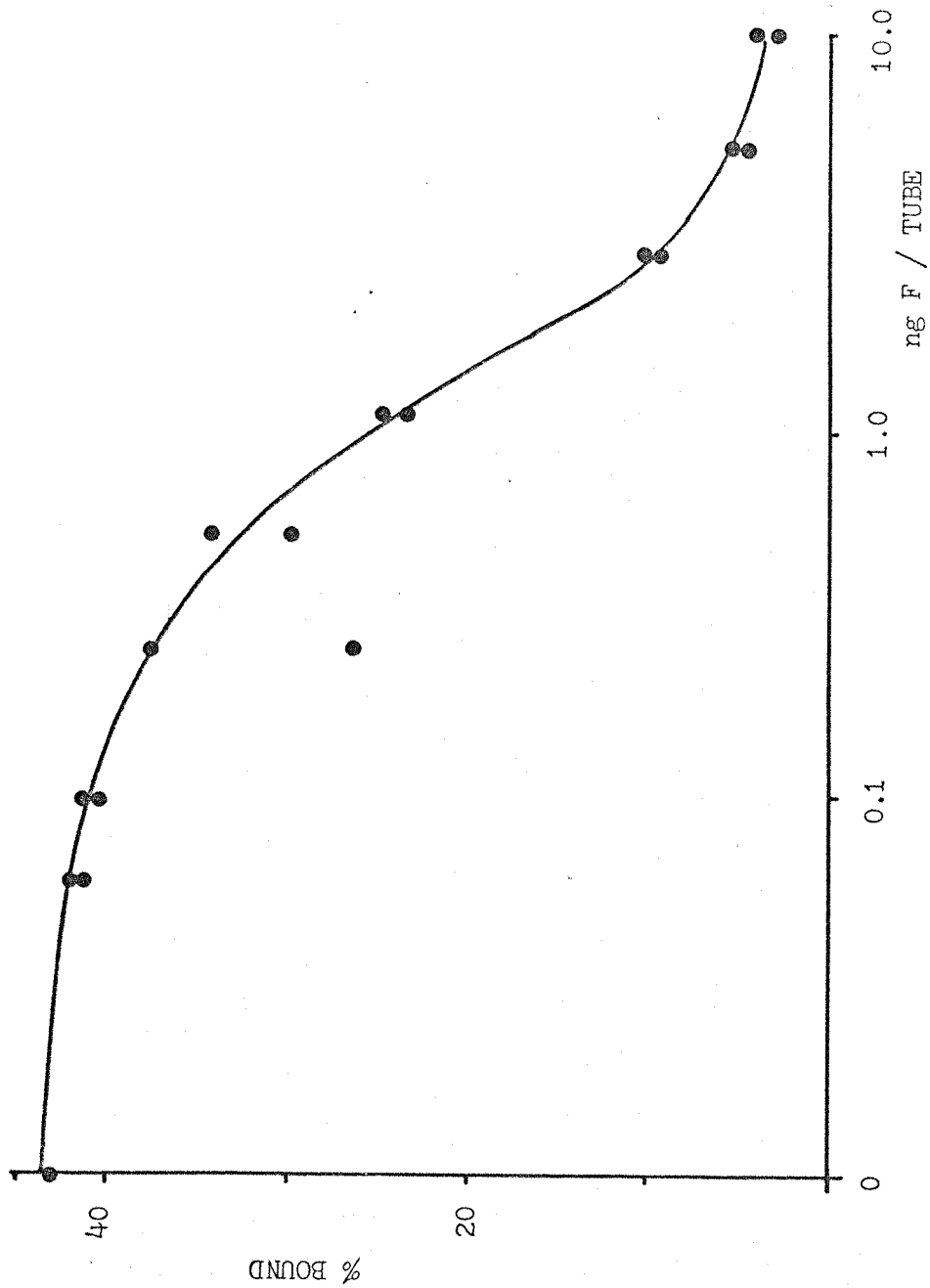


Fig.M.16. A standard curve for F

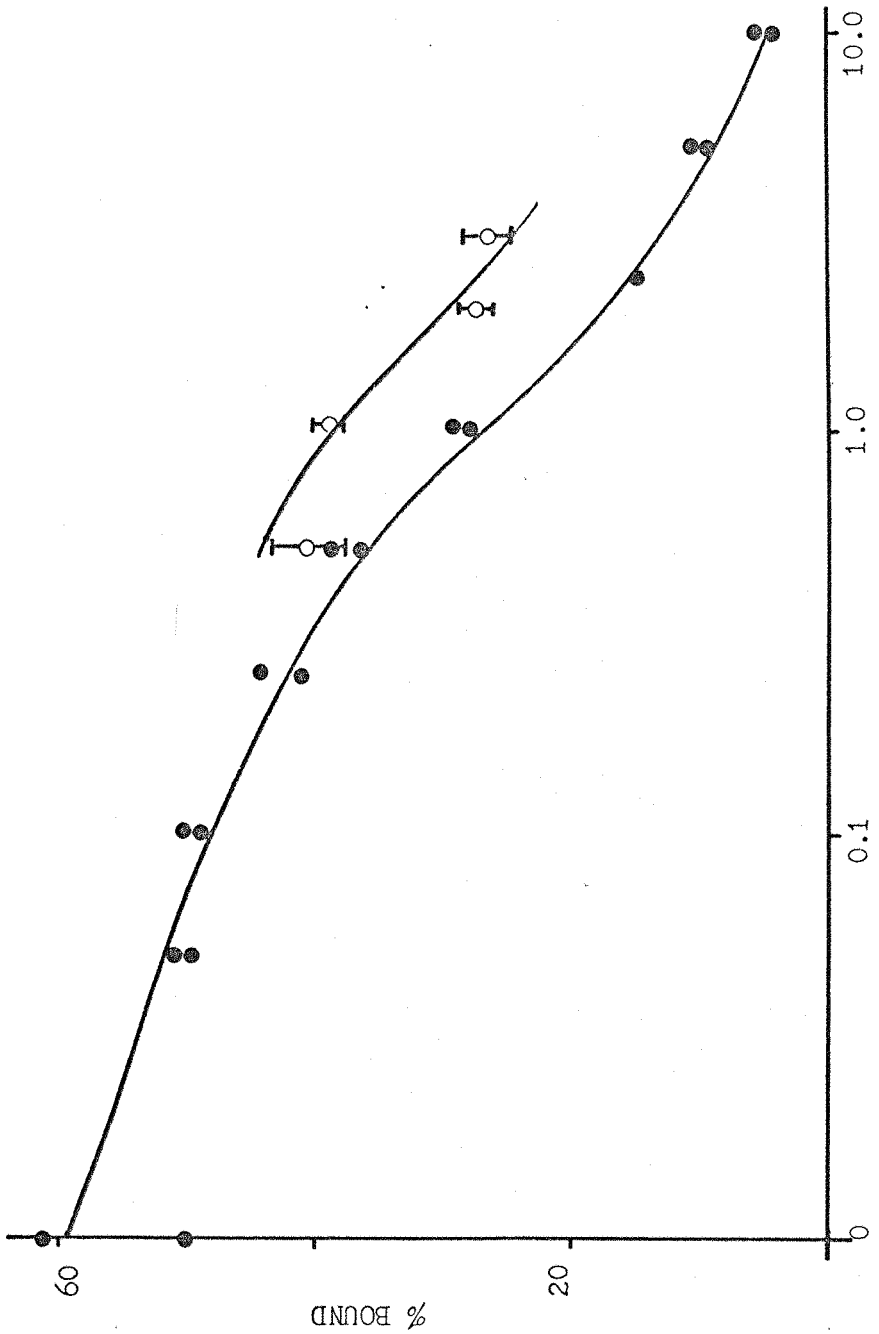


Fig. M.17. Parallelism between the standard curve for F (●—●) and extracted plasma (○—○).

7. Measurement of Plasma Thyroxine Concentration

Plasma thyroxine concentrations were assayed using a modification of the method of Murphy and Patte (1964). Plasma samples (200 ul) were extracted, in duplicate, with 2 ml of ethanol, centrifuged to remove the protein precipitate and the supernatant was evaporated to dryness under air in a water bath at 45°C. Suitable aliquots of a solution of pure thyroxine (0.01 ug/ml) in ethanol were also evaporated to form a standard curve. When dry, 2 ml of a solution of plasma and radioactive thyroxine (2.5 ml 1% phenol, 7.5 ml of human plasma, 2.5 ml of propylene glycol, 20 uCi of ^{125}I thyroxine made up to 250 ml with 0.1 M sodium barbital buffer pH 8.6) was added to each tube. The tubes were vortexed, incubated in a water bath at 45°C for 5 minutes and then transferred to an ice tray for 10 minutes. 200 mg of Amberlite resin was then added quickly to each tube using a calibrated plastic spoon. The tubes were then shaken vigorously for 2 minutes and returned to the ice bath where a further 3 ml of ice cold barbital buffer was added to each tube. The resin was allowed to settle for 10 minutes and then 1 ml aliquots were transferred to counting vials. The tubes were counted in a Beckman Biogamma counter for 5 minutes each. Fig. M.18 shows a typical standard curve.

8. Measurement of Plasma Glucose Concentration

The plasma glucose concentration was determined using a glucose oxidase method. The principle of the method is that glucose oxidase specifically oxidises D-glucose to gluconolactone with the production of hydrogen peroxide. In the presence of peroxidase and hydrogen peroxide a redox dye can be oxidised to give colour. The colour can be read spectrophotometrically.

Materials

The glucose oxidase reagent was prepared by mixing 9.6 mg peroxidase with 600 mg glucose oxidase and 1.2 g of ABTS in 1200 mls of sodium phosphate buffer 0.1 M pH 7.0. This was stored at 4°C until required.

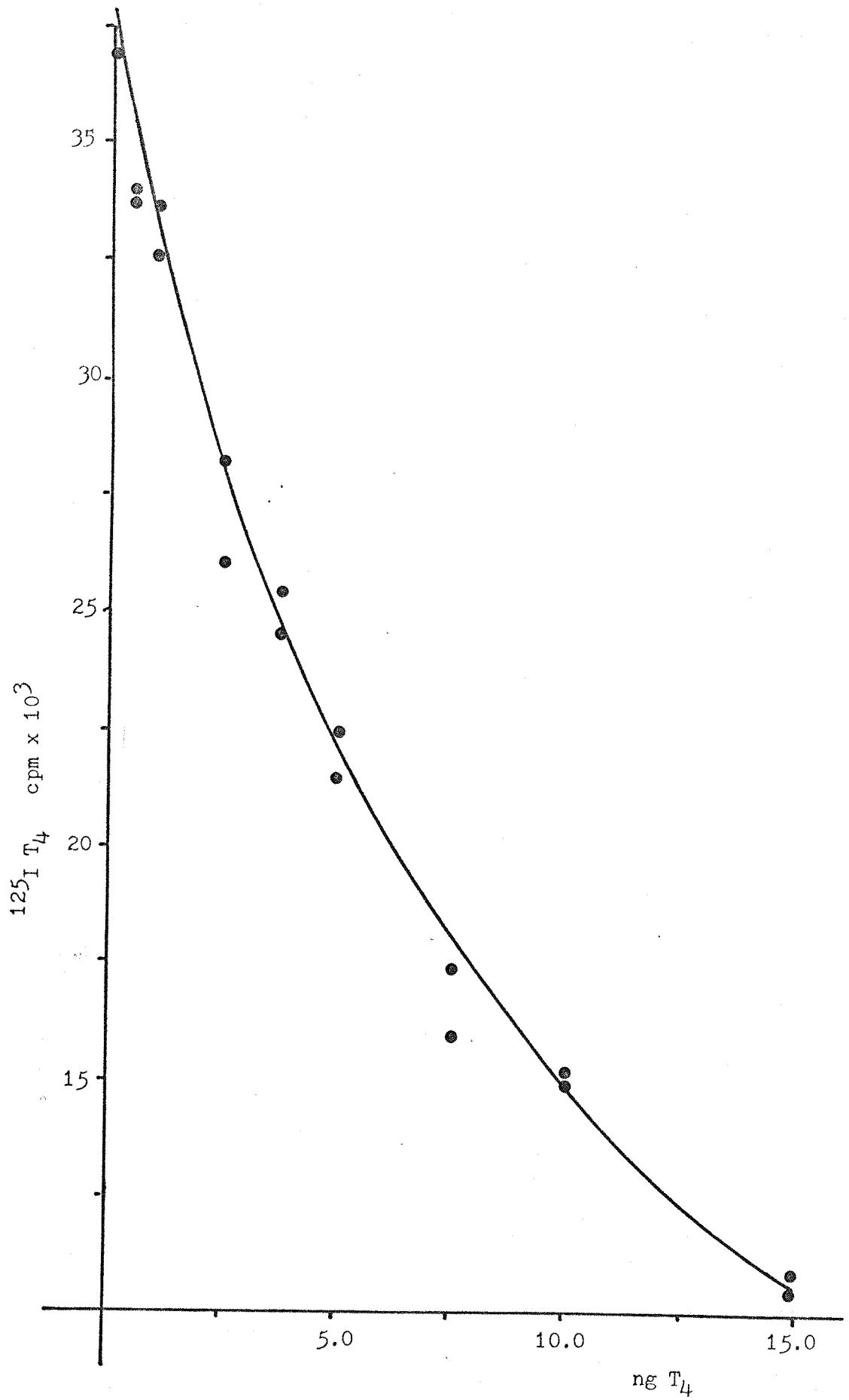


Fig. M.18. A standard curve for T_4.

Assay procedure

50 ul plasma samples were mixed with 450 ul 6% (w/v) perchloric acid. The tubes were vortexed and the precipitated protein was removed by centrifugation for 10 minutes in a bench MSE centrifuge. A 100 ul aliquot of the supernatant was added to 2.5 ml of the glucose oxidase reagent. At the same time, a standard curve was set up (in duplicate) to give a range of 0-100 mg% glucose. The standards were added to 2.5 ml of the assay reagent. Both the samples and the standard curve were incubated at 37°C for 15 minutes. The absorption was determined on an SP 500 spectrophotometer at 620 nm. Fig. M.19 shows a typical standard curve.

9. Measurement of Total Plasma Protein

This was measured using the Bradford method (1976). The principle of the method is that the dye Coomassie Brilliant Blue G-250 shifts its absorption from 465 nm to 595 nm when it binds to protein.

Materials

The protein reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue in 50 mls of 95% ethanol. 100 mls of phosphoric acid (85%) was added. Then the solution was diluted to 1 litre with water. The reagent was stored in the dark and mixed thoroughly before use. The protein standard (BSA) was stored at -20°C at a concentration of 1 mg/ml.

Assay Procedure

The standard curve was constructed by dispensing different volumes of protein standard to give a range of 0-100 ug of protein. All volumes were then made up to 100 ul with water. The plasma samples were diluted 1:10 with water before 50 ul aliquots were added to test-tubes with 50 ul of water. 5 ml of the protein reagent were added to both standards and samples. The tubes were vortexed

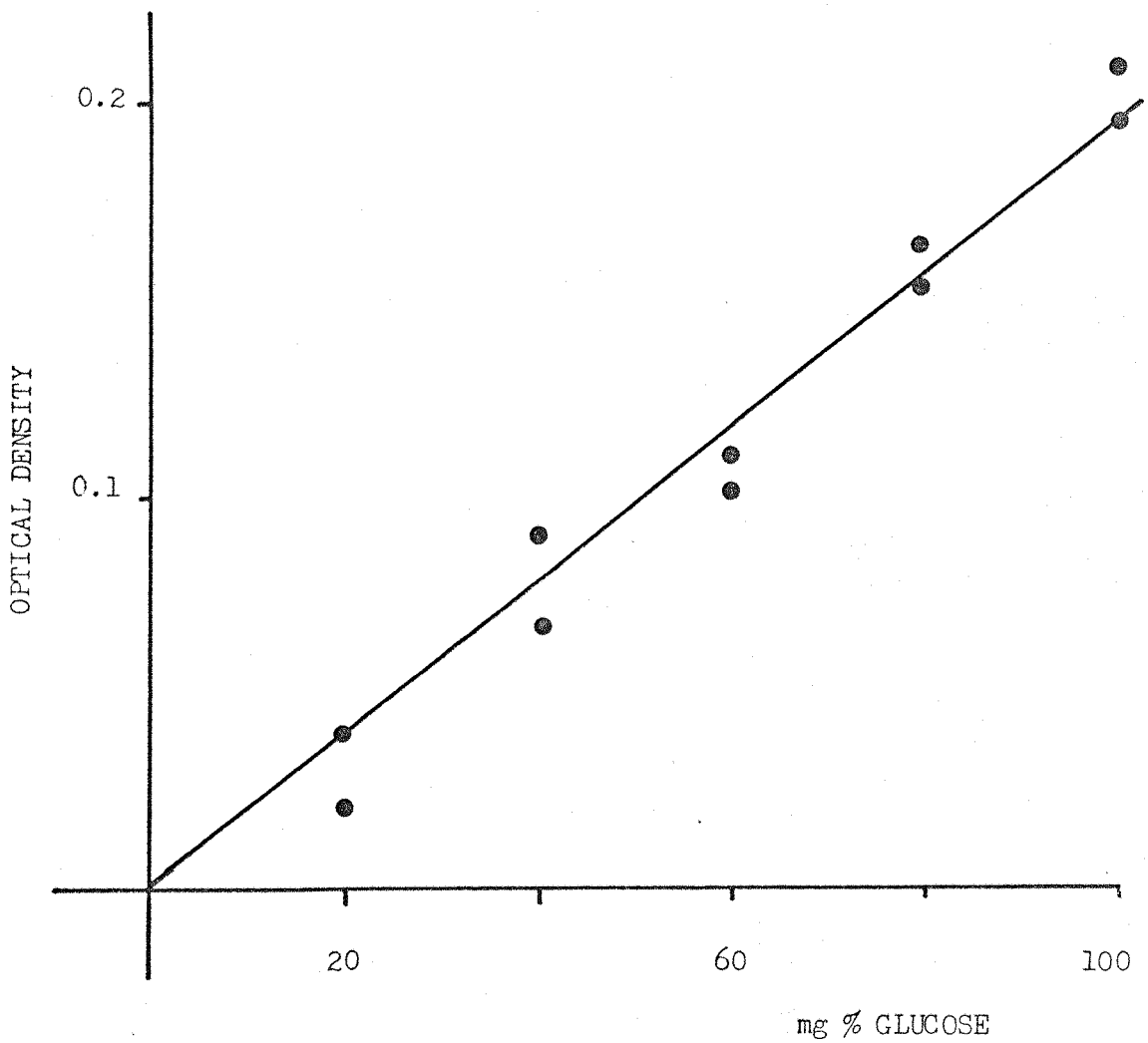


Fig. M.19. A standard curve for glucose.

and the absorption was determined at 595 nm on a SP 600 spectrophotometer. The colour developed fully in 2 minutes and remained stable for 1 hour. Fig. M.20 shows a typical standard curve.

10. Preparation of Sephadex G-100 Superfine Column

Preparation of Column

15 g of Sephadex G-100 superfine were allowed to swell with 400 mls of sodium phosphate buffer pH 7.4, 0.01 M with 0.15 M sodium chloride containing 0.01% sodium azide for 72 hours at room temperature. The Sephadex was cooled to 4°C and poured carefully into a 2.5 cm x 70 cm column. When all the Sephadex had settled, 1 ml of 10% BSA was applied to the top of the column and eluted with 500 mls of buffer. This procedure was 'to saturate binding sites in the Sephadex' (Andrews, 1966). The flow rate and the packing of the column was checked using blue dextran. 1 ml of 2 mg/ml blue dextran was applied to the column. The distance the band travelled each hour was noted along with the volume of elute collected. Fig. M.21 demonstrates that the flow rate of blue dextran through the column was linear indicating that the column was packed evenly. The void volume (the volume of elute which ran in front of the blue dextran) was 75 mls and the flow rate was approximately 6 mls/hour.

Validation

Fig. M.22 shows the elution profile of adult rat plasma containing ^{3}HB, and Fig. M.23 demonstrates that in the presence of an excess of cold B the first peak, corresponding to the bound peak, can be displaced to the second peak, the unbound peak.

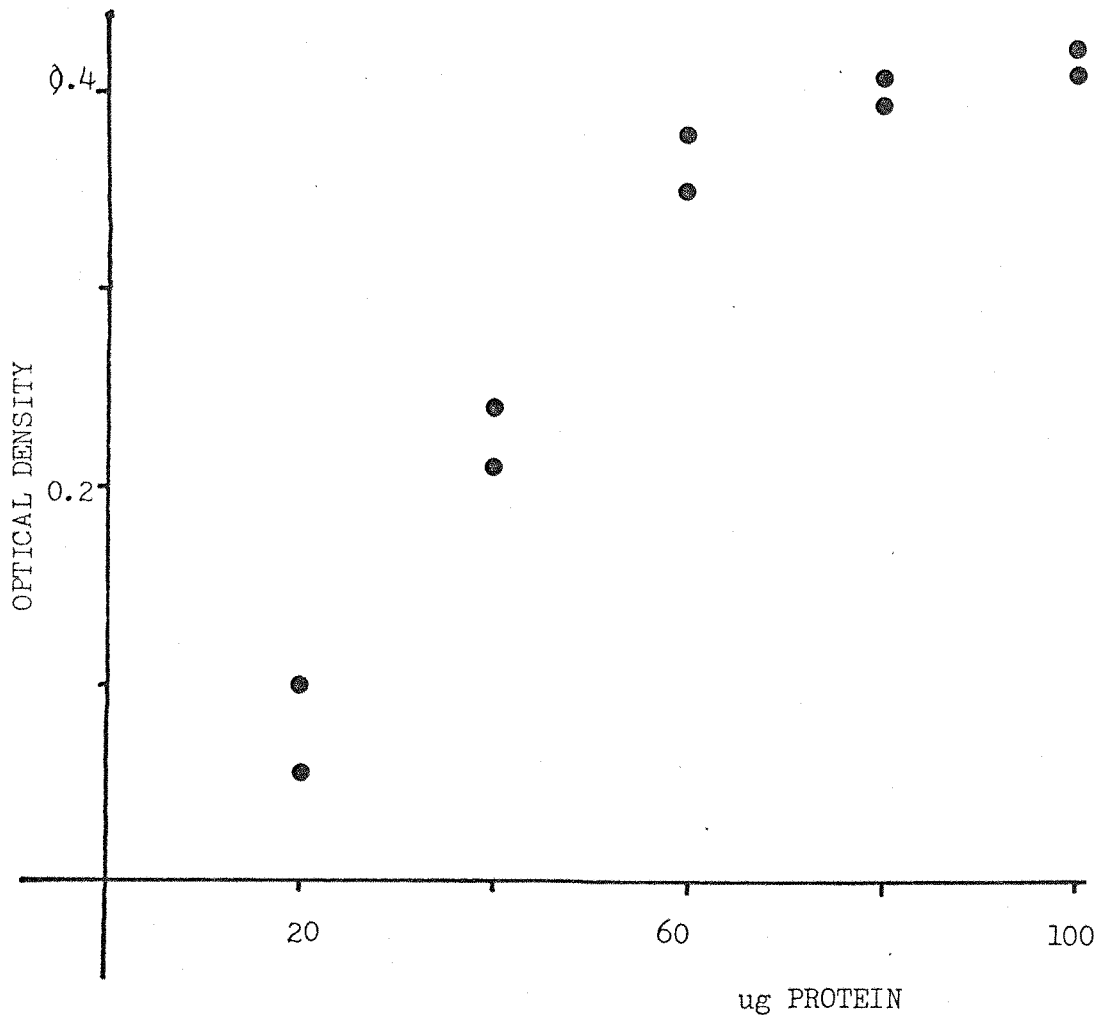


Fig. M.20 A standard curve for protein using Coomassie Brilliant Blue.

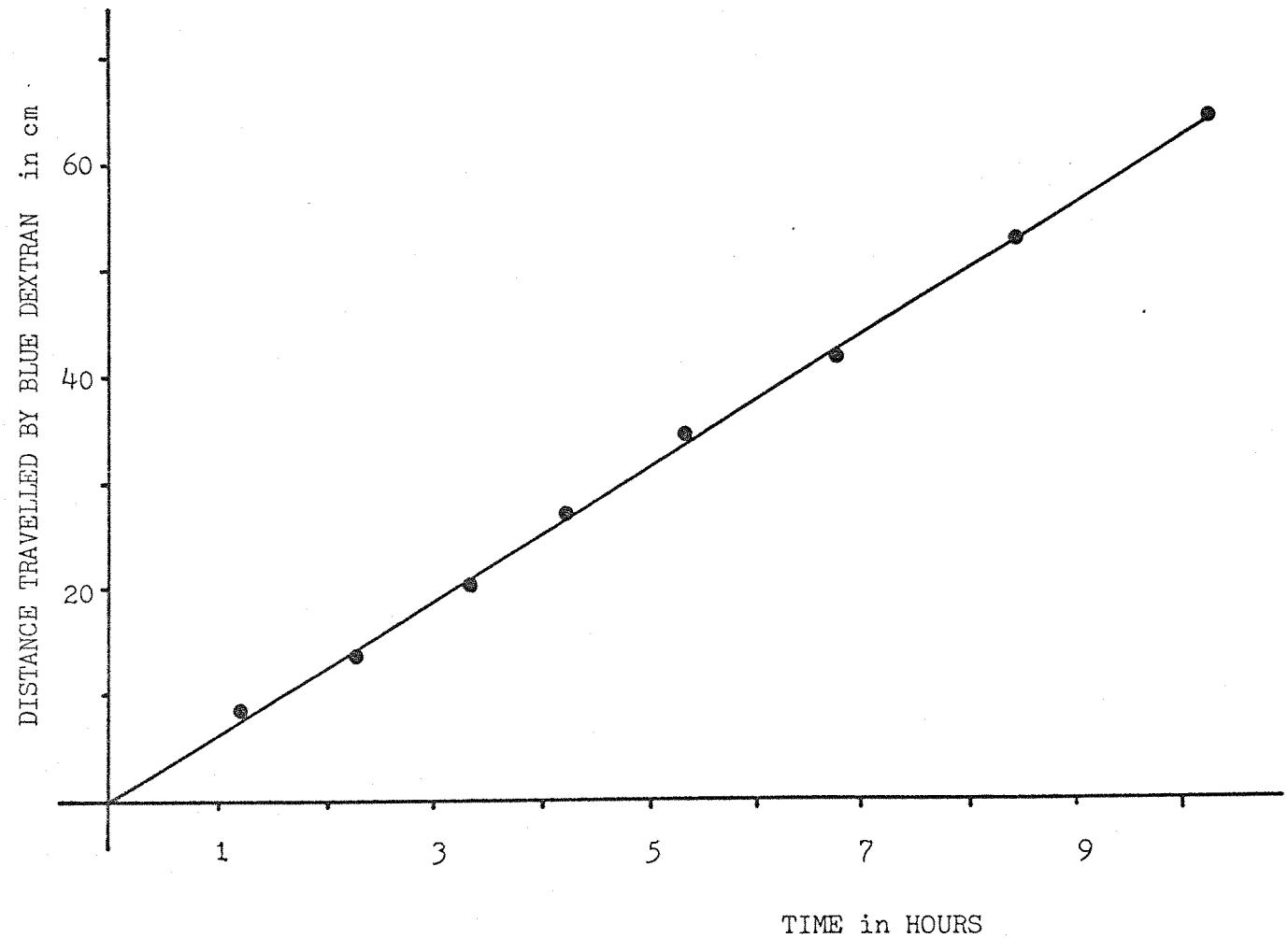


Fig. M.21. The rate blue dextran travelled through the Sephadex column.

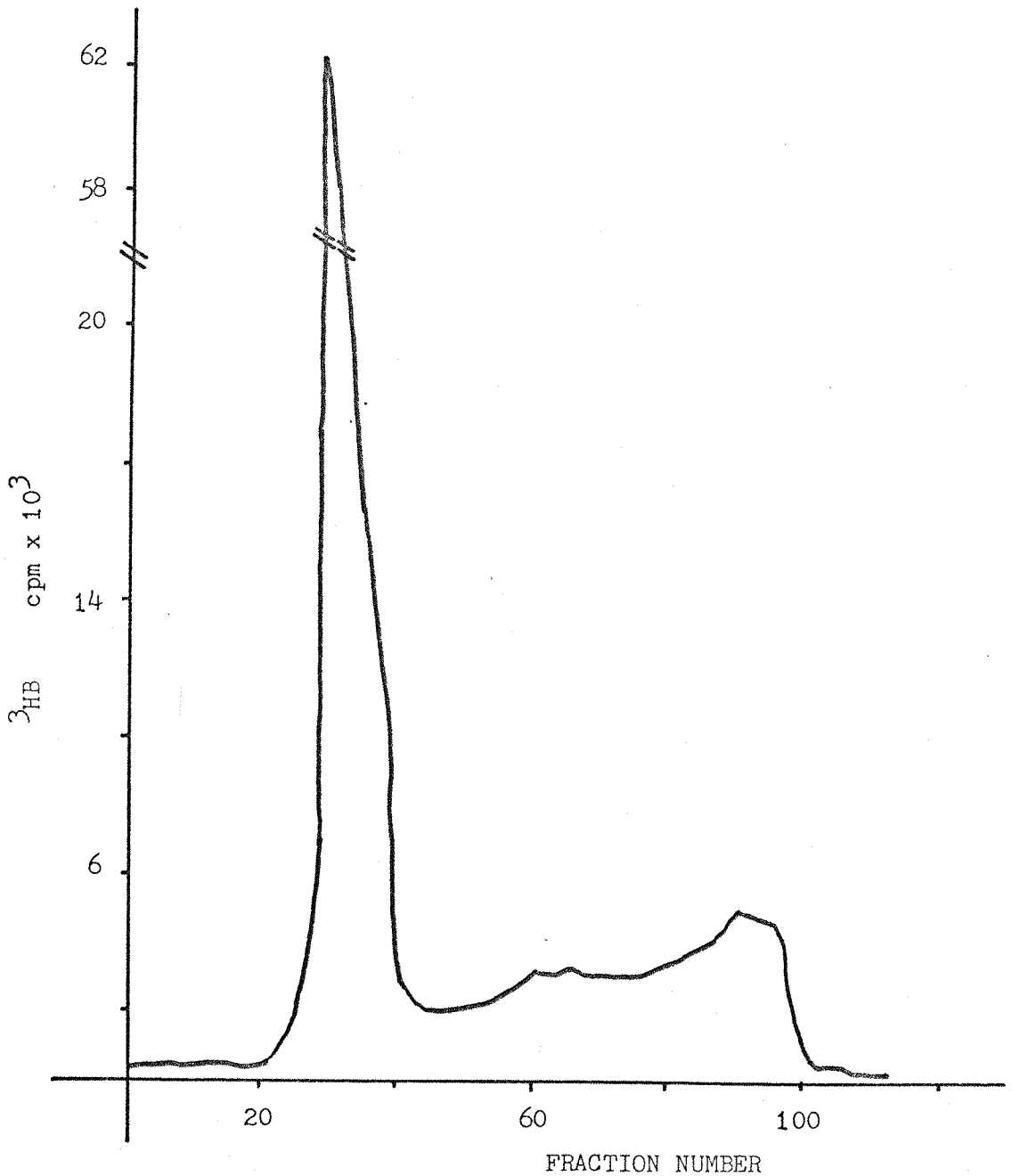


Fig. M.22. The profile of adult rat plasma with ^{3}HB on the Sephadex column.

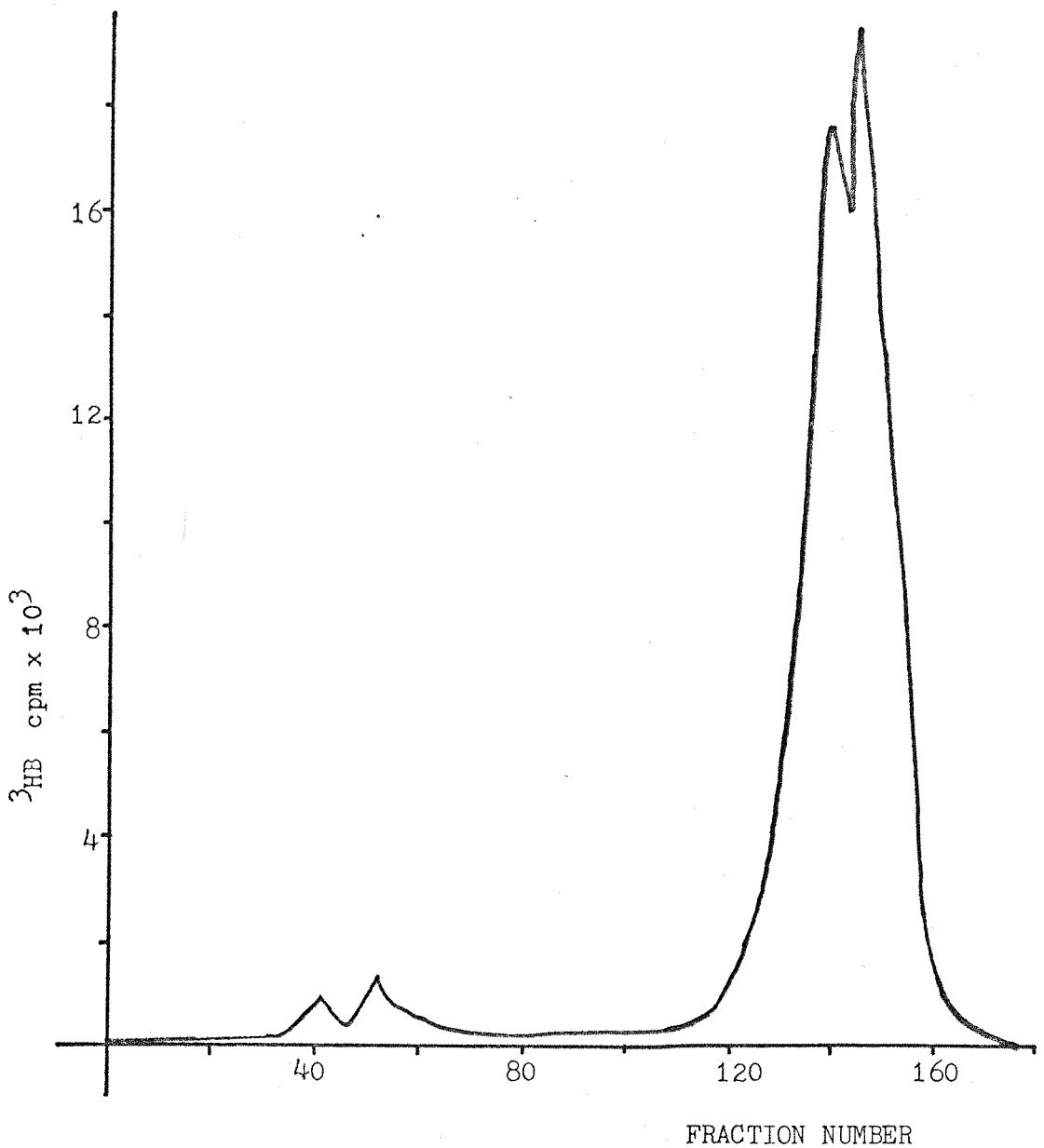


Fig. M.23. The profile of adult rat plasma with $^{3}_{HB}$ and an excess of cold B on the Sephadex column.

11. Measurement of the Amount of DNA in the Cell Suspensions

The simple rapid method of Lebarca and Paigen (1980) was used to measure the quantity of DNA in the cell suspensions. The method is based on the enhancement of fluorescence seen when bisbenzimide binds to DNA. The chromatin is dissociated in high salt buffer to ensure accurate determination of DNA.

Materials

The buffer used was 0.05 M sodium phosphate with 2.0 M sodium chloride pH 7.4. A stock solution of bisbenzimide of 200 ug/ml was made up in water. This was stable at 4°C for up to 6 months. Calf Thymus DNA was used as the standard and a stock solution of 40 ug/ml in phosphosaline buffer was stored at 4°C.

Assay Procedure

Serial dilutions of the DNA standard were made to give a standard curve with the range of 0-10 ug of DNA. 1 ml of buffer was placed in a test tube containing either the buffer standards, 20 ul of Krebs Ringer bicarbonate with 5% BSA and 2% glucose (the reagent blank) or 20 ul of the cell suspension. The final volume was the same for all tubes. The cell suspensions were sonicated for 30 seconds. 1 ml of bisbenzimide (1 ug/ml) solution was added, the tubes were vortexed and the fluorescence determined on a Perkin Elmer fluorimeter, excitation wavelength 356 nm and emission wavelength 458 nm. Fig. M.24 shows a typical standard curve.

12. Measurement of Plasma CBG Activity

CBG activity was assessed using a modification of the absorption method of Trapp and West (1969).

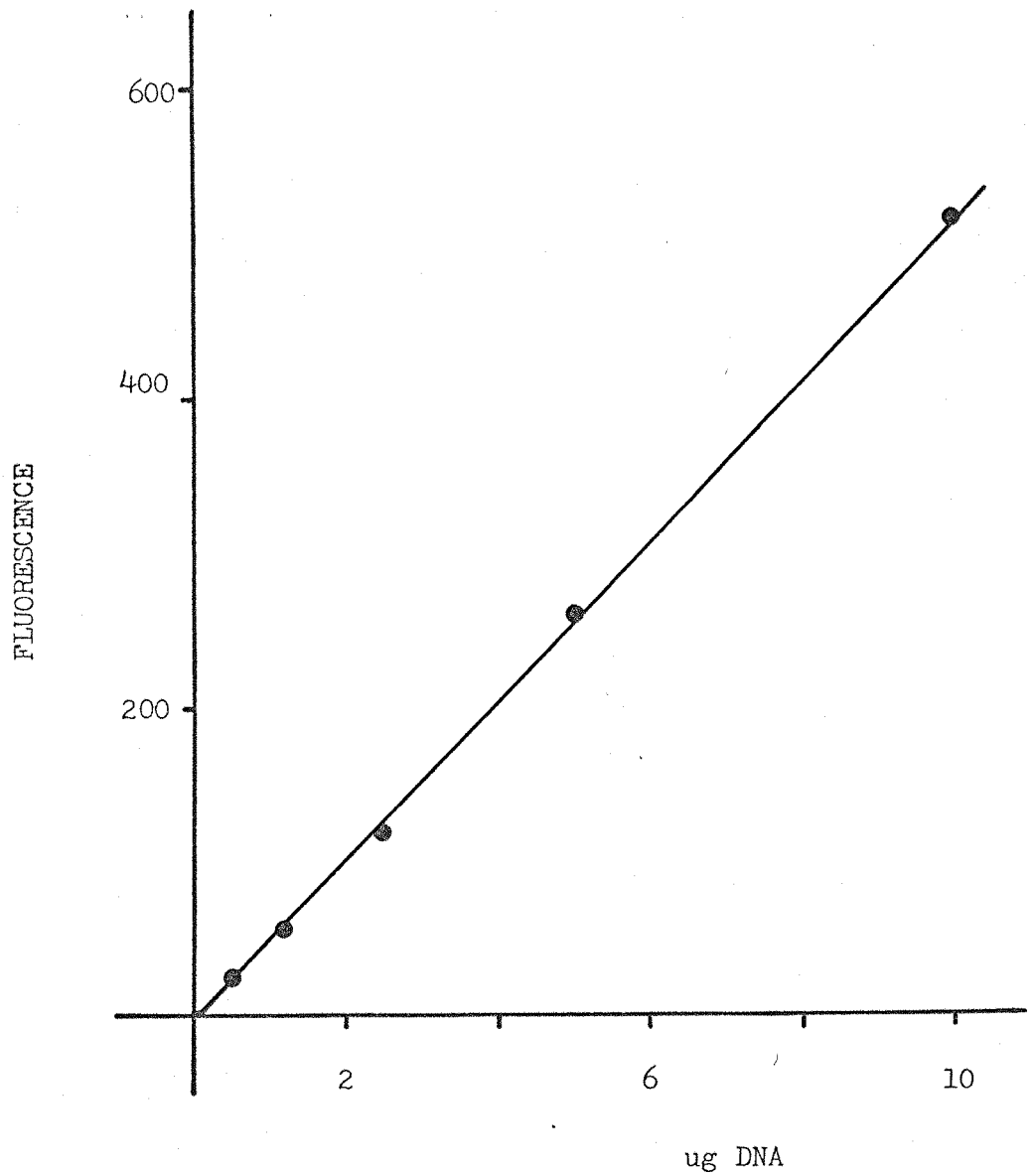


Fig. M. 24. A standard curve for DNA.

Materials

The buffer used was 0.1 M sodium phosphate with 0.1 M sodium chloride pH 7.4. The F standard was hydrocortisone sodium succinate (1 mg/ml in water). Subsequent dilutions were made using buffer. The label solution of ^{3}HF in ethanol (10 u Ci /ml) was diluted with buffer to give a solution of approximately 50 000 cpm/ml.

Assay Procedure

200 ul plasma samples, where the endogenous steroid had been removed using 10 mg/ml charcoal, were aliquoted into test-tubes. 900 ul of the stock solution containing 500 ul of ^{3}HF/50 ml buffer was added to this along with 100 ul of cold F in buffer. The proportion of the binding activity that was due to albumin was calculated by heating an aliquot of plasma to 60°C for 10 minutes before it was assayed. The tubes were incubated at 45°C for 5 minutes, placed on ice for 15 minutes before 1 spoonful of Florisil (200 mg) was added. The tubes were shaken for 2 minutes and returned to ice for a further 10 minutes. A 500 ul sample was removed from each tube and placed in a scintillation vial with 10 mls of tritoscint. The radioactivity added to the system was determined using tubes containing plasma, stock and cold F with no Florisil.

Validation

Fig. M.25 shows curves for plasma dilutions of 1:1, 1:5 and 1:10 with either 5 ug, 1 ug or 0.5 ug per ml of plasma of cold F. A plasma dilution of 1.5 with 1 ug/ml plasma of F was used routinely.

13. Statistics

Groups have been compared with saline-treated litter-mates as controls wherever possible. All results have been presented as the mean $\pm$ the standard error of the mean, where n = the number of samples in each group. Significance limits have been determined

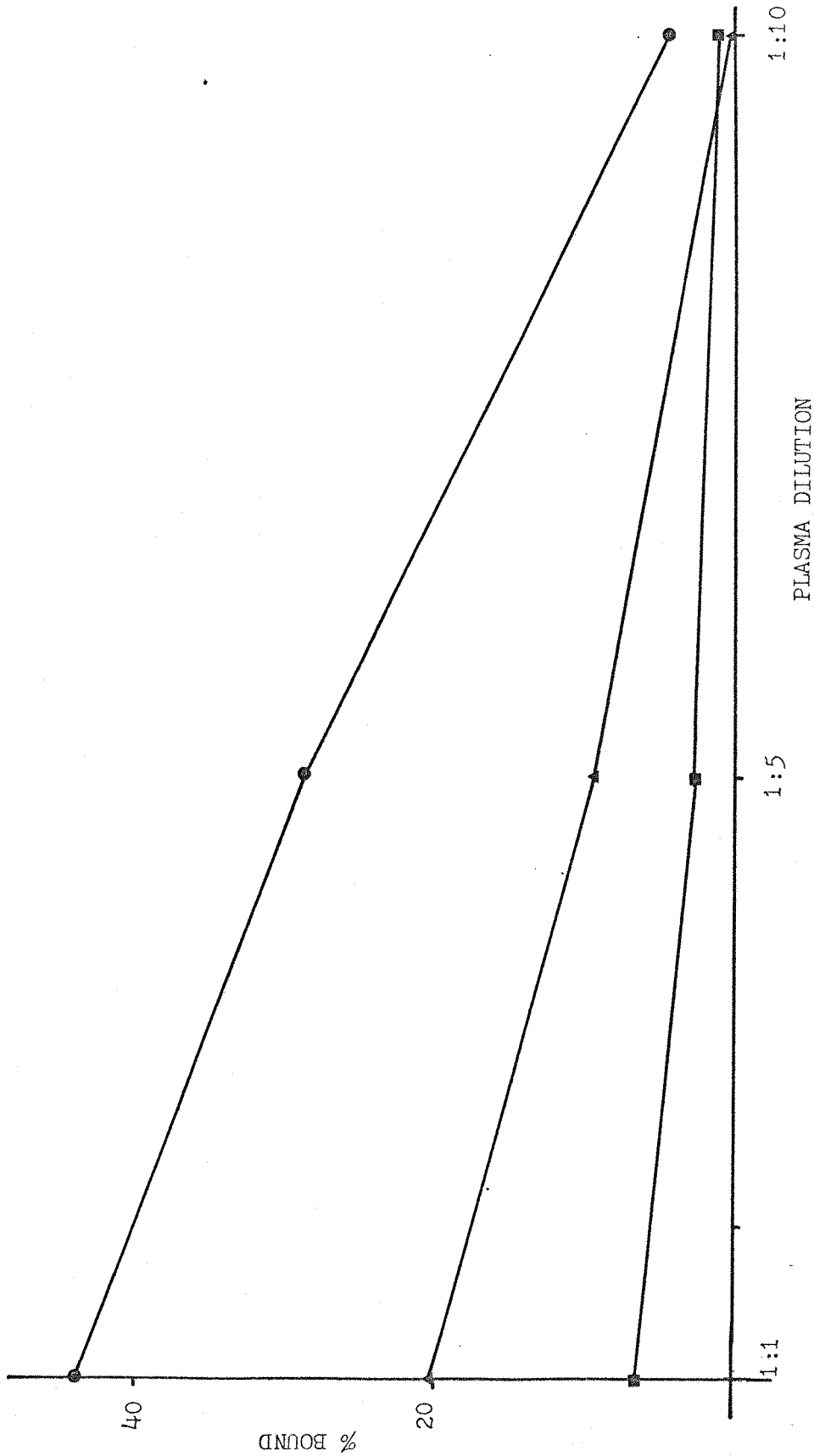


Fig. M.25. Plasma dilution curves at 500ng/ml F (●—●), 1000ng/ml F(▲—▲), and 5000ng/ml F (■—■).

using Student's t test unless otherwise stated. All probabilities of < 0.05 have been taken to be significant.

On the figures  = Mean $\pm$ SEM

*	=	$p < 0.05$
**	=	$p < 0.01$
***	=	$p < 0.001$

Regressions have been calculated on a Hewlett Packard computer programme using the least squares method.

RESULTS

SECTION 1

In this section, measurements of % free B, plasma protein, B and F concentrations and adrenal to body weight ratio in young rats, during the postnatal period, are described.

Experimental Procedure

Litters were taken from their mothers at the appropriate ages, weighed and killed as quickly as possible. Trunk blood was collected and kept on ice until centrifugation. The plasma was separated and stored at -20°C until assay. The left adrenal was removed from each rat cleared of fat and connective tissue and weighed using a torsion balance. Samples were collected from rats of the following ages:- 1, 3, 5, 6, 7, 9, 11, 13, 15, 17, 19 and 21 days old. Blood samples were pooled together in younger litters so that adequate plasma could be collected for assay. The % free B, plasma protein, B and F concentrations were determined.

Results

Table 1.1: Body Weight, Adrenal Weight and Adrenal to Body Weight Ratio During the Postnatal Period

Age	n	Body Weight g	Adrenal Weight mg	$\frac{\text{Adrenal Weight}}{\text{Body Weight}}$ $\frac{mg}{g} \times 10^{-2}$
1	15	5.0 ± 0.2	0.9 ± 0.1	17.0 ± 1.3
3	21	6.6 ± 0.2	1.2 ± 0.8	18.1 ± 1.0
6	26	9.9 ± 0.3	1.3 ± 0.1	13.3 ± 0.9
9	20	15.1 ± 0.2	1.4 ± 0.1	9.9 ± 0.8
13	16	22.0 ± 0.3	2.5 ± 0.2	11.2 ± 0.8
15	6	23.0 ± 0.3	2.6 ± 0.3	10.8 ± 1.2
19	5	42.3 ± 1.2	6.6 ± 0.7	15.5 ± 1.6
21	7	36.7 ± 0.4	5.7 ± 0.8	15.6 ± 1.4
Adult	14	273.6 ± 10.4	35.2 ± 3.2	16.5 ± 1.4

There was a ~~linear~~ increase in body weight during the first 3 weeks after birth AND the adrenals grew slowly during the first 2 weeks and then more rapidly during the 3rd. Thus, the adrenal to body weight ratio fell during the 1st week post-partum remained low during the 2nd week and increased in the 3rd week to reach adult values by day 21 (Fig. 1.1).

Table 1.2: Total Plasma B Concentration During the Postnatal Period

Age	n	Total Plasma B Concentration ng/ml
1	6	89.0 ± 22.4
3	4	15.6 ± 6.6
6	8	3.9 ± 0.4
9	8	5.1 ± 2.4
13	10	7.1 ± 0.7
15	7	41.4 ± 10.0
19	4	70.0 ± 10.0
21	9	73.0 ± 8.0

Table 1.3: Total Plasma F Concentration During the Postnatal Period

Age	n	Total Plasma F Concentration ng/ml
3	6	6.8 ± 1.8
5	3	7.1 ± 1.7
7	9	9.5 ± 3.5
9	8	9.5 ± 3.5
11	4	10.4 ± 1.4
13	11	8.5 ± 1.3
19	4	22.5 ± 1.5
21	8	19.5 ± 1.5

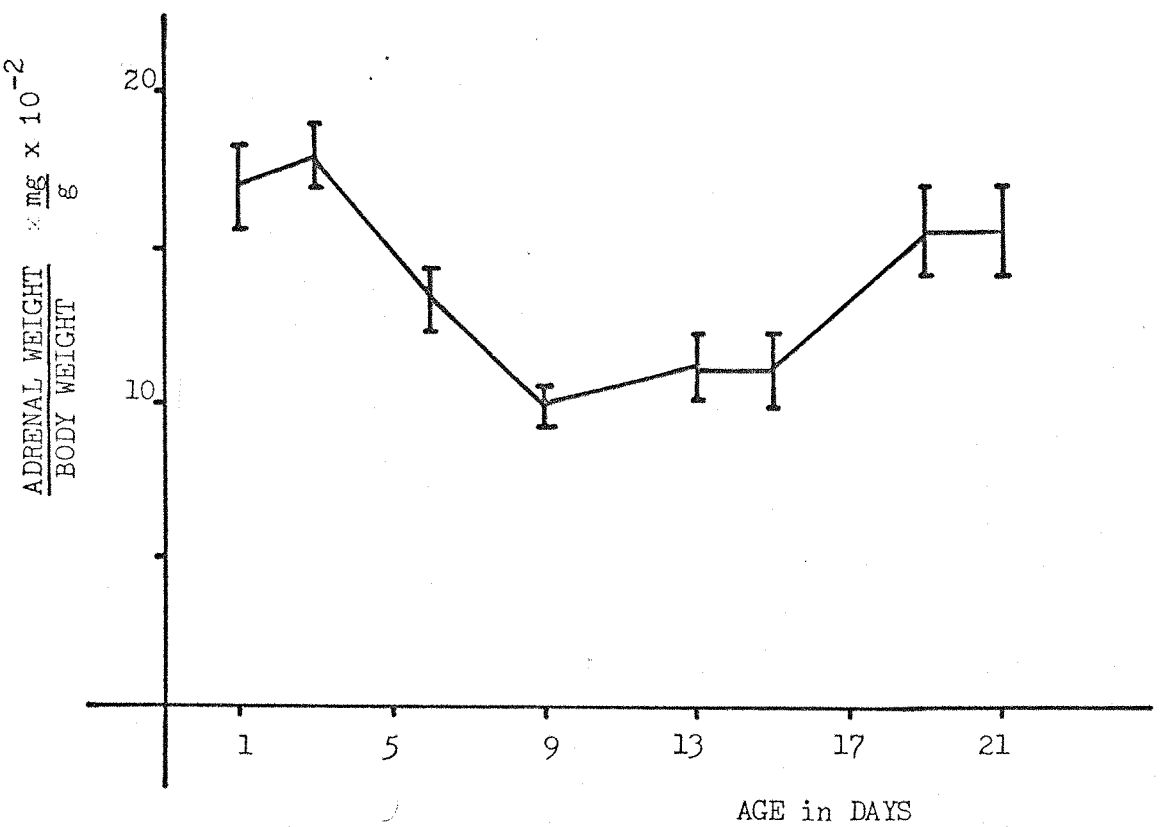


Fig. 1.1 Adrenal to body weight ratio over the postnatal period.

The total plasma B concentration was high on day 1, decreased rapidly to a low value by day 6, remained low until day 13 and then began to increase to reach a value of 70.0 ± 10.0 ng/ml at day 19 (Fig. 1.2). The adrenal to body weight ratio and the total plasma B concentration showed a linear relationship from day 13 to day 19 (Fig. 1.3) but there was no clear relationship from day 1 to day 9 post-partum. The plasma F concentration remained low (<11 ng/ml) during the first 2 weeks after birth but increased to 22.8 ± 0.8 ng/ml by day 19 (Fig. 1.2). The ratio of B/F over this period was 2.3 on day 3, decreased to 0.5 by day 9 and then increased to 3.7 by day 21.

Table 1.4: % Free B and Total Plasma Protein Concentration During the Postnatal Period

Age	n	% Free B	n	Total Bradford Protein Concentration g/1
1	4	44.5 ± 5.4		
3	4	57.0 ± 3.3	5	25.0 ± 5.4
5	7	80.1 ± 6.9		
7	17	92.0 ± 3.2		
9	16	91.0 ± 2.4	6	26.3 ± 1.5
11	18	82.5 ± 4.3		
13	8	90.2 ± 3.1		
15	6	84.5 ± 4.2		
17	9	70.2 ± 4.5		
21	9	42.6 ± 6.2	6	32.8 ± 3.8

The % free B was low at day 1, increased to reach a maximum value by day 7, remained high until day 13 and then decreased to a value of 42.6 ± 6.2 at day 21 (Fig. 1.4). There was an inverse linear relationship between the adrenal to body weight ratio and the % free B from day 5 to day 21 (Fig. 1.5). Despite the changes in the % free B observed there was no significant change in the total plasma protein concentration over the first 3 weeks post-partum.

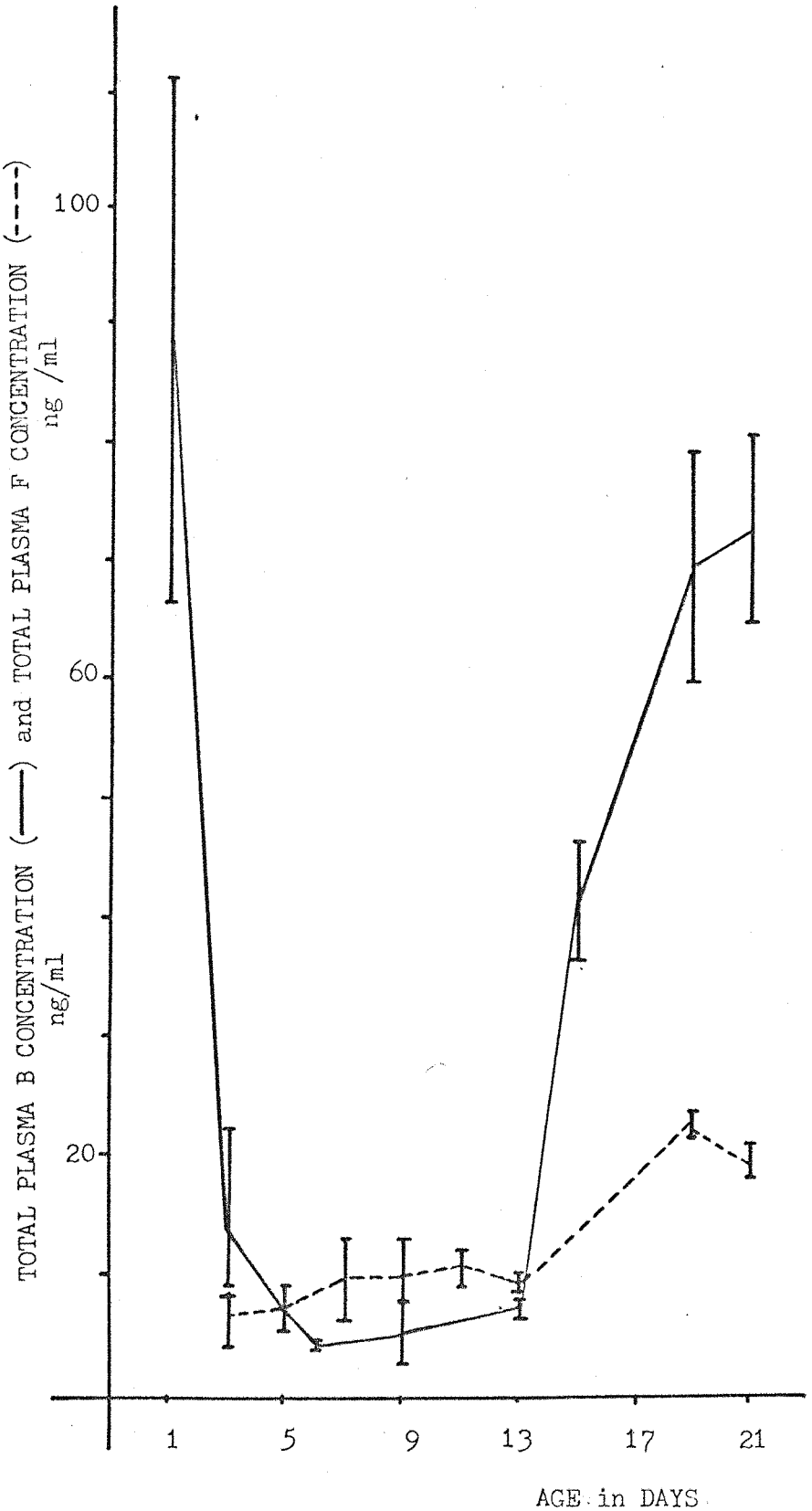


Fig. 1.2 Total plasma B concentration and total plasma F concentration over the postnatal period.

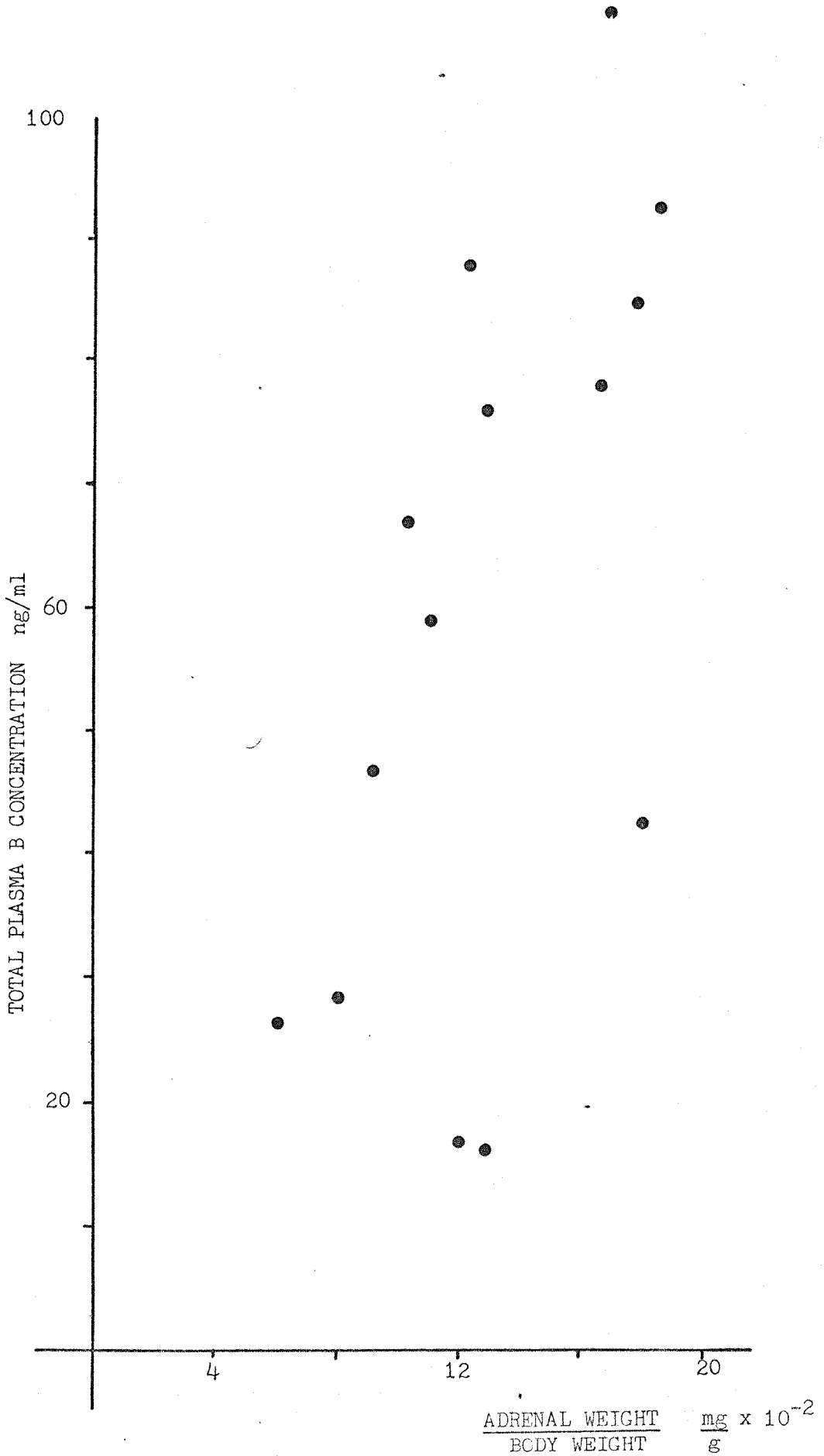


Fig. 1.3 Total plasma B concentration against adrenal to body weight during the postnatal period.
slope = 7.9, intercept = 4.5, $R^2 = 0.49$.

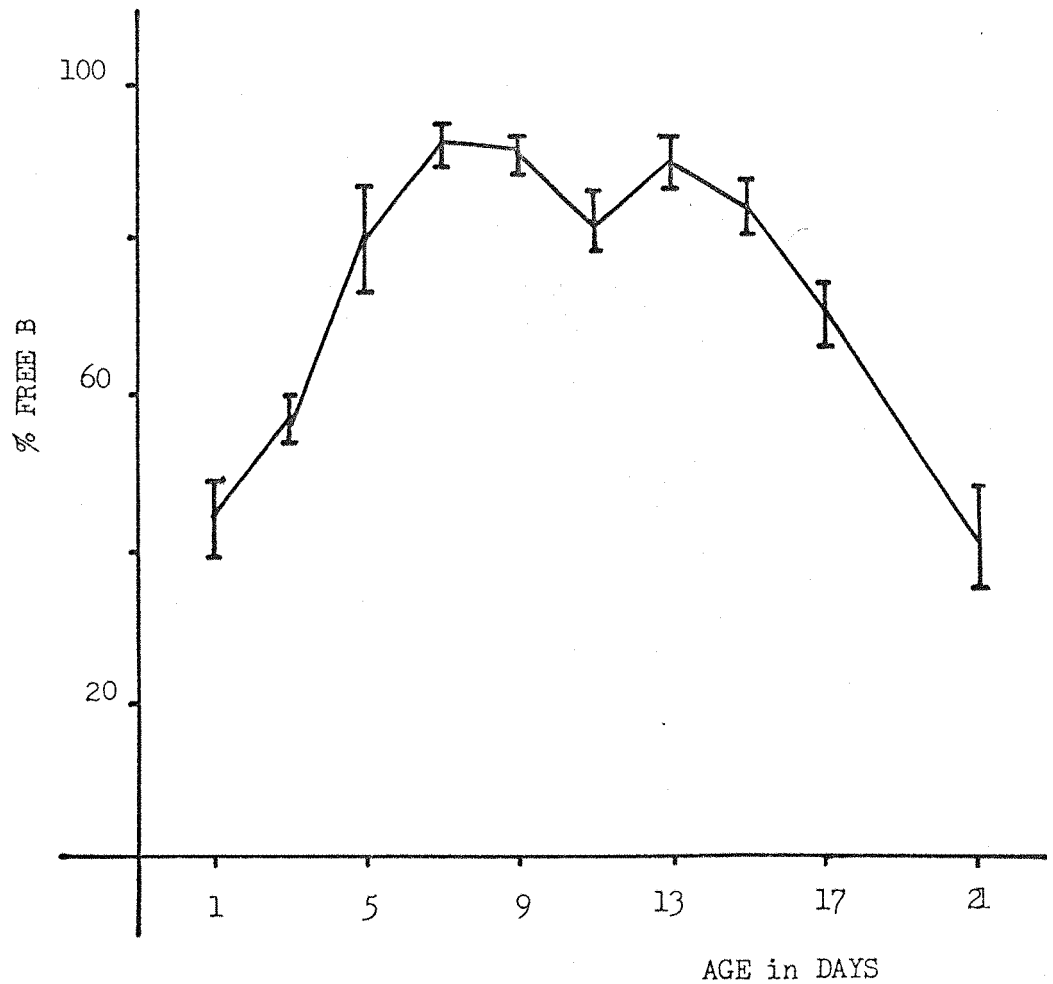


Fig. 1.4 % free B during the postnatal period.

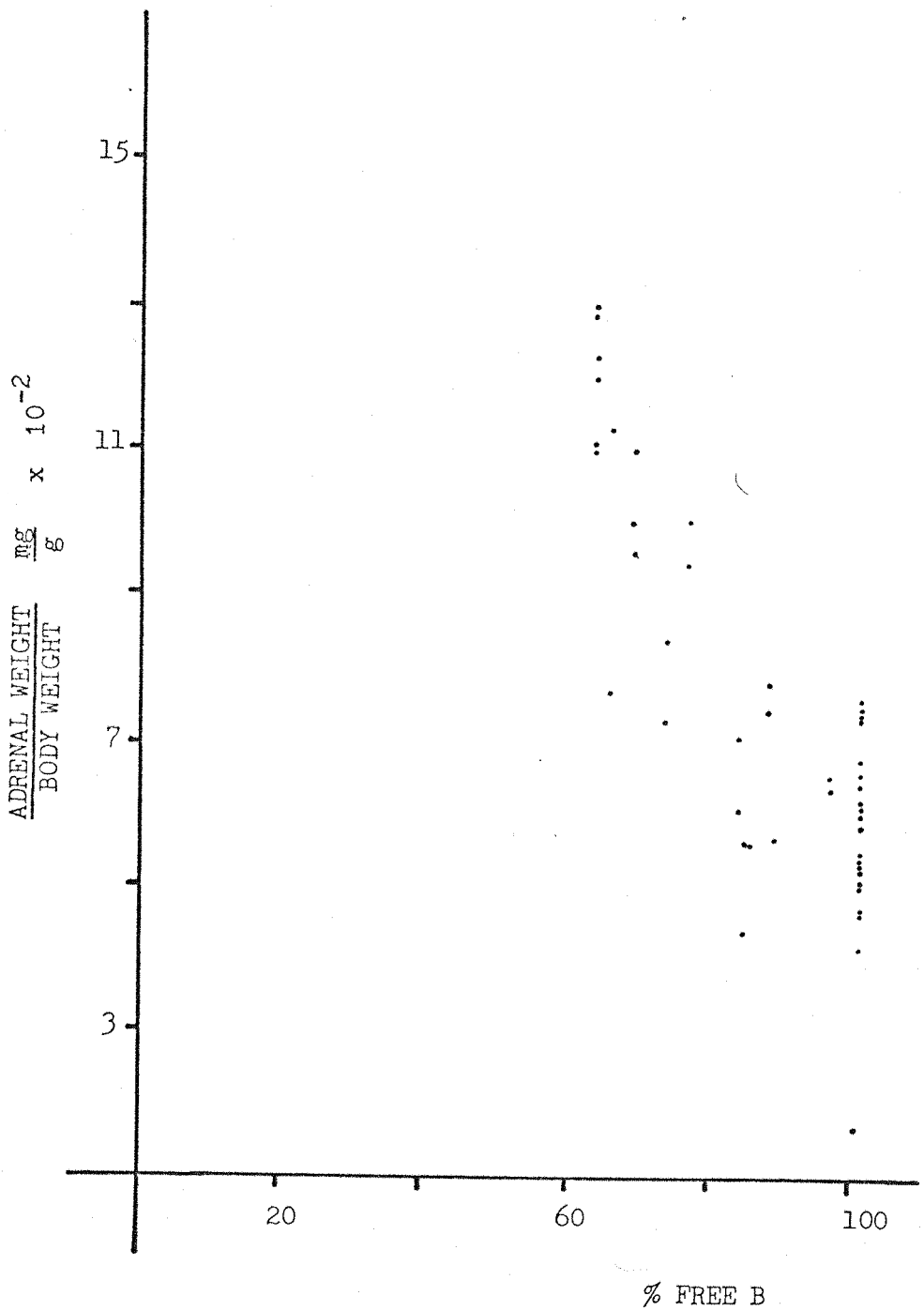


Fig. 1.5 Adrenal to body weight ratio against % free B during the postnatal period
slope =-20.6, intercept = 15.2, R^2 = 0.68

COMMENT

The relative adrenal weight and the total plasma B concentration decreased during the 1st postnatal week, remained low during the 2nd week and then increased to reach adult values by day 21. The % free B changed in an inverse manner over the same period. The total plasma F concentration varied little over this period thus, at day 3 and day 21 there was more B present in the plasma than F but at day 9 there was more F present than B (see Fig. 1.2).

A change in % free B indicates a change in the proportion of the hormone concentration which is physiologically active. Thus, as the % free B increased from birth to day 9 there was an increase in the free active plasma B concentration. It is probable that this high active plasma B concentration will feed back to the pituitary to suppress ACTH secretion and this decrease in ACTH secretion may reduce the rate of adrenal growth and secreting capacity. The % free B decreased from day 9 to day 21 thus there was a decrease in the proportion of the hormone which was active and so there may be a reduction in the negative feedback inhibition on the pituitary. The resulting increase in the plasma ACTH concentration may be responsible for the increased adrenal growth and secretion seen over this period.

SECTION 2

Introduction

The activity of the pituitary adrenal axis can be monitored at 3 levels by measuring the changes in plasma B concentration in response to acute challenges. Insulin induced hypoglycaemia can be used to test the responsiveness of the entire axis. The effect of releasing the animals own ACTH can be examined using ADH and exogenous ACTH can be used to test the responsiveness of the adrenal itself. The responsiveness of the adrenal axis in 3, 9 and 21 day old rats to these agents was investigated.

Experimental Procedure

Litters were removed from their mothers at the appropriate ages. They were weighed and injected with either the test substance or saline ip. Insulin (BDH Chemicals) was given in a saline suspension. 3 day old rats received 1 mU, 9 day old rats received 2 mU and 21 day old rats received 5 mU. Synthetic ADH (Pitressin, Parke Davis) was administered at a dose of 1 mU to day 3 rats, 2 mU to day 9 rats and 5 mU to day 21 rats. Synthetic ACTH (Synacthen, CIBA) was given at a dose of 0.25 ug/rat at day 3, 0.5 ug/rat at day 9 and 1.25 ug/rat at day 21. The rats were killed by decapitation 30 minutes after the ACTH and the ADH injection and 60 minutes after the insulin injection. Trunk blood was collected and after centrifugation the plasma stored at -20°C until assay. For the ACTH treated animals only, when all the animals from one litter had been killed, the left adrenal was removed from each rat, cleared of fat and connective tissue and weighed. Samples from 4-6, 3 day old rats and 2-3, 9 day old rats were pooled together. At day 21 no pooling was necessary as adequate plasma could be collected for assay. The % free B and the total plasma B concentration were determined for each plasma sample. The plasma glucose concentration was measured for the insulin treated groups only.

Results

The response of young rats to an acute ACTH challenge

Table 2.1: % Free B

	n	% Free	
day 3	10	56.2 ± 2.0	$p < 0.001$
day 9	19	93.0 ± 0.2	
day 21	26	49.4 ± 4.6	$p < 0.001$

Table 2.2: Adrenal to Body Weight Ratio

	n	$\frac{\text{Adrenal Weight}}{\text{Body Weight}}$ $\frac{mg}{g} \times 10^{-2}$	
day 3	35	16.9 ± 0.8	$p < 0.001$
day 9	34	6.2 ± 0.3	
day 21	19	21.9 ± 0.5	$p < 0.001$

Table 2.3: Total Plasma B Concentration After Saline or ACTH

		n	Total Plasma B Concentration ng/ml	
day 3	saline	6	21.5 ± 5.8	$p < 0.001$
	ACTH	7	81.2 ± 7.3	
day 9	saline	9	4.4 ± 1.1	$p < 0.02$
	ACTH	9	11.1 ± 2.2	
day 21	saline	12	132.4 ± 8.5	NS
	ACTH	14	150.8 ± 12.3	

Table 2.4: Increment in Total Plasma B Concentration Caused by ACTH (ACTH Treated Animals - Saline Treated Controls)

	n	Increment Caused by ACTH ng/ml	
day 3	7	59.7 ± 7.3	p < 0.01
day 9	9	6.7 ± 2.2	
day 21	14	18.4 ± 12.3	NS

The % free B was low at day 3, increased to a high value by day 9 and then decreased by day 21. The adrenal to body weight ratio changed in a similar but inverse way to the % free B. 3 day old rats responded to ACTH injection with a large increment in total plasma B concentration whilst 9 and 21 day old rats responded with a small increment in total plasma B concentration (Fig. 2.1).

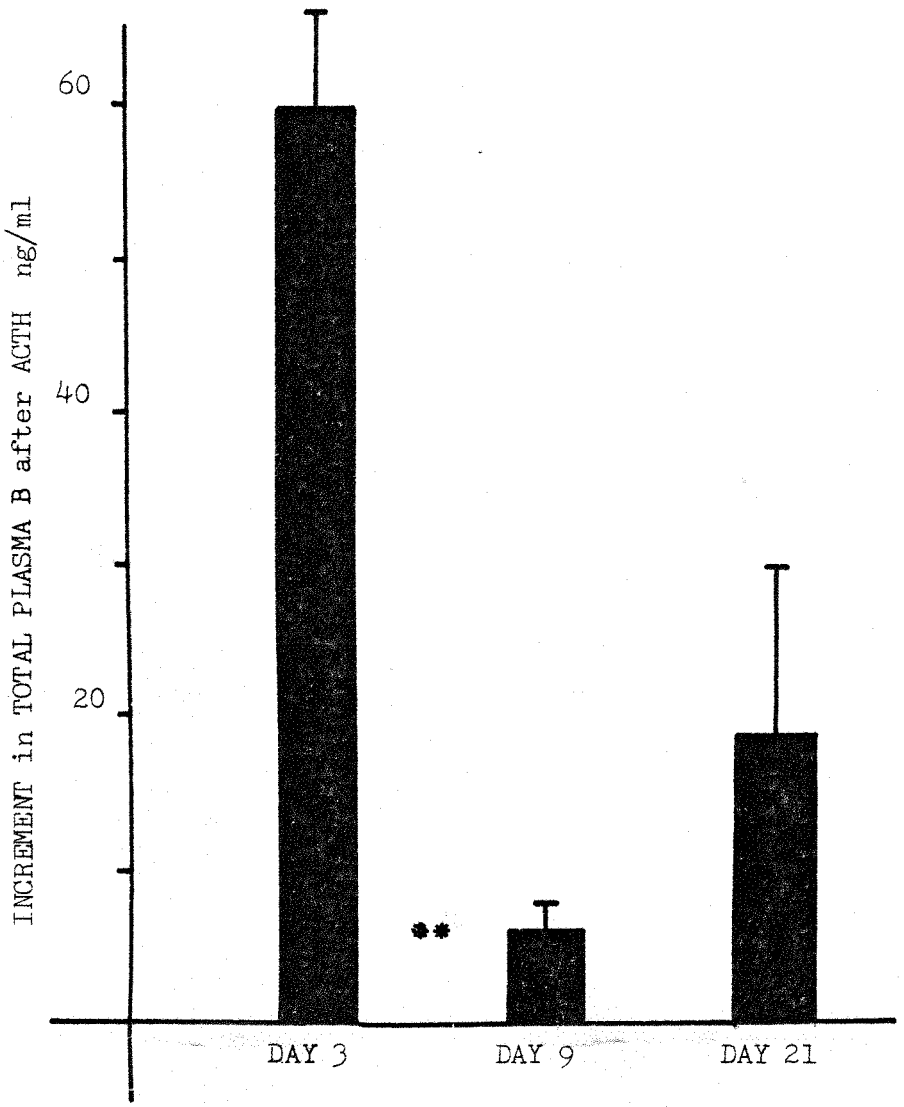


Fig. 2.1 Increment in total plasma B concentration caused by exogenous ACTH in 3, 9 and 21 day old rats.

The response of young rats to an acute ADH challenge

Table 2.5: % Free B

	n	% Free B	
day 3	11	38.9 ± 4.5	$p < 0.001$
day 9	15	82.3 ± 3.3	
day 21	17	37.3 ± 2.7	$p < 0.001$

Table 2.6: Total Plasma B Concentration After Saline or ADH

		n	Total Plasma B Concentration ng/ml	
day 3	saline	6	14.2 ± 3.3	$p < 0.001$
	ADH	6	80.7 ± 9.7	
day 9	saline	7	5.6 ± 1.0	NS
	ADH	9	6.3 ± 1.5	
day 21	saline	7	117.2 ± 6.7	NS
	ADH	8	110.2 ± 7.8	

Table 2.7: Increment in Total Plasma B Concentration Caused by ADH

	n	Increment Caused by ADH ng/ml	
day 3	9	66.5 ± 9.7	$p < 0.001$
day 9	9	0.7 ± 1.5	NS
day 21	9	- 7.0 ± 7.9	

Again similar values of % free B have been observed at day 3, 9 and 21. At day 3 the rat responded to an acute ADH challenge with a large increment in total plasma B concentration but at day 9 and day 21 no response was observed (Fig. 2.2).

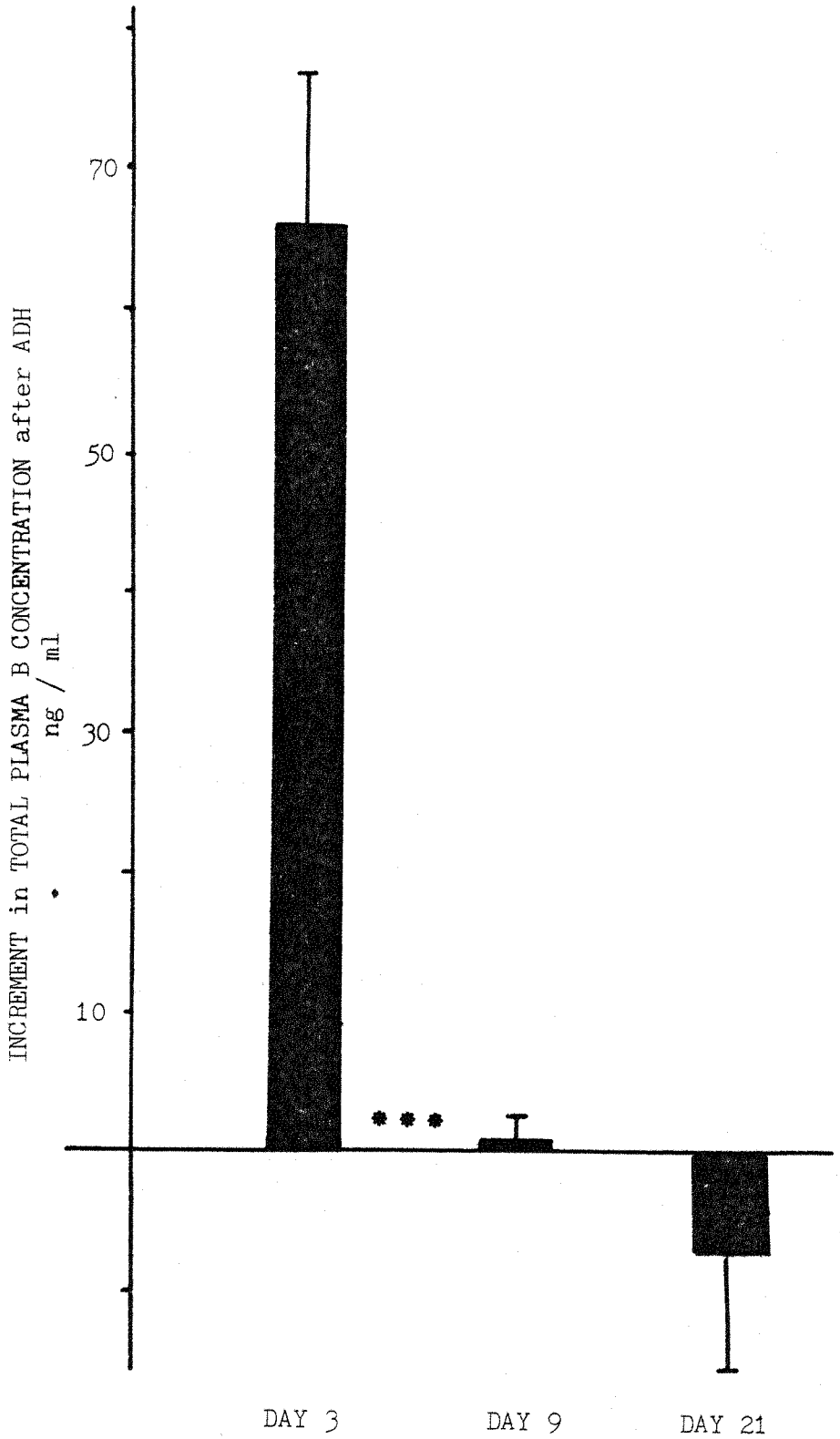


Fig. 2.2 Increment in total plasma B concentration caused by ADH in 3, 9 and 21 day old rats.

The response of young rats to an acute insulin challenge.

Table 2.8: % Free B

	n	% Free B	
day 3	5	72.8 ± 4.7	
day 9	7	91.7 ± 2.0	$p < 0.01$
day 21	6	73.8 ± 0.6	$p < 0.001$

Table 2.9: Total Plasma B Concentration and Plasma Glucose Concentration After Saline or Insulin

		n	Total Plasma B Concentration ng/ml		n	Plasma Glucose Concentration mg%	
day 3	saline	6	37.7 ± 4.8	$p < 0.01$	6	79.8 ± 8.2	$p < 0.01$
	insulin	6	76.4 ± 10.1		6	37.2 ± 8.6	
day 9	saline	7	8.0 ± 1.8	$p < 0.001$	7	104.0 ± 9.1	$p < 0.001$
	insulin	7	33.5 ± 3.3		7	40.3 ± 9.1	
day 21	saline	6	134.0 ± 10.4	$p < 0.01$	6	81.7 ± 6.8	$p < 0.01$
	insulin	6	182.6 ± 10.5		5	20.0 ± 14.9	

Table 2.10: Increment in Total Plasma B Concentration and Decrement In Plasma Glucose Concentration Caused by Insulin

	n	B Increment ng/ml		n	Glucose Decrement mg%	
day 3	6	38.7 ± 10.1	NS	6	42.6 ± 8.6	$p < 0.05$
day 9	7	25.5 ± 3.3	$p < 0.05$	7	63.7 ± 9.1	
day 21	6	48.6 ± 10.5		5	61.7 ± 14.9	NS

Insulin treatment caused a fall in plasma glucose concentration at day 3, 9 and 21. Insulin was less effective at day 3 than at day 9 ($p < 0.05$). The increments in the total plasma B concentration produced at day 21 and day 3 were similar but greater than that observed at day 9 (Fig. 2.3).

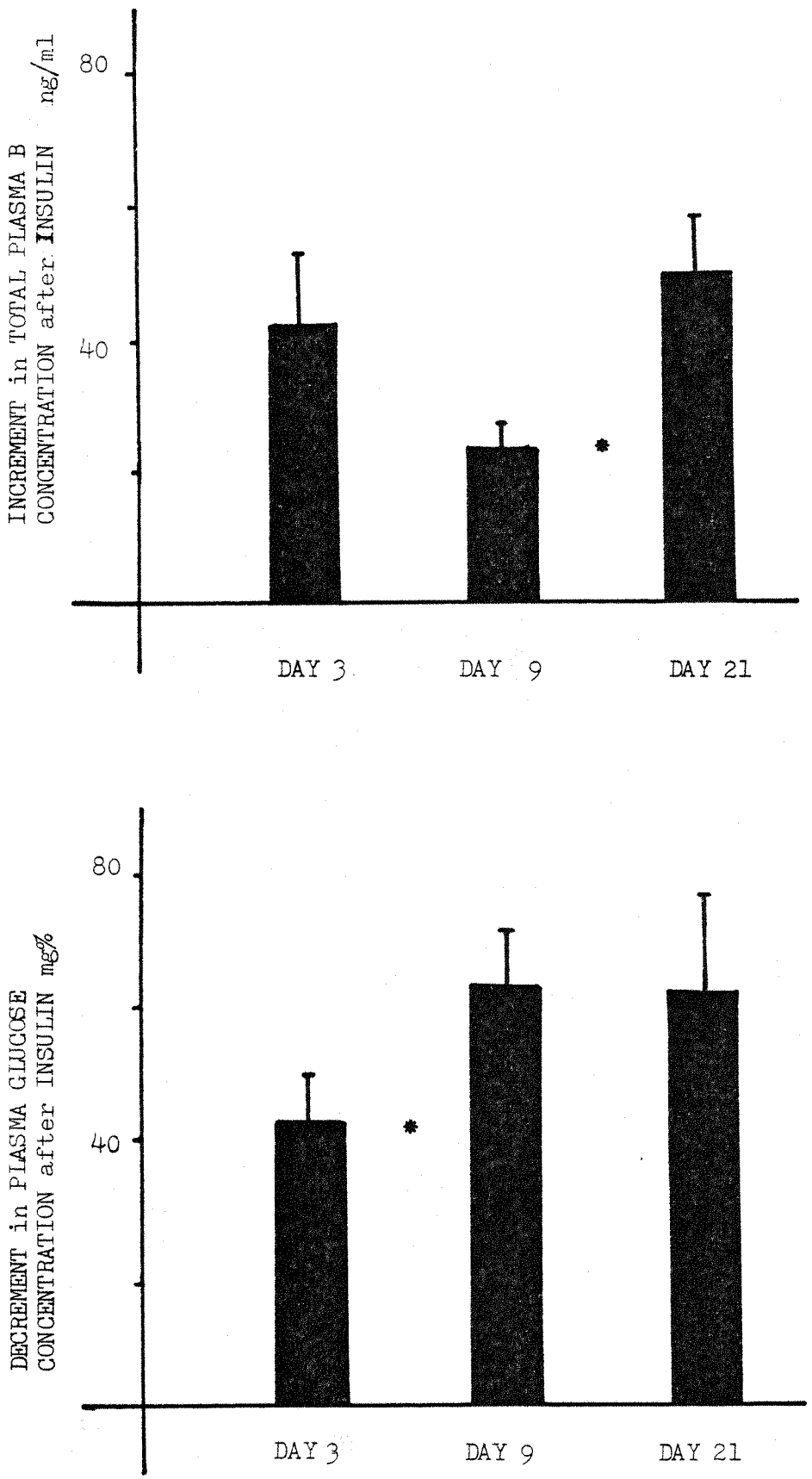


Fig. 2.3 Increment in total plasma B concentration and decrement in plasma glucose concentration caused by insulin in 3, 9 and 21 day old rats.

COMMENT

The % free B and relative adrenal weight showed the same trends observed in Section 1 thus establishing that both the % free B and relative adrenal weight were showing typical changes in these animals. Administration of ACTH, ADH or insulin gave a similar pattern of responsiveness during the postnatal period. The day 3 rat adrenal was very responsive but this response was lost by day 9 and did not return by day 21. However, as illustrated in Table 2.11, at day 21 an injection of saline itself caused a considerable increment in plasma B concentration, whilst this effect was not observed at day 3 or day 9.

Table 2.11: The Effect of Saline Injection Alone on the Total Plasma B Concentration, ng/ml

Age		Saline Injection 30 Mins Prior to Killing	n	Untreated (From Section 1)	
day 3	6	21.5 ± 5.8	4	15.6 ± 6.6	NS
day 9	9	4.4 ± 1.1	8	5.0 ± 2.4	NS
day 21	12	132.4 ± 8.5	9	73.0 ± 8.0	$p < 0.001$

Therefore the effect of exogenous ACTH was re-examined in 21 day old rats which had received 100 ug/rat dexamethasone phosphate ip (Decadron, Merck, Sharp and Dohme) 4 hours prior to ACTH or saline injection. Dexamethasone is a potent synthetic glucocorticoid and therefore would be expected to reduce ACTH secretion. When this secretion was blocked, 21 day old rats were seen to be very responsive to exogenous ACTH (Table 2.12, Fig. 2.4) and the increment in total plasma B concentration became 120.9 ± 36.0.

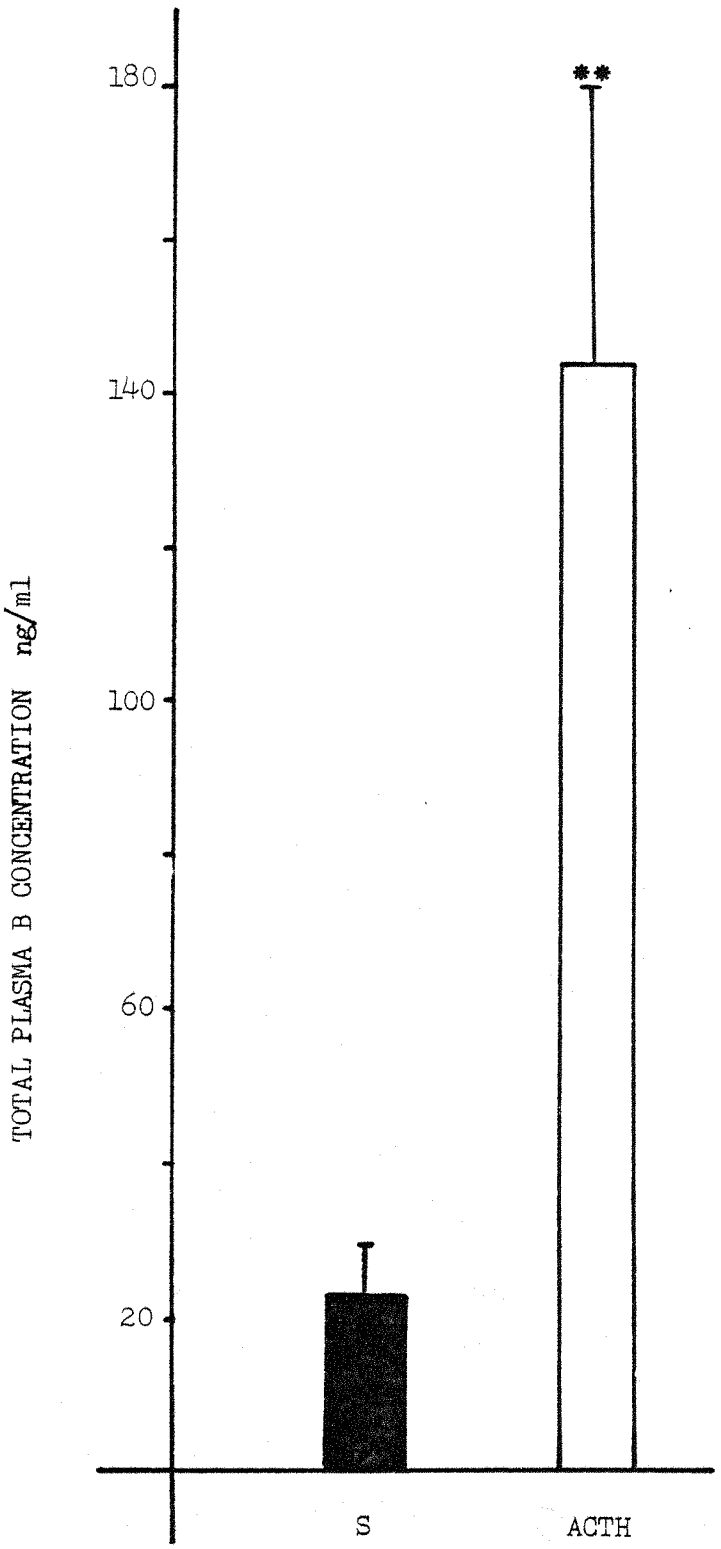


Fig. 2.4 Total plasma B concentration after saline or ACTH with pretreatment with dexamethasone in 21 day old rats.

Table 2.12: Total Plasma B Concentration After Saline or ACTH With Pretreatment with Dexamethasone

See Fig. 2.4

	n	Total Plasma B Concentration ng/ml	
saline	6	23.1 ± 7.5	
ACTH	7	144.0 ± 36.0	$p < 0.01$

The results indicate that there was an 8 fold decrease in the increment in total plasma B concentration in response to ACTH from day 3 to day 9 but a 17 fold increase in the increment from day 9 to day 21. The total secretion per mg of adrenal tissue also changed during this period. This can be seen if the increment in total plasma B concentration is expressed as a fraction of the adrenal to body weight ratio. At day 3 the value is 3.6, at day 9 1.1 and at day 21 5.5. Thus, it appears that the adrenal axis was losing activity during the 1st week post-partum and that there was a return of response during the 2nd week. However, over the same period the % free B changed considerably indicating a change in the proportion of the total plasma hormone concentration that was free and active. Thus, the adrenal axis during the postnatal period attempts to keep a constant response to stress in terms of the free plasma B concentration by altering the adrenal B production. From day 3 to day 9 as the % free B increased the total secretion decreased whilst from day 9 to day 21 when the % free B decreased the total secretion increased.

SECTION 3

Introduction

The decrease in total plasma B concentration and the loss of adrenal responsiveness to exogenous ACTH seen during the 1st week post-partum makes the rat adrenal axis appear as if there were little or no ACTH drive to stimulate adrenal growth and secretion.

The plasma ACTH concentration during the postnatal period was measured. The effect of supplying an ACTH drive, by administering depot ACTH, on the responsiveness of the adrenal to an acute ACTH challenge, was investigated.

Experimental Procedure

a. For the Plasma ACTH Measurements

Litters were taken from their mothers at the appropriate ages and killed by decapitation as quickly as possible. Trunk blood was collected and after centrifugation the plasma was separated, flash frozen using liquid nitrogen and stored at -20°C until assay. 1 plasma sample was collected per litter at day 3 and day 9 and 2 samples per litter at day 21.

b. For the Depot ACTH Experiments

6.25 ug of depot ACTH (Synacthen Depot, Ciba) was injected into 4 day old rats. At day 9 the litters were taken from their mothers, weighed and injected with 0.5 ug of ACTH (Synacthen, Ciba) or saline ip. 30 minutes later they were killed by decapitation. Trunk blood was collected, centrifuged and the plasma stored at -20°C until assay. Samples from 2-3 rats were pooled together. The left adrenal was removed from each rat, cleared of fat and connective tissue and weighed. Plasma B concentration and % free B were determined.

Results

Table 3.1: Plasma ACTH Concentration During the Postnatal Period in the Rat

See Fig. 3.1

Age	n	Plasma ACTH Concentration pg/ml	
3	3	151 ± 56	NS
9	4	241 ± 26	p < 0.02
21	4	451 ± 56	

Table 3.2: % Free B After Saline or Depot ACTH IN 9 DAY OLD RATS

	n	% Free	
saline day 4	14	80.9 ± 3.0	NS
depot ACTH day 4	9	84.2 ± 2.3	

Table 3.3: Adrenal to Body Weight Ratio After Saline or Depot ACTH on Day 4 AT DAY 9

	n	$\frac{\text{Adrenal Weight}}{\text{Body Weight}}$	$\frac{mg}{g} \times 10^{-2}$
saline day 4	19	7.0 ± 0.4	p < 0.001
depot ACTH day 4	15	13.0 ± 0.5	

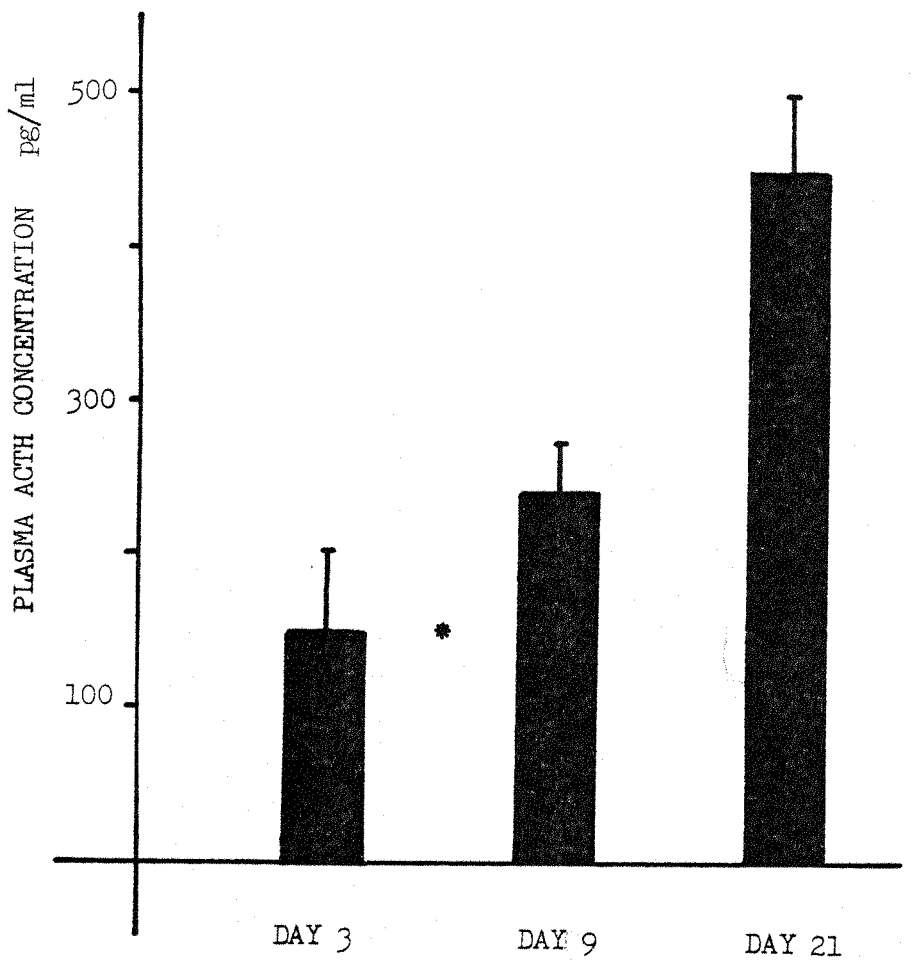


Fig. 3.1 Plasma ACTH concentration at day 3, day 9 and day 21 post-partum.

Table 3.4: Total Plasma B Concentration After ACTH or Saline on Day 9 with Pretreatment with Depot ACTH or Saline on Day 4

		n	Total Plasma B Concentration ng/ml	
saline day 4	saline	7	10.0 ± 2.3	$p < 0.02$
	ACTH	7	17.0 ± 1.2	
depot ACTH	saline	7	29.3 ± 2.9	$p < 0.01$
	ACTH	8	57.8 ± 4.6	

Table 3.5: The Increment in Total Plasma B Concentration Caused by ACTH After Pretreatment with Saline or Depot ACTH

See Fig. 3.2

	n	Increment Caused by ACTH ng/ml	
saline day 4	7	7.0 ± 1.2	$p < 0.001$
depot ACTH day 4	8	28.5 ± 4.6	

Table 3.6: The Effect of Saline Injection on the Total Plasma B Concentration After Pretreatment with Depot ACTH on Day 4

	n	Total Plasma B Concentration ng/ml	
untreated	8	5.6 ± 0.5	$p < 0.001$
saline treated	7	29.3 ± 2.9	

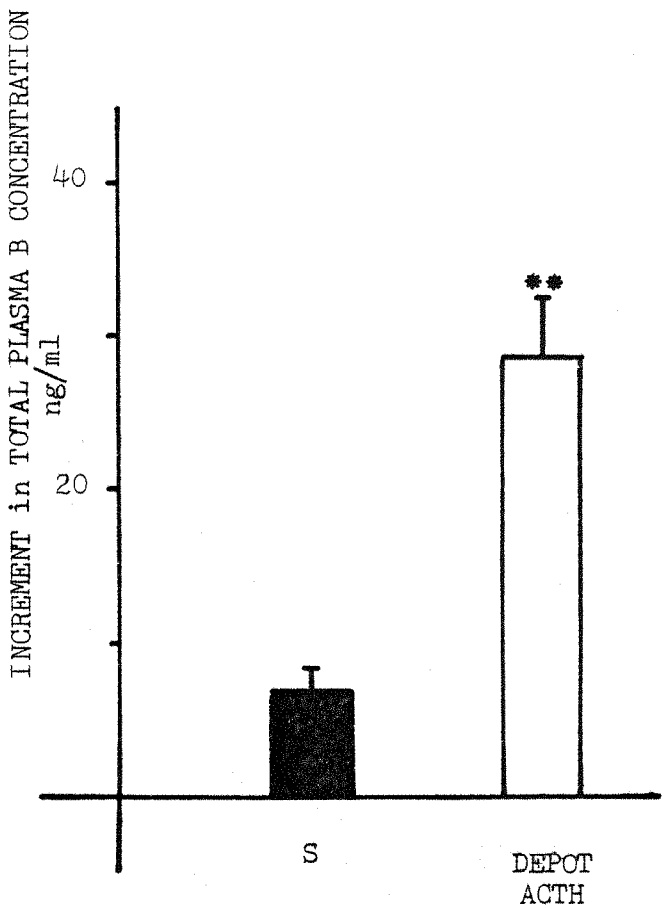


Fig. 3.2 Increment in total plasma B concentration caused by exogenous ACTH in 9 day old rats after either saline or depot ACTH on day 4.

COMMENT

The plasma ACTH concentration was significantly lower at day 3 and day 9 compared with day 21. This suggests that there is a reduced ACTH drive during the 1st postnatal week.

The ability of depot ACTH to cause adrenal growth and restore adrenal responsiveness to exogenous ACTH during the 1st week post-partum also suggests that the young rat adrenal is normally lacking pituitary stimulation.

SECTION 4

Introduction

Section 1 demonstrated changes in % free B with inverse but similar changes in total plasma B concentration and adrenal to body weight ratio during the postnatal period. It was possible to show correlations between these parameters suggesting that % free B plays an important role in determining the total plasma B concentration and the rate of adrenal growth. If there is a causal relationship between these parameters it should be possible to modify the total plasma B concentration and adrenal to body weight ratio by changing the % free B.

The fall in % free B after day 9 is the result of an increase in plasma corticosterone binding protein (CBG) concentration (Van Baelen, Vandoren, and De Moor, 1977), probably through an increase in neonatal CBG synthesis. Thyroid hormones are known to increase the plasma binding capacity for B in the adult rat (Labrie, Raynaud, Ducommun, Fortier, 1964; Gala and Westphal, 1966). They achieve this by stimulating de novo CBG synthesis in the liver (Feldman, Mondon, Horner, Weiser, 1979). The activity of the rat thyroid is low at birth, and begins to increase at the beginning of the second week (Dussault and Labrie, 1975). This increase in the activity of the thyroid could be the physiological stimulus for the decrease in % free B through stimulation of CBG synthesis, seen at the beginning of the second postnatal week.

Therefore the effect of T_4 administration was studied in the 9 day old rat, when the % free B is normally high, to see if the % free could be decreased prematurely with compensatory increases in total plasma B concentration and relative adrenal weight. Firstly the administration of different doses of T_4 was studied. Schapiro and Norman (1967) established that 1.0 ug.gm body weight^{-1} of T_4 given daily to young rats from day 3 abolished the stress non-responsive period seen in 4-11 day old rats. They attributed this to the neural effects of T_4. This effect of T_4 administration might also result from an action on CBG synthesis. T_4 was administered to young rats at 3 different doses, (10 ug/rat - the same dose as Schapiro and Norman), 5 times this does (50 ug/rat) and a tenth of this dose

(1.0 ug/rat). The effect on % free B, total plasma B concentration and adrenal to body weight ratio was examined.

Experimental Procedure

For all experiments the litters were divided into 2 groups, one the experimental group and one the control group. The control group received saline with 10% ethanol whenever the experimental group received a dose of T_4 in saline with 10% ethanol. All injections were given subcutaneously and handling kept to a minimum to avoid maternal rejection of the litter. On day 9, litters were removed from their mothers, weighed and decapitated as quickly as possible. Trunk blood was collected and after centrifugation the plasma was stored at -20°C until assay. The left adrenal was removed from each rat, cleared of fat and connective tissue and weighed. The plasma samples were assayed for % free B and total plasma B concentration. T_4 treatment of the litters was carried out as follows:-

	Dose of T_4	Day of Injection				
		4	5	6	7	8
GROUP A	50 ug/rat	x	x	x	x	x
	10 ug/rat	x	x	x	x	x
	1 ug/rat	x	x	x	x	x
GROUP B	10 ug/rat	x		x		x
	10 ug/rat	x	x	x		
	10 ug/rat			x	x	x
GROUP C	10 ug/rat	x				
	10 ug/rat		x			
	10 ug/rat			x		
	10 ug/rat				x	
	10 ug/rat					x

All animals were killed on day 9.

Results

Group A: The effect of 3 different doses of T_4 given for 5 days on body weight, adrenal to body weight ratio, % free B and total plasma B concentration in the 9 day old rat

Table 4.1: The Effect of 5 Days of T_4 on Body Weight, (g)

See Fig. 4.1

Dose of T_4	n	Saline	n	T_4	
50 ug/rat	17	20.4 ± 0.8	19	18.9 ± 0.5	NS
10 ug/rat	20	17.2 ± 0.2	24	15.5 ± 0.4	$p < 0.01$
1 ug/rat	8	18.4 ± 0.8	10	19.1 ± 0.9	NS

Table 4.2: The Effect of 5 Days of T_4 on the Adrenal to Body Weight Ratio, $(\frac{mg}{g} \times 10^{-2})$

See Fig. 4.2

Dose of T_4	n	Saline	n	T_4	
50 ug/rat	28	5.7 ± 0.5	32	6.9 ± 0.5	NS
10 ug/rat	25	2.8 ± 0.5	22	5.5 ± 0.5	$p < 0.001$
1 ug/rat	8	5.6 ± 0.6	10	5.3 ± 0.7	NS

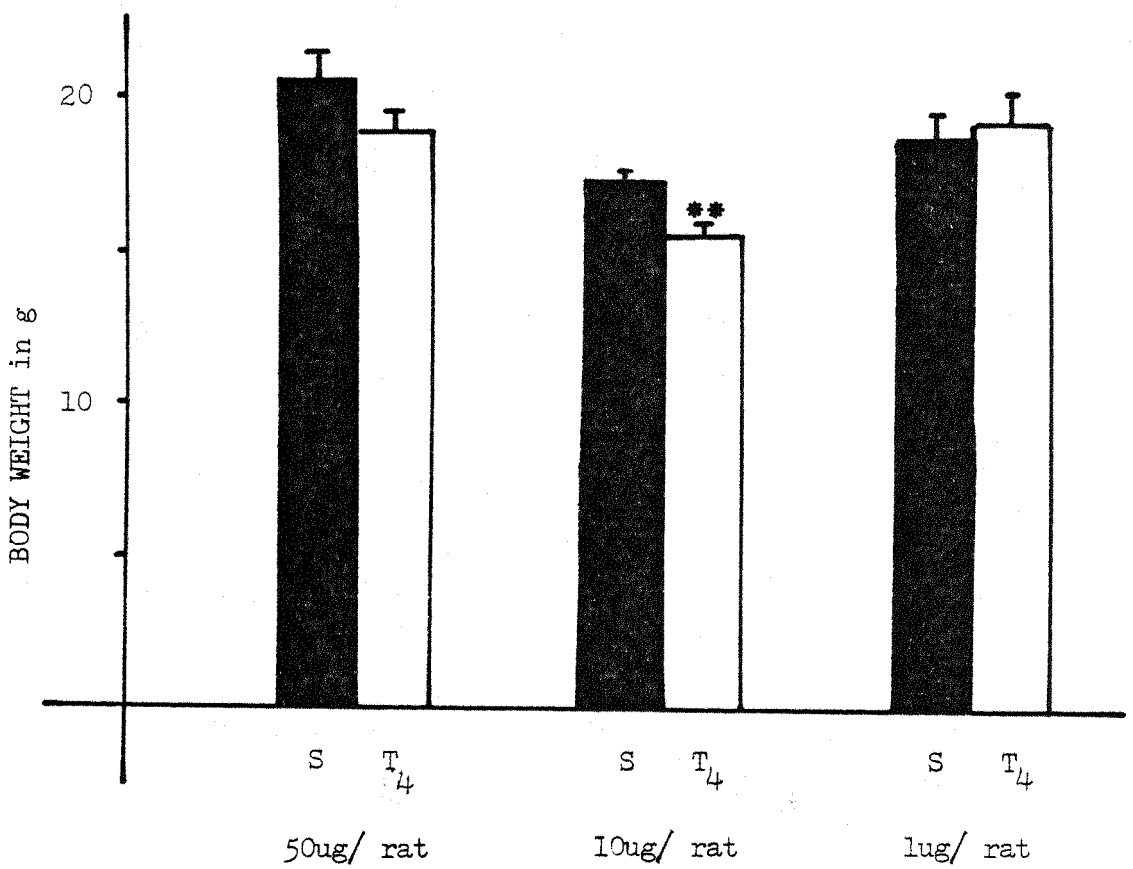


Fig. 4.1 The effect of 5 days of T_4 treatment on body weight (see text for details).

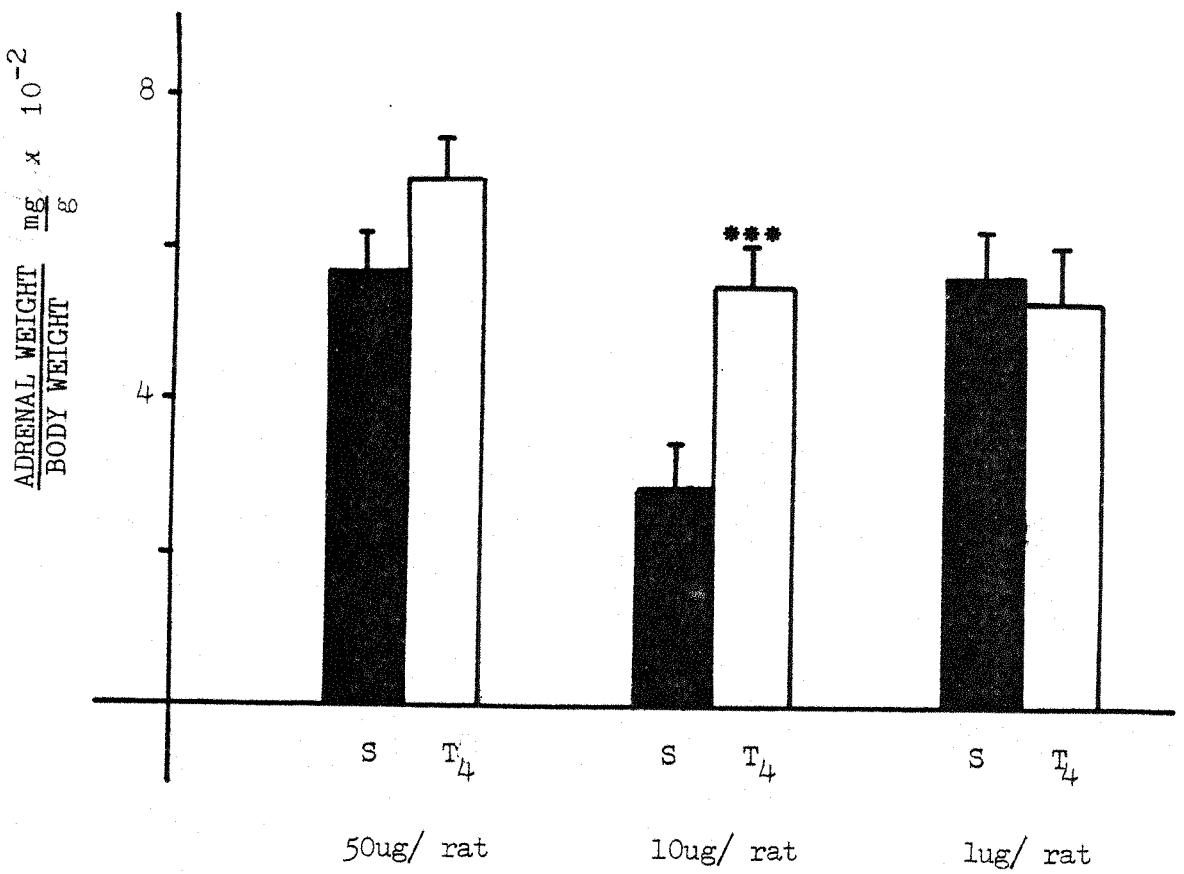


Fig. 4.2 The effect of 5 days of T_4 treatment on adrenal to body weight ratio (see text for details)

Table 4.3: The Effect of 5 Days of T_4 on % Free B

See Fig. 4.3

Dose of T_4	n	Saline	n	T_4	
50 ug/rat	15	83.8 ± 4.9	18	50.7 ± 1.7	$p < 0.001$
10 ug/rat	9	89.5 ± 1.8	11	44.6 ± 4.9	$p < 0.001$
1 ug/rat	4	74.9 ± 8.6	5	68.7 ± 9.2	NS

Table 4.4: The Effect of 5 Days of T_4 on the Total Plasma B Concentration (ng/ml)

See Fig. 4.4

Dose of T_4	n	Saline	n	T_4	
50 ug/rat	16	7.2 ± 1.5	17	32.2 ± 8.6	$p < 0.01$
10 ug/rat	8	5.0 ± 1.1	8	45.1 ± 7.7	$p < 0.001$
1 ug/rat	4	2.5 ± 0.8	5	4.4 ± 1.1	NS

Thus, 5 days of 1 ug T_4/rat had no significant effect on body weight, % free B, total plasma B concentration or adrenal to body weight ratio. 10 times this dose, 10 ug/rat caused significant changes in all the parameters. This was a maximal effect because there were no significant differences between the action of 50 ug/rat and 10 ug/rat on the changes in % free and total plasma B concentration. 50 ug/rat, however was less effective in causing changes in body weight and adrenal weight. Plasma T_4 concentrations were significantly greater after 5 days of 10 ug T_4/rat (111.5 ± 11.2 ng/ml, n = 6) compared with saline treated litter mates (21.5 ± 6.6 ng/ml, n = 6), $p < 0.001$.

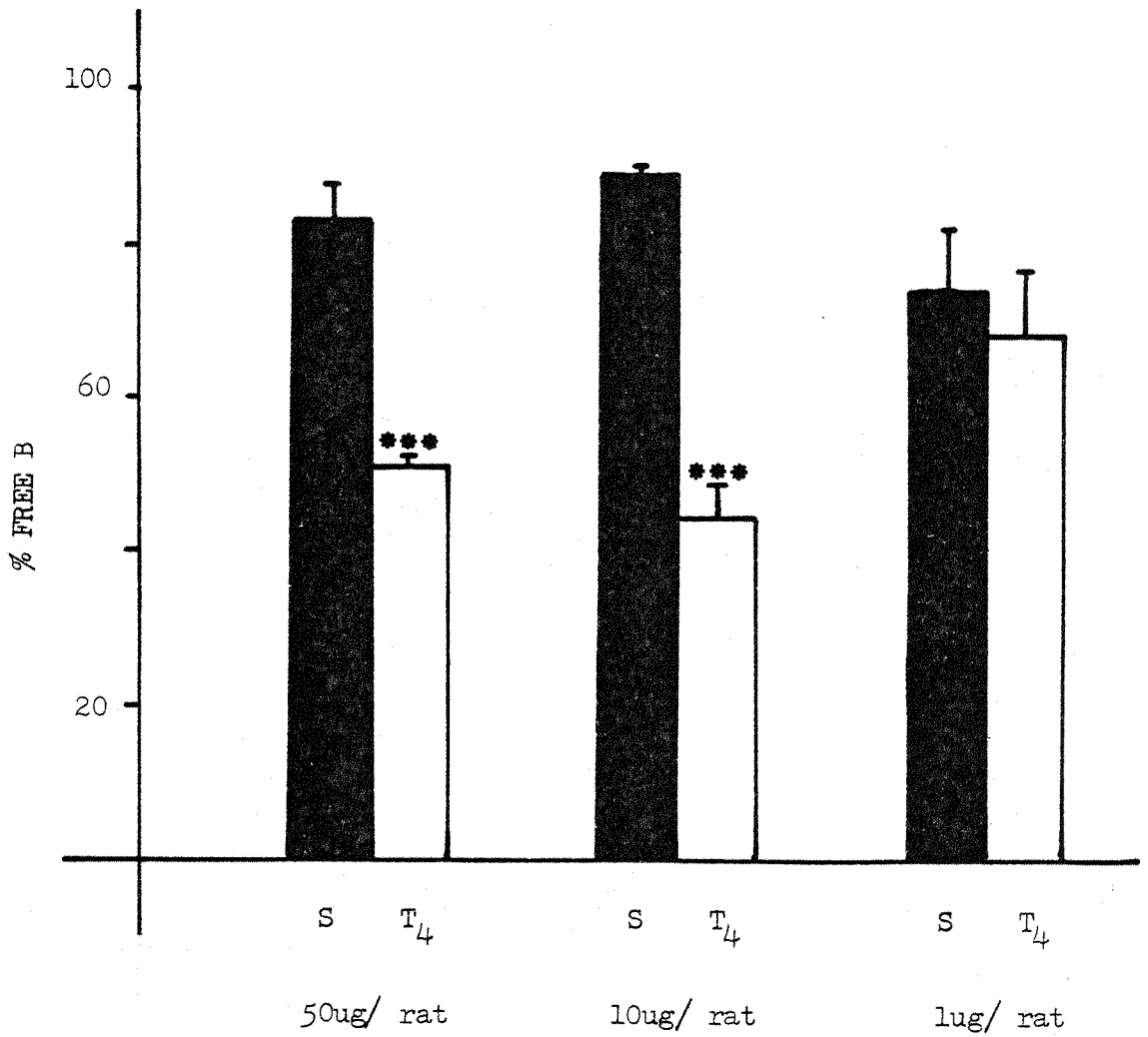


Fig. 4.3 The effect of 5 days of T_4 treatment on % free B (see text for details).

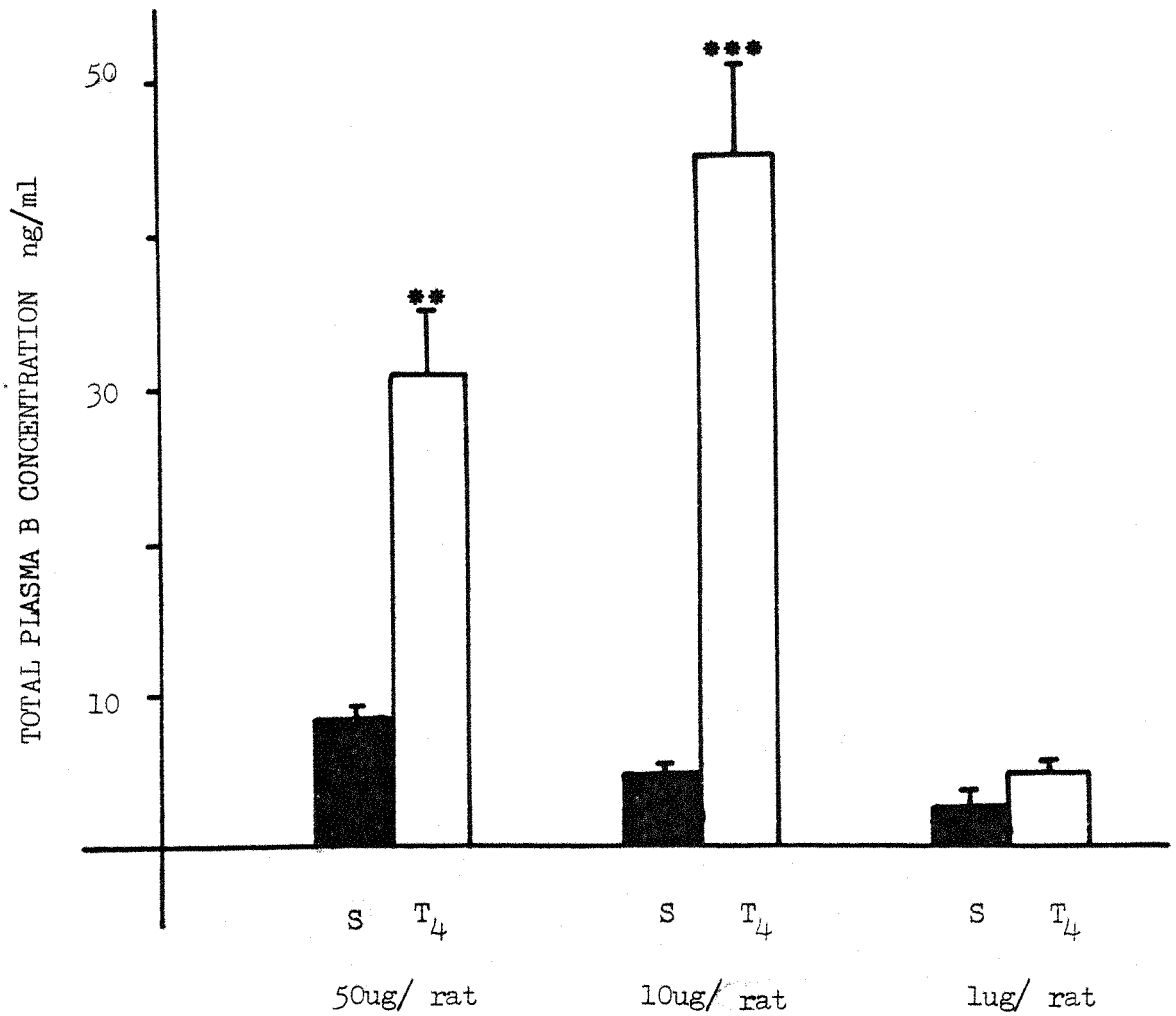


Fig. 4.4 The effect of 5 days of T_4 treatment on total plasma B concentration (see text for details).

Group B: The effect of 10 ug/rat of T_4 given for 3 days on % free B, total plasma B concentration and adrenal to body weight ratio

Table 4.5: The Effect of 3 Days of T_4 on % Free B

See Fig. 4.5

Day of Injection	n	Saline	n	T_4	
4, 5, 6	6	86.8 ± 3.3	6	32.6 ± 7.7	$p < 0.001$
6, 7, 8	7	98.5 ± 1.0	10	68.1 ± 5.5	$p < 0.001$
4, 6, 8	6	90.9 ± 2.5	7	30.2 ± 2.5	$p < 0.001$

Table 4.6: The Effect of 3 Days of T_4 on Total Plasma B Concentration, (ng/ml)

See Fig. 4.6

Day of Injections	n	Saline	n	T_4	
4, 5, 6	6	3.9 ± 0.9	6	15.9 ± 3.1	$p < 0.01$
6, 7, 8	9	15.7 ± 5.9	7	27.5 ± 6.6	NS
4, 6, 8	6	7.8 ± 1.5	7	32.7 ± 3.8	$p < 0.001$

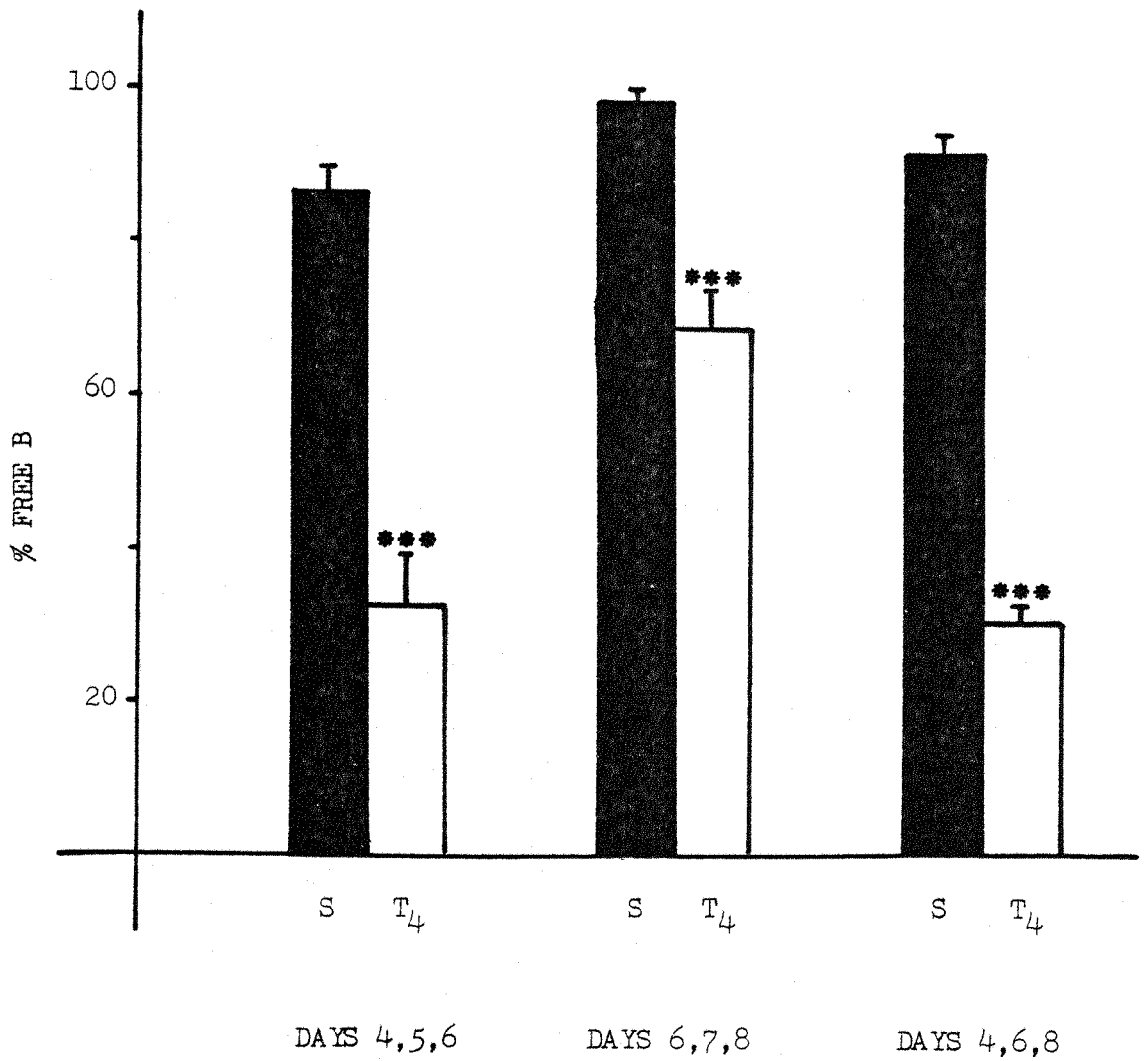


Fig. 4.5 The effect of 3 days of T_4 treatment on % free B (see text for details)

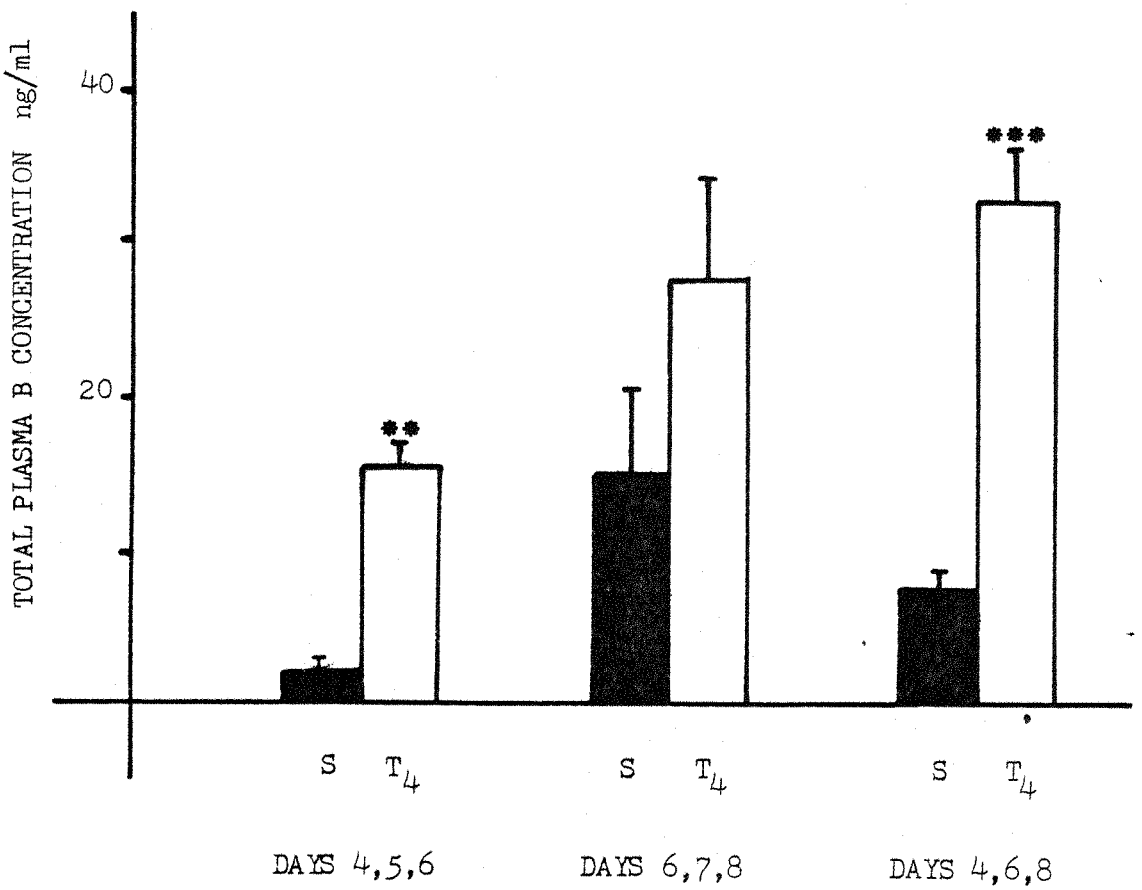


Fig. 4.6 The effect of 3 days of T_4 treatment on total plasma B concentration (see text for details).

Table 4.7: The Effect of 3 Days of T_4 on Adrenal to Body Weight Ratio, $(\frac{mg}{g} \times 10^{-2})$

See Fig. 4.7

Day of Injection	n	Saline	n	T_4	
4, 5, 6	10	15.1 ± 1.0	12	16.6 ± 0.4	NS
6, 7, 8	16	6.5 ± 0.4	22	7.8 ± 0.6	NS
4, 6, 8	16	9.3 ± 0.7	16	10.2 ± 0.7	NS

3 days of T_4 administration caused a significant decrease in % free B compared with saline-treated litter-mates. There was no significant difference between the groups injected on days 4, 5, 6 and 4, 6, 8 but both groups had a significantly greater response than T_4 given on days 6, 7, 8 ($p < 0.01$ and $p < 0.001$ respectively). T_4 given on days 6, 7, 8 caused no significant increase in total plasma B concentration whilst T_4 on days 4, 5, 6 and 4, 6, 8 caused a significant increase in total plasma B concentration. T_4 on days 4, 6, 8 had a significantly greater effect than T_4 on days 4, 5, 6 ($p < 0.01$). There was no significant effect of any of these T_4 treatments on adrenal to body weight ratio.

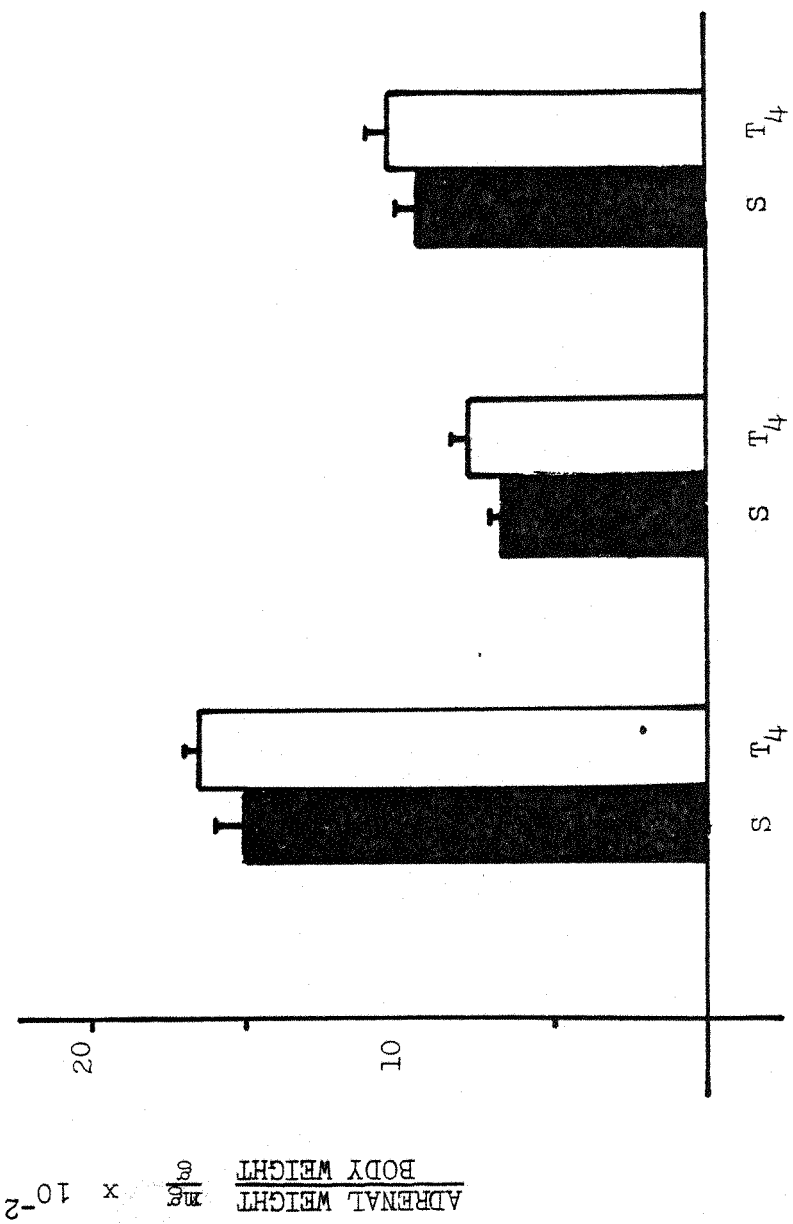


Fig. 4.7 The effect of 3 days of T_4 treatment on adrenal to body weight ratio (see text for details).

Group C: The effect of a single 10 ug/rat dose of T_4 on % free, total plasma B concentration and adrenal to body weight ratio

Table 4.8: The Effect of T_4 on % Free B

See Fig. 4.8

Day of Injection	n	Saline	n	T_4	
4	6	87.8 ± 4.9	6	43.2 ± 5.0	$p < 0.001$
5	6	86.1 ± 4.6	10	54.2 ± 5.9	$p < 0.01$
6	7	88.8 ± 2.4	6	69.3 ± 3.9	$p < 0.01$
7	6	92.4 ± 3.8	8	88.4 ± 4.0	NS
8	7	89.4 ± 6.3	6	83.3 ± 7.4	NS

Table 4.9: The Effect of T_4 on Total Plasma B Concentration, (ng/ml)

See Fig. 4.9

Day of Injection	n	Saline	n	T_4	
4	8	7.0 ± 2.4	5	13.2 ± 2.0	NS
5	5	6.2 ± 3.1	8	12.5 ± 2.7	NS
6	7	3.8 ± 0.4	6	11.2 ± 1.1	$p < 0.001$
7	4	6.3 ± 2.0	8	10.6 ± 1.7	NS
8	10	3.8 ± 1.7	11	3.6 ± 1.4	NS

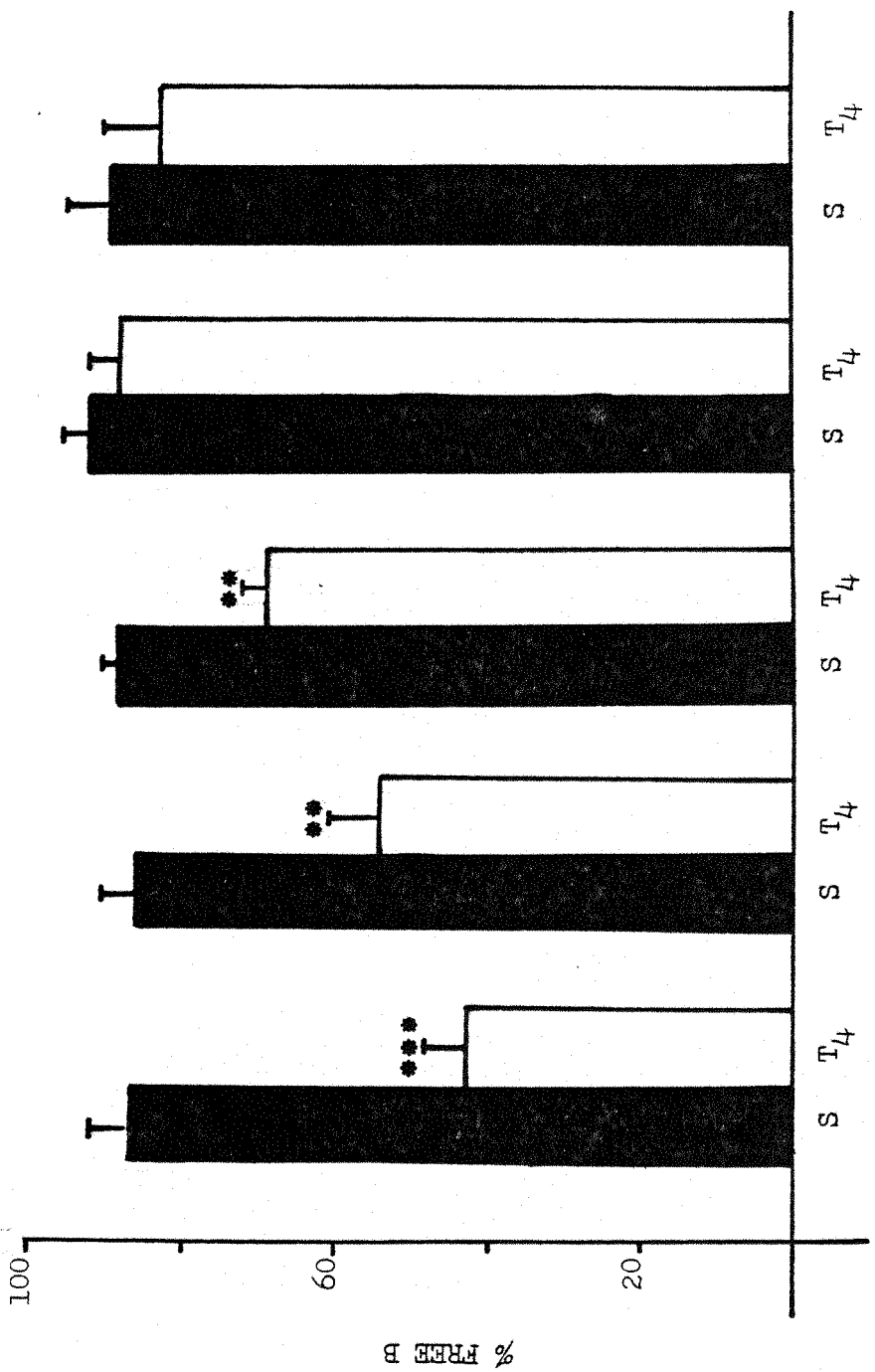


Fig. 4.8 The effect of the day of injection of a single dose of 10ug T_4/rat on % free B at day 9.

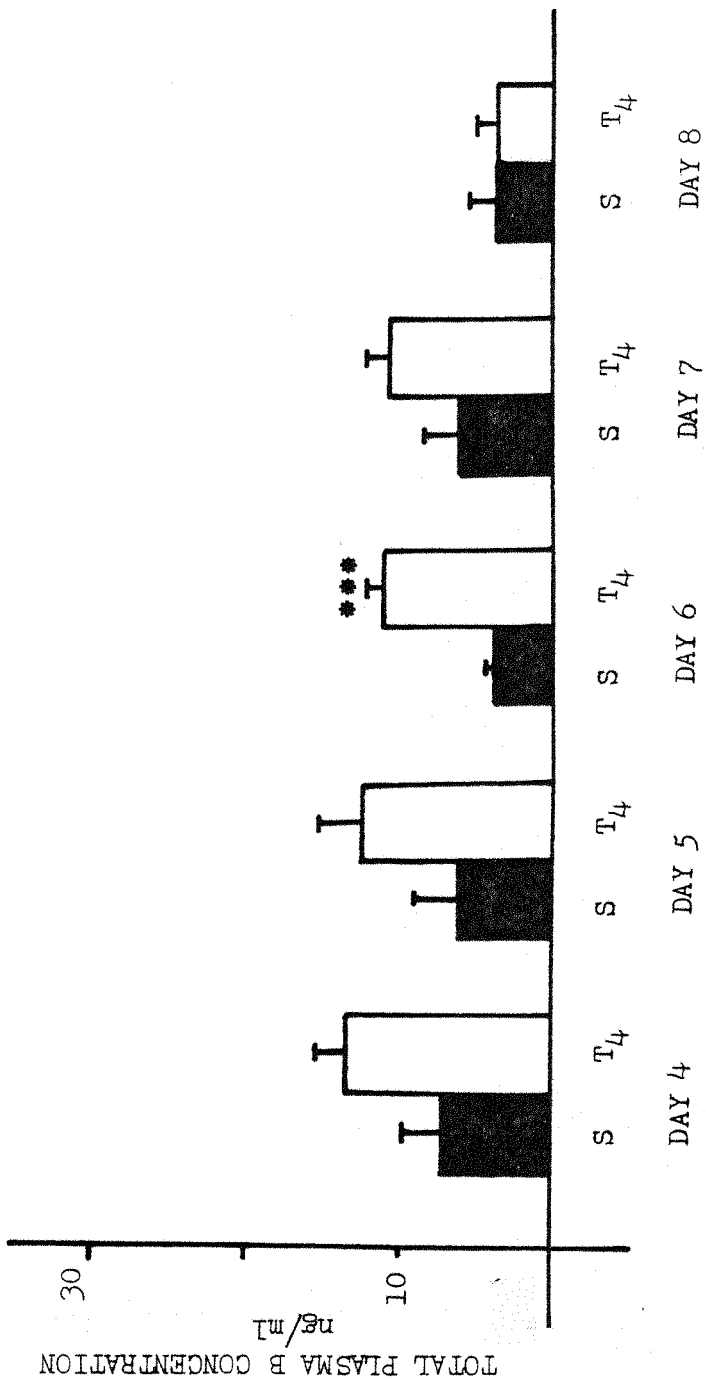


Fig. 4.9 The effect of the day of injection of a single dose of 10ug T_4/rat on total plasma B concentration at day 9.

Table 4.10: The Effect of T_4 on Adrenal to Body Weight Ratio, $(\frac{mg}{g} \times 10^{-2})$

See Fig. 4.10

Day of Injection	n	Saline	n	T_4	
4	11	8.8 ± 0.9	17	8.9 ± 0.6	NS
5	5	8.1 ± 0.5	21	8.5 ± 0.6	NS
6	10	5.1 ± 0.5	10	5.5 ± 0.4	NS
7	11	8.5 ± 0.8	4	10.9 ± 1.0	NS
8	7	8.9 ± 1.4	10	11.8 ± 1.3	NS

One single injection of T_4 had no significant effect given on day 7 or later, but injections given before day 7 caused a decrease in % free B. The effect of 10 ug/rat of T_4 on day 4 was significantly greater than the effect on the same dose of T_4 on day 6 ($p < 0.01$). These single injections of T_4 caused no significant increase in total plasma B concentration with the exception of T_4 given on day 6. There was no effect of single T_4 injections on adrenal to body weight ratio.

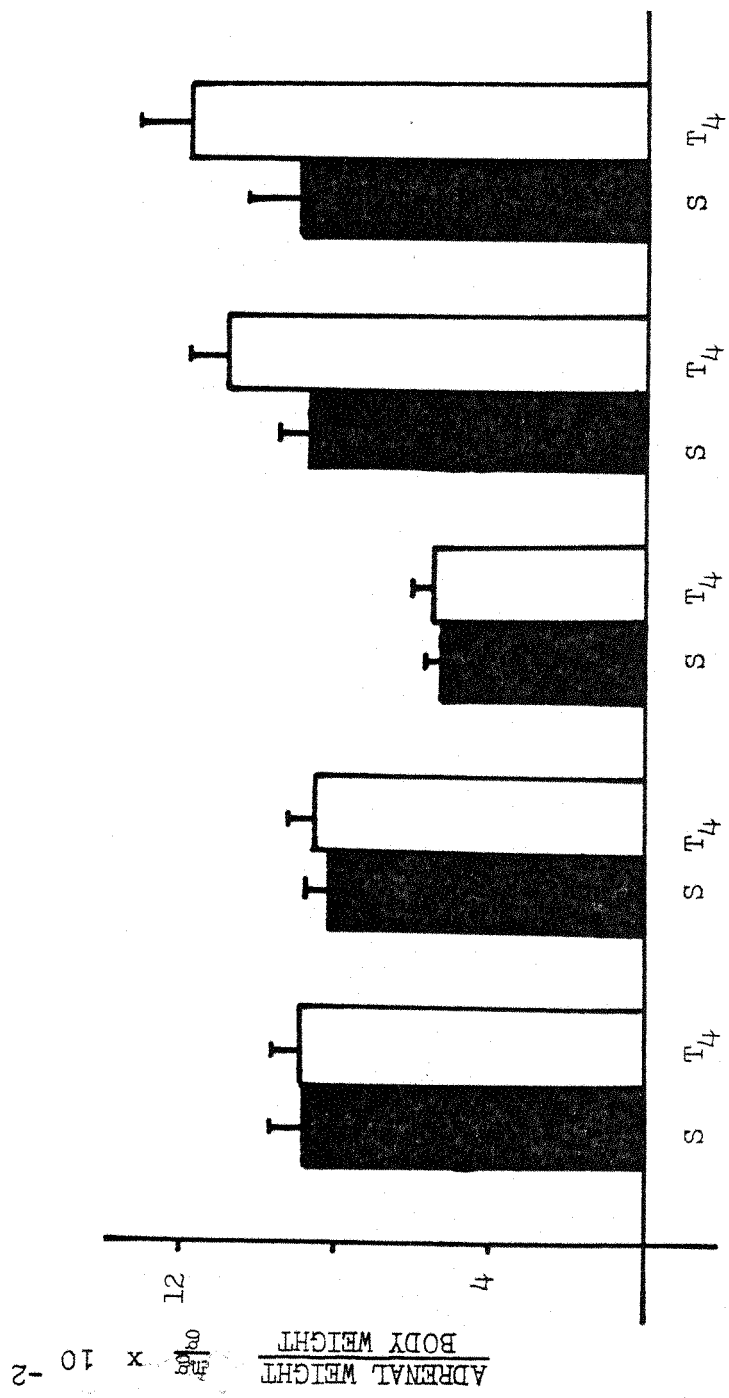


Fig. 4.10 The effect of the day of injection of a single dose of 10ug T_4/rat on adrenal to body weight ratio at day 9.

COMMENT

It has been possible to show that T_4 administration caused a premature fall in % free B. A single injection of 10 ug/rat of T_4 was sufficient to switch on CBG synthesis. There was no significant difference in the % free B between groups injected on day 4 only and groups which had received daily T_4 injections beginning on day 4. The magnitude of the fall in % free depended on the timing of the initial injection. Thus, a rat given T_4 on day 4 had a much greater response than a rat given T_4 on day 6. This effect probably results from the longer time available for CBG synthesis.

When there was a decrease in % free there was sometimes an increase in total plasma B concentration. However these changes were small unless there was an accompanying increase in adrenal to body weight ratio. Increases in adrenal to body weight ratio were only seen when the dose of T_4 was sufficiently large and was administered continuously for more than 4 days. The results can be broken into 4 main groups.

1. In this group the dose of T_4 caused no change in % free B, total plasma B concentration or adrenal to body weight ratio, e.g. 1 ug/rat T_4 given for 5 days

2. In this group there was a decrease in % free B but no change in total plasma B concentration or adrenal to body weight ratio, e.g. 10 ug/rat given on day 4 only

3. In this group there was a decrease in % free B, a small increase in total plasma B concentration but no change in relative adrenal weight, e.g. 10 ug/rat given on days 4, 5, 6

4. In this group there was a decrease in % free B, a substantial increase in total plasma B concentration with an increase in relative adrenal weight, e.g. 10 ug/rat given for 5 days.

Thus, T_4 caused a fall in the % free B, through initiating CBG synthesis. The decrease in % free B meant that there was a fall in the proportion of the total plasma B concentration which was free and active. Therefore, there may be a fall in the strong negative feedback on the pituitary ACTH secretion which is normally present in the 9 day old rat. The resulting increase in ACTH secretion may lead to a stimulation of adrenal growth and secretion. The total plasma B concentration would rise in an attempt to restore the active plasma B concentration in the presence of the greater binding activity.

In addition, T_4 appears to increase the total B secretion per mg of adrenal tissue. When 10 ug/rat of T_4 was administered on days 4, 6, 8 there was a fall in the % free B and a considerable increase in total plasma B concentration with no change in relative adrenal weight.

SECTION 5

Introduction

The effect of T_4 treatment on the responsiveness of the adrenal axis was examined using the 3 agents used previously, that is, insulin induced hypoglycaemia, ADH and ACTH.

Experimental Procedure

After the appropriate saline or T_4 administration, on day 9, litters were removed from their mothers, weighed and injected with either saline or the test substance ip. Insulin was given in a saline suspension, at a dose of 2 mU/rat, synthetic ADH was administered at a dose of 2 mU/rat and ACTH was given at a dose of 0.5 ug/rat. The rats were killed by decapitation 30 minutes after the ACTH or ADH injection and 60 minutes after the insulin injection. Trunk blood was collected and after centrifugation the plasma stored at -20°C until assay. For the ACTH treated groups only, when all the animals from each litter had been killed, the left adrenal was removed, cleared of fat and connective tissue and weighed. Blood from 2-3 rats was pooled together so adequate plasma could be collected for assay. The % free B and total plasma B concentration were determined for each sample. The plasma glucose concentration was determined for samples in the insulin treated groups only.

T_4 Treatment

Litters were injected with 10 ug/rat of T_4 in saline with 10% ethanol sc for either 5 days from day 4 to 8 or on day 4 only. Controls were litters injected with saline containing 10% ethanol using the same protocol.

Results

The effect of T_4 administration on the response of a 9 day old rat to an acute ACTH challenge.

Table 5.1: % Free B After Saline or T_4

	n	% Free	
saline day 4	12	80.9 ± 3.0	$p < 0.001$
T_4 day 4	14	55.8 ± 2.4	
5 days of saline	12	92.3 ± 1.6	$p < 0.001$
5 days of T_4	11	46.0 ± 2.2	

Table 5.2: Adrenal to Body Weight Ratio After Saline or T_4

	n	$\frac{\text{Adrenal Weight}}{\text{Body Weight}} \frac{mg}{g} \times 10^{-2}$	
saline day 4	19	7.0 ± 0.4	NS
T_4 day 4	37	7.7 ± 0.4	
5 days of saline	33	7.9 ± 0.4	$p < 0.05$
5 days of T_4	23	9.4 ± 0.5	

		n	Total Plasma B Concentration ng/ml	
saline day 4	saline	7	10.0 ± 2.3	$p < 0.02$
	ACTH	7	17.0 ± 1.2	
T_4 day 4	saline	6	12.8 ± 2.2	$p < 0.001$
	ACTH	7	32.6 ± 2.6	
5 days of saline	saline	6	9.7 ± 3.0	NS
	ACTH	6	18.0 ± 2.6	
5 days of T_4	saline	5	29.3 ± 10.3	$p < 0.001$
	ACTH	6	121.6 ± 13.5	

Table 5.4: Increment in Total Plasma B Concentration Caused by ACTH After Pretreatment With T_4 or Saline

See Figs 5.1, 5.2

	n	Increment Caused by ACTH ng/ml	
saline day 4	7	7.0 ± 1.2	$p < 0.01$
T_4 day 4	7	19.8 ± 2.6	
5 days of saline	6	8.3 ± 2.6	$p < 0.001$
5 days of T_4	6	93.3 ± 13.5	

A single injection of T_4 on day 4 caused a significant decrease in % free B with no compensatory change in adrenal to body weight ratio. These changes caused a small but significant increase in the increment in total plasma B concentration caused by ACTH. After 5 days of T_4 administration there was a significant decrease in the % free B, with an increase in adrenal to body weight ratio. This caused a large increase in the increment in total plasma B concentration seen after ACTH.

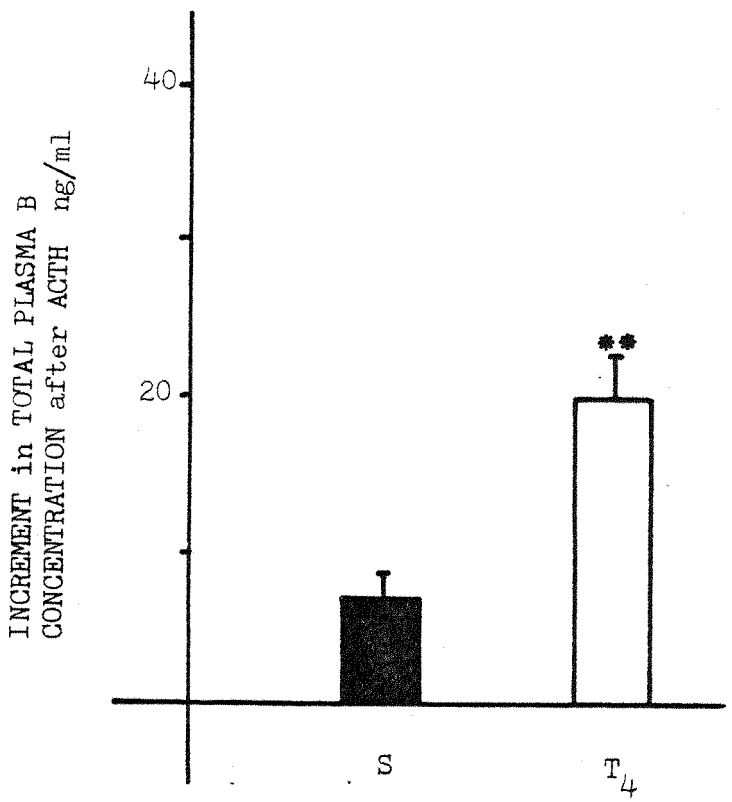


Fig. 5.1 Increment in total plasma B concentration caused by ACTH injection in 9 day old rats after either saline or 10ug T_4/rat on day 4.

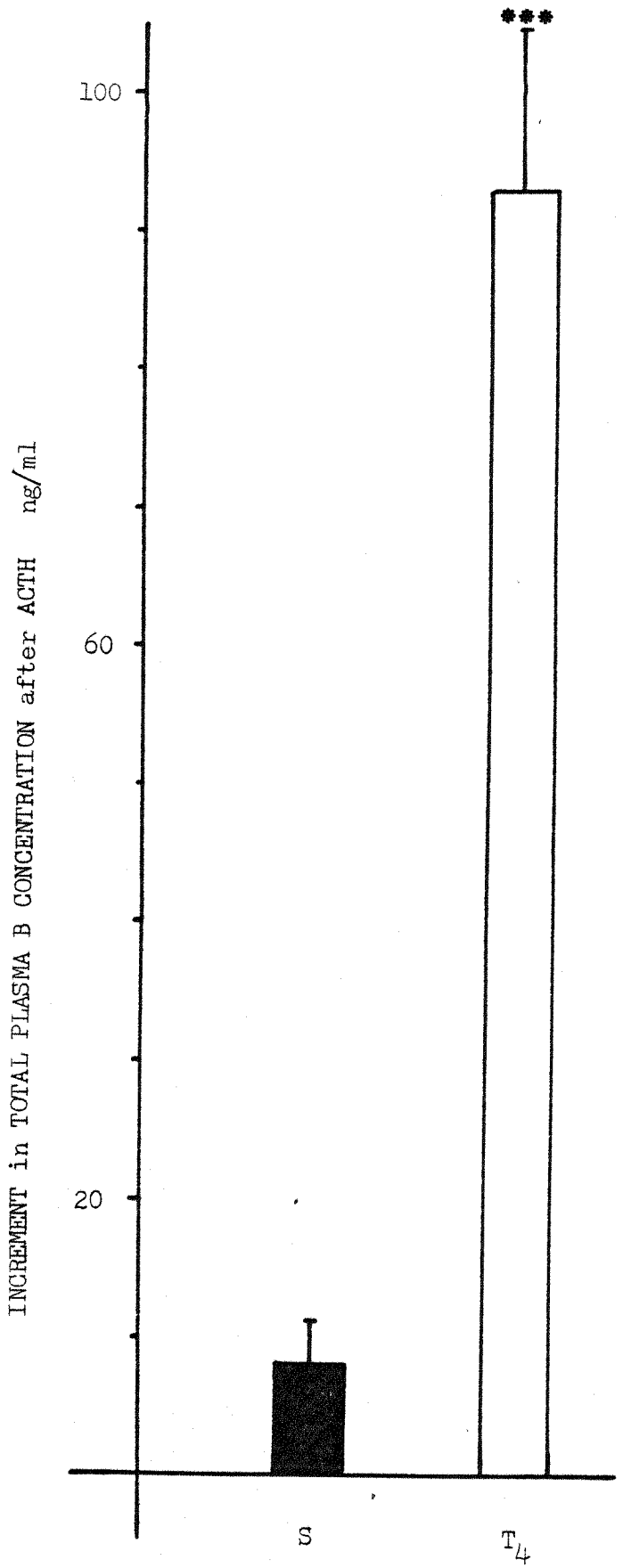


Fig. 5.2 Increment in total plasma B concentration caused by ACTH injection in 9 day old rats after either saline or 10ug T_4 for 5 days.

The effect of 5 days of T_4 treatment on the response of the 9 day old rat to an acute ADH challenge.

Table 5.5: % Free B After Saline or T_4

	n	% Free	
saline	8	75.0 ± 4.3	$p < 0.01$
T_4	24	36.9 ± 3.1	

Table 5.6: Total Plasma B Concentration After ADH or Saline with Pretreatment with T_4 or Saline

		n	Total Plasma B Concentration ng/ml	
saline	saline	5	6.9 ± 1.8	NS
	ADH	6	4.4 ± 0.7	
T_4	saline	12	69.5 ± 10.0	NS
	ADH	13	109.2 ± 22.6	

Table 5.7: Increment in Total Plasma B Concentration Caused by ADH

See Fig. 5.3

	n	Increment in Total Plasma B Concentration Caused by ADH ng/ml	
saline	6	- 2.5 ± 0.7	$p < 0.05$
T_4	13	39.7 ± 22.7	

After 5 days of T_4 administration there was a significant fall in the % free B. This resulted in an increase in the increment in total plasma B concentration caused by ADH in the 9 day old rat.

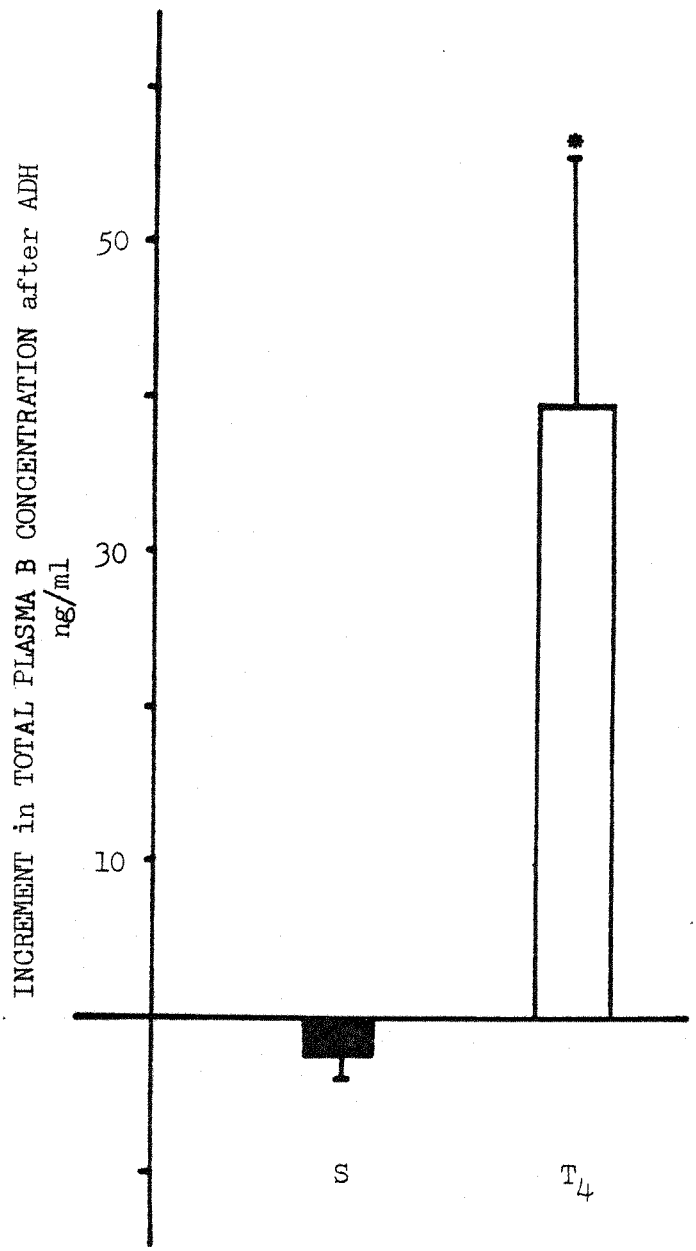


Fig. 5.3 Increment in total plasma B concentration caused by ADH after either saline or 10ug T_4 for 5 days.

The effect of 5 days of T_4 treatment on the response of the 9 day old rat to insulin induced hypoglycaemia.

Table 5.8: % Free B

	n	% Free B	
saline	6	87.8 ± 2.1	$p < 0.01$
T_4	5	63.8 ± 1.9	

Table 5.9: Total Plasma B Concentration and Plasma Glucose Concentration After Insulin or Saline with Pretreatment with T_4 or Saline

		n	Total Plasma B Concentration ng/ml		n	Plasma Glucose Concentration mg%	
saline	saline	6	9.3 ± 4.3	NS	6	93.5 ± 6.0	$p < 0.01$
	insulin	7	17.3 ± 3.3		7	57.3 ± 8.3	
T_4	saline	6	35.4 ± 14.0	$p < 0.001$	6	103.0 ± 7.2	$p < 0.001$
	insulin	8	192.0 ± 20.4		8	36.3 ± 6.1	

Table 5.10: Increment in Total Plasma B Concentration and Decrement in Plasma Glucose Concentration After Insulin with Pretreatment with Saline or T_4

See Fig. 5.4

	n	B Increment ng/ml		n	Glucose Decrement mg%	
saline	7	8.0 ± 3.3	$p < 0.001$	7	36.2 ± 8.3	$p < 0.02$
T_4	8	156.6 ± 20.4		8	66.7 ± 6.1	

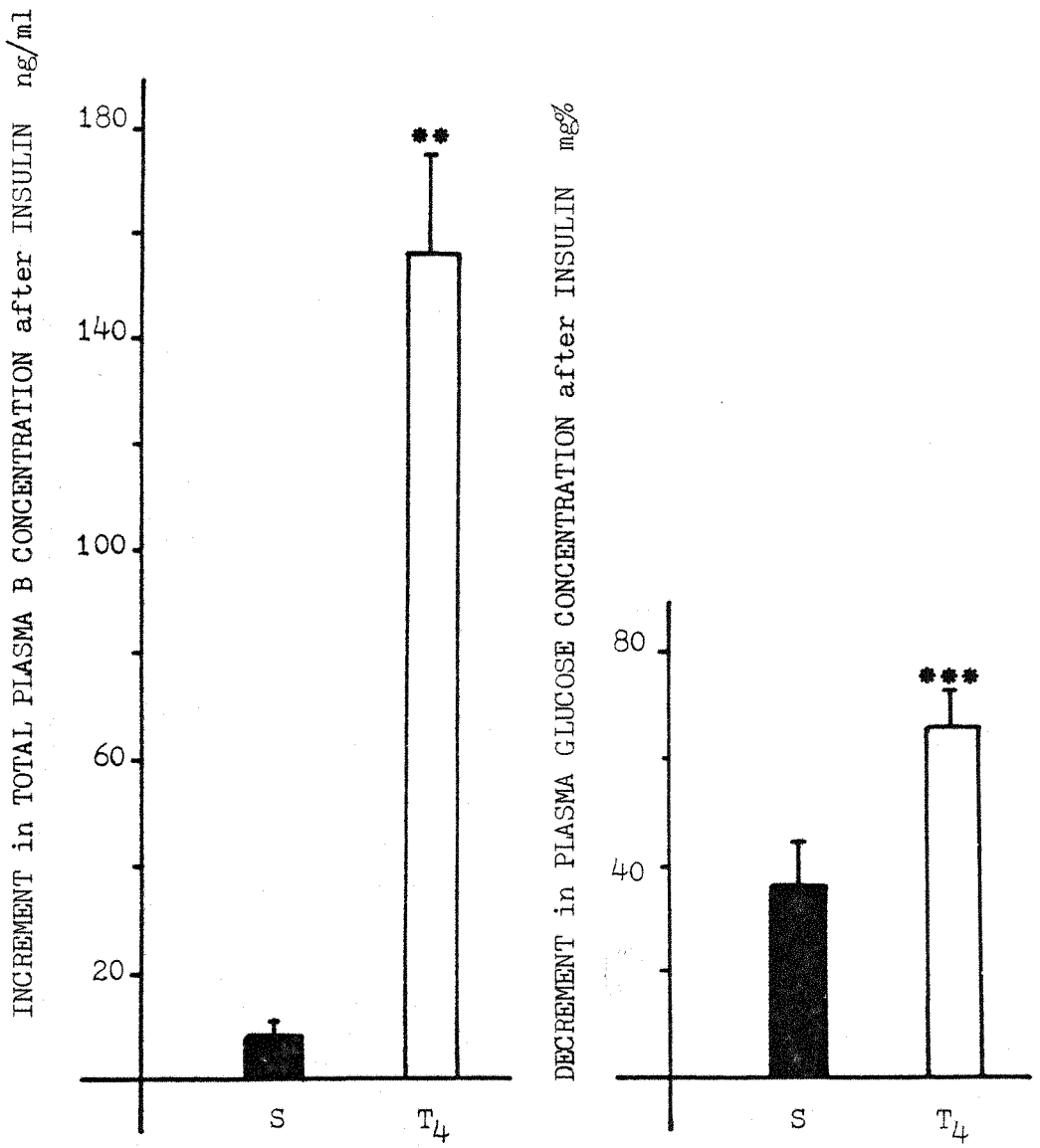


Fig. 5.4 Increment in total plasma B concentration and decrement in plasma glucose concentration caused by insulin after either saline or 10ug T_4 for 5 days.

T_4 treatment caused a significant fall in % free B in the 9 day old rat. Insulin induced a significant decrease in plasma glucose concentration in both saline and T_4 treated animals. T_4 treated rats had a greater response. The hypoglycaemia induced by insulin caused a small increment in total plasma B concentration in saline-treated controls and a large increment in total plasma B concentration in the T_4 treated animals.

COMMENT

The responsiveness of the adrenal axis of the 9 day old rat to all 3 agents increased after T_4 treatment. This suggests that the final site of action was at the level of the adrenal itself, since insulin induced hypoglycaemia, which tests the activity of the entire axis, gave similar results to exogenous ACTH, which tests the activity of the adrenal directly. The % free B decreased after T_4 decreasing the proportion of the total plasma hormone concentration which was active, thus, the total plasma B concentration increased in an attempt to maintain a constant response to stimulation.

The effect of ADH and ACTH were similar indicating that after T_4 treatment the endogenous secretion from the pituitary caused the same response from the adrenal as exogenous ACTH. This suggests that T_4 treatment was not altering the composition of pituitary secretion. After T_4 administration the production of B per mg of adrenal tissue in response to an acute ACTH challenge was increased. This can be seen if the increment in total plasma B concentration is expressed as a fraction of the adrenal to body weight ratio. After 5 days of T_4 treatment this value was 10 compared with a value of 1.1 for saline-treated controls. Therefore the production of B from the adrenal was increased in an attempt to maintain a constant response to stimulation in the presence of the greater plasma binding activity.

SECTION 6

Introduction

T_4 treatment has been shown to cause a premature decrease in the % free B and this may lead to an increase in the total plasma B concentration and relative adrenal weight (see Section 4). If the rise in plasma T_4 concentration at about day 7 is the physiological stimulus for the increase in B binding seen after day 9 in the rat, then treatment with antithyroid drugs should be able to delay these changes. The effect of pretreatment with the antithyroid drug propylthiouracil (PTU) was studied in 21 day old rats to see if PTU treatment could inhibit the decrease in % free B and the increase in total plasma B concentration which occurs during the 2nd and 3rd week post-partum. The effect of PTU on the responsiveness of the adrenal to exogenous ACTH was also studied in the 21 day old rat.

Experimental Procedure

a. For the Effect of PTU on the Static Aspects of Adrenal Function in 21 Day Old Rat

Each litter was divided into 2 groups. 1 group received 50 ug PTU/rat in alkaline saline and the control group received alkaline saline only. Injections were given s.c. on alternate days beginning on day 4. On day 21 the rats were weighed and killed by decapitation. Trunk blood was collected, centrifuged and the plasma stored at -20°C until assay. The plasma was assayed for % free B and total plasma B concentration.

b. For the Effect of PTU on the ACTH Responsiveness in the 21 Day Old Rat

Litters were either injected with 50 ug PTU/rat in alkaline saline or alkaline saline only. Injections were given s.c. on alternate days beginning on day 4. At day 21, the rats were given 100 ug/rat dexamethasone phosphate ip 4 hours prior to either a saline or ACTH injection. The dose of ACTH used was 1.25 ug/rat. 30 minutes after the injections had been given the animals were killed by decapitation, trunk blood was collected, centrifuged and the plasma stored at -20°C until assay. The total plasma B concentration was determined for each sample.

Results

The effect of PTU treatment on body weight, % free and total plasma B concentration.

Table 6.1: The Effect of PTU on Body Weight

	n	Body Weight in g	
saline	7	34.2 ± 0.7	$p < 0.05$ using the signed rank test
PTU	8	32.8 ± 0.6	

Table 6.2: The Effect of PTU on % Free B

See Fig. 6.1

	n	% Free B	
saline	7	31.4 ± 1.8	$p < 0.001$
PTU	8	59.1 ± 5.8	

Table 6.3: The Effect of PTU on Total Plasma B Concentration

See Fig. 6.2

	n	Total Plasma B Concentration ng/ml	
saline	6	67.9 ± 9.4	$p < 0.01$
PTU	7	33.7 ± 5.5	

PTU treatment caused a small but significant decrease in body weight, a significant increase in % free B and a significant decrease in total plasma B concentration.

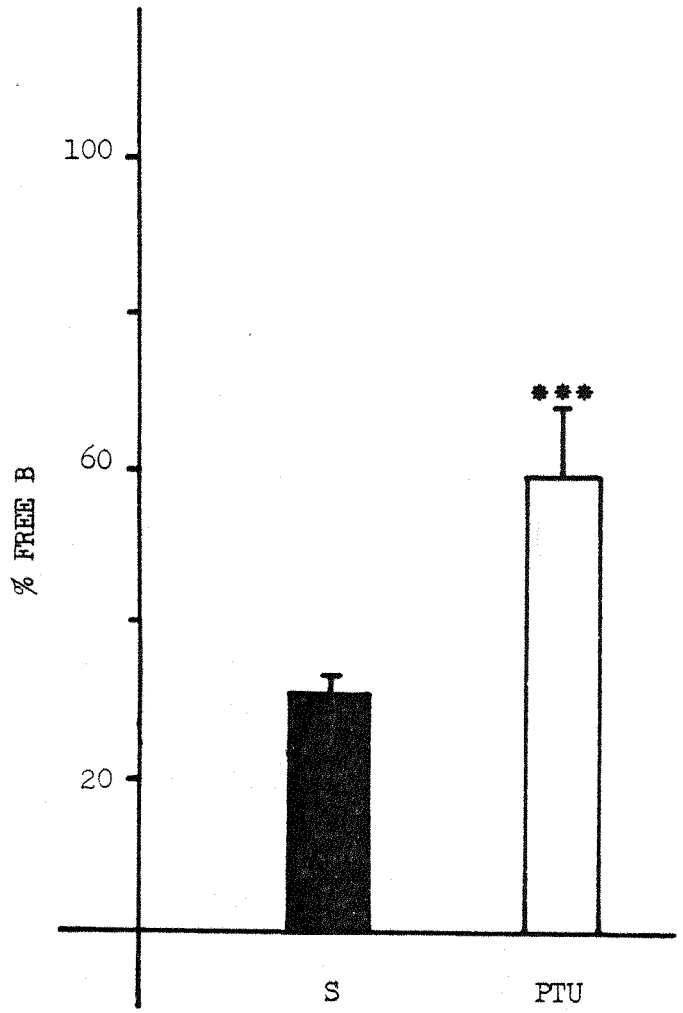


Fig. 6.1 The effect of PTU treatment from day 4 on % free B in 21 day old rats.

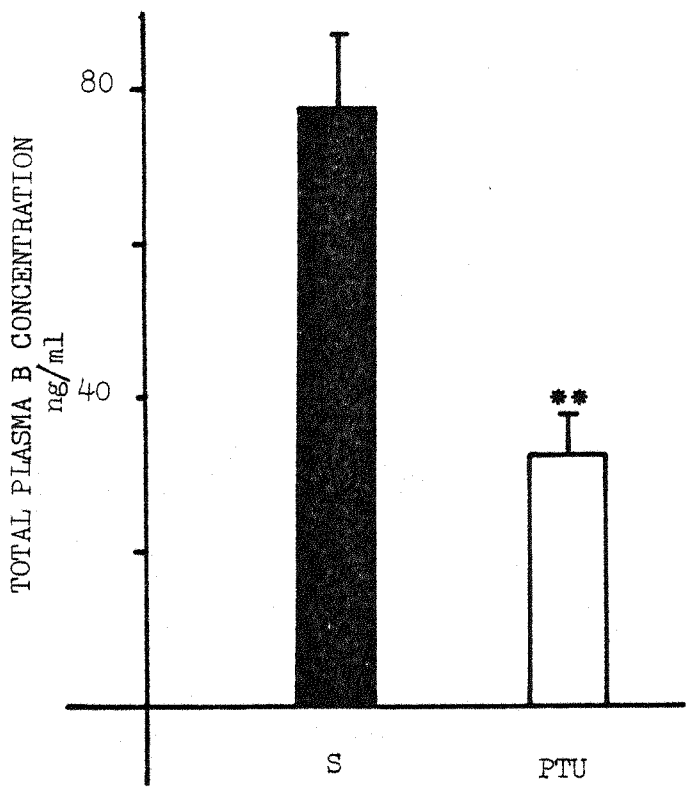


Fig. 6.2 The effect of PTU treatment from day 4 on total plasma B concentration in 21 day old rats

The effect of PTU on the responsiveness of the adrenal to an acute ACTH challenge in the 21 day old rat.

Table 6.4: The Effect of PTU and Dexamethasone on Total Plasma B Concentration After Saline or ACTH

	n	Total Plasma B Concentration ng/ml
saline	5	19.8 ± 2.1
ACTH	6	96.3 ± 10.7

The increment in total plasma B concentration caused by ACTH was 76.5 ± 10.7 ng/ml. The total plasma B concentration was 18.1 ± 2.1 ng/ml (n = 5) with only PTU treatment and dexamethasone injection 4 hours prior to killing.

COMMENT

PTU is a drug which has many sites of action. It blocks iodide uptake into the thyroid, T_4 secretion by the thyroid (Morreale de Escobar, and Escobar del Rey, 1962) and its peripheral conversion to T3 (Kaplan and Utiger, 1978). The effect of PTU administration is therefore to reduce plasma T_4 and T_3 concentrations (Westgren, Melander, Wahlin and Lingren, 1977). Wysocki and Segal (1972) have shown that the dose of PTU used in the present study reduces plasma T_4 concentration in the young rat.

This suppression of thyroid function decreased the fall in % free B normally seen by day 21. PTU was also able to reduce the rise in total plasma B concentration and the increase in the adrenal's response to exogenous ACTH normally seen by this time. This gives further support to the suggestion that the rise in plasma thyroid hormone concentration is the physiological stimulus for the fall in % free B seen during the 2nd and 3rd postnatal week and that the % free B plays an important role in determining the total plasma B concentration and the responsiveness of the adrenal to exogenous ACTH at this time.

SECTION 7

Introduction

The decrease in % free B caused by T_4 administration in the 9 day old rat provides a simple unique system to study the activity of other potential thyroid hormones *in vivo*. In this section the activities of tri-iodothyronine (T_3) and reverse tri-iodothyronine (rT_3) are described.

T_3 is formed from T_4 by β deiodination in the peripheral tissues. It is the most potent thyroid hormone and is thought to be the major route through which T_4 exerts all its effects. The actions of T_4 and T_3 on the change in % free B, total plasma B concentration and relative adrenal weight were compared in the 9 day old rat.

rT_3 is formed from T_4 by α deiodination. It is unclear whether it has an inhibitory or weakly stimulatory action on thyroid dependent systems. Its action on CBG synthesis was assessed in 9 and 19 day old rats. Its action on the T_4 and T_3 response in the 9 day old rat was also investigated to see if either or both were inhibited.

Experimental Procedure

T_4 and T_3 Relative Potencies

Litters were divided into two groups, one group received 10 ug/rat of T_4 in ethanolic saline when the other group received a dose of T_3 in ethanolic saline. Injections were given s.c. on days 4, 6 and 8 post-partum. The doses of T_3 were 5 ug/rat, 1 ug/rat and 0.5 ug/rat. On day 9 animals were weighed and killed by decapitation and trunk blood was collected. The left adrenal was removed from each rat, cleared of fat and connective tissue and weighed. The plasma was assayed for % free B and total plasma B concentration.

Action of rT_3

As before all litters were divided into 2 groups. At the appropriate age the animals were killed, blood collected, centrifuged and the plasma stored at -20°C until assay. % free B was determined for each sample.

a. 10 ug rT_3/rat was injected on days 4, 5 and 6 post-partum. The animals were killed on day 9.

b. The effect of rT_3 in 19 day old rats was examined. 10 ug rT_3/rat was injected on day 8, 9, 10 and then 20 ug was injected on days 12, 14, 16 and 18. The rats were killed on day 19.

c. The effect of rT_3 on T_4 action in the 9 day old rat was studied. 10 ug T_4/rat was given to one half of the litter and the other half received 10 ug of both T_4 and rT_3. Injections were given s.c. on days 4, 5 and 6 and the rats were killed on day 9.

d. The effect of rT_3 on the T_3 response in 9 day old rats was examined. The protocol here was as for c. except 1 ug T_3/rat was given in place of 10 ug T_4/rat.

Results

The potency of T_3 compared with T_4

Table 7.1: The Effect of Different Doses of T_3 Compared with 10 ug/rat T_4 on Body Weight

See Fig. 7.1

Dose of T_3		n	Body Weight g	
5 ug/rat	T_3	15	14.4 ± 0.8	NS
	T_4	16	14.3 ± 0.7	
1 ug/rat	T_3	15	14.5 ± 0.4	NS
	T_4	12	13.6 ± 0.7	
0.5 ug/rat	T_3	10	15.6 ± 1.4	NS
	T_4	14	16.3 ± 0.9	

Table 7.2: The Effect of Different Doses of T_3 Compared with 10 ug/rat T_4 on % Free B

See Fig. 7.2

Dose of T_3		n	% Free B	
5 ug/rat	T_3	6	50.0 ± 3.1	$p < 0.001$
	T_4	6	77.0 ± 7.9	
1 ug/rat	T_3	6	69.0 ± 1.4	NS
	T_4	8	70.0 ± 4.3	
0.5 ug/rat	T_3	8	54.7 ± 7.9	NS
	T_4	9	44.4 ± 4.5	

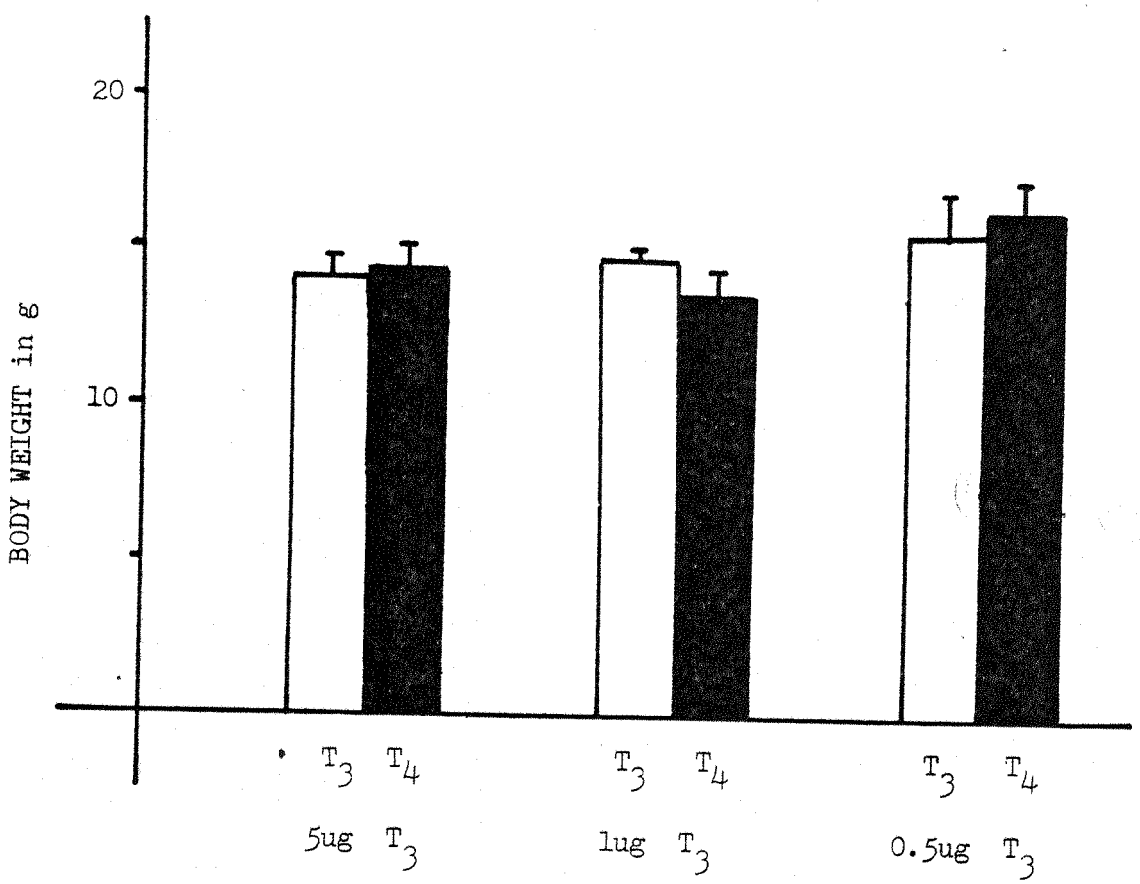


Fig. 7.1 The changes in body weight in response to T_3 administration compared with the response to 10ug T_4/rat.

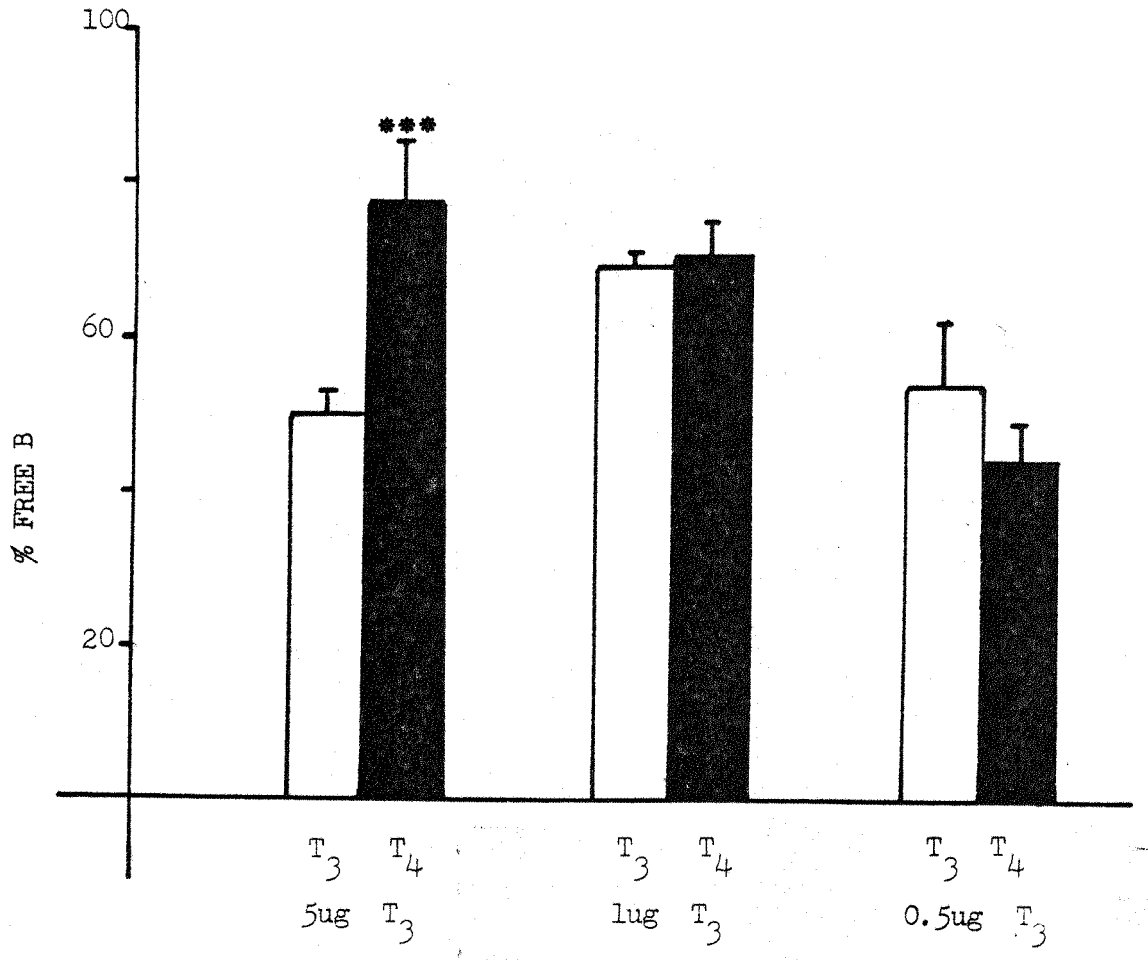


Fig. 7.2 The changes in % free B in response to T_3 administration compared with the response to 10ug T_4/rat.

Table 7.3: The Effect of Different Doses of T_3 Compared with 10 ug/rat T_4 on Total Plasma B Concentration

See Fig. 7.3

Dose of T_3		n	Total Plasma B Concentration ng/ml	
5 ug/rat	T_3	6	127.3 ± 25.0	$p < 0.05$
	T_4	6	54.9 ± 11.2	
1 ug/rat	T_3	8	35.7 ± 6.6	NS
	T_4	5	44.5 ± 4.5	
0.5 ug/rat	T_3	9	9.9 ± 1.0	$p < 0.001$
	T_4	8	36.8 ± 5.3	

Table 7.4: The Effect of Different Doses of T_3 Compared with 10 ug/rat T_4 on Adrenal to Body Weight Ratio

See Fig. 7.4

Dose of T_3		n	$\frac{\text{Adrenal Weight}}{\text{Body Weight}}$ $\frac{mg}{g} \times 10^{-2}$	
5 ug/rat	T_3	15	12.0 ± 0.2	$p < 0.001$
	T_4	16	10.9 ± 0.1	
1 ug/rat	T_3	11	11.4 ± 0.7	NS
	T_4	11	11.1 ± 0.8	
0.5 ug/rat	T_3	17	10.7 ± 0.5	NS
	T_4	14	10.6 ± Q.7	

These results show T_3 to be 10 times more potent than T_4 in causing a decrease in % free B and an increase in both adrenal to body weight ratio and total plasma B concentration in the 9 day old rat.

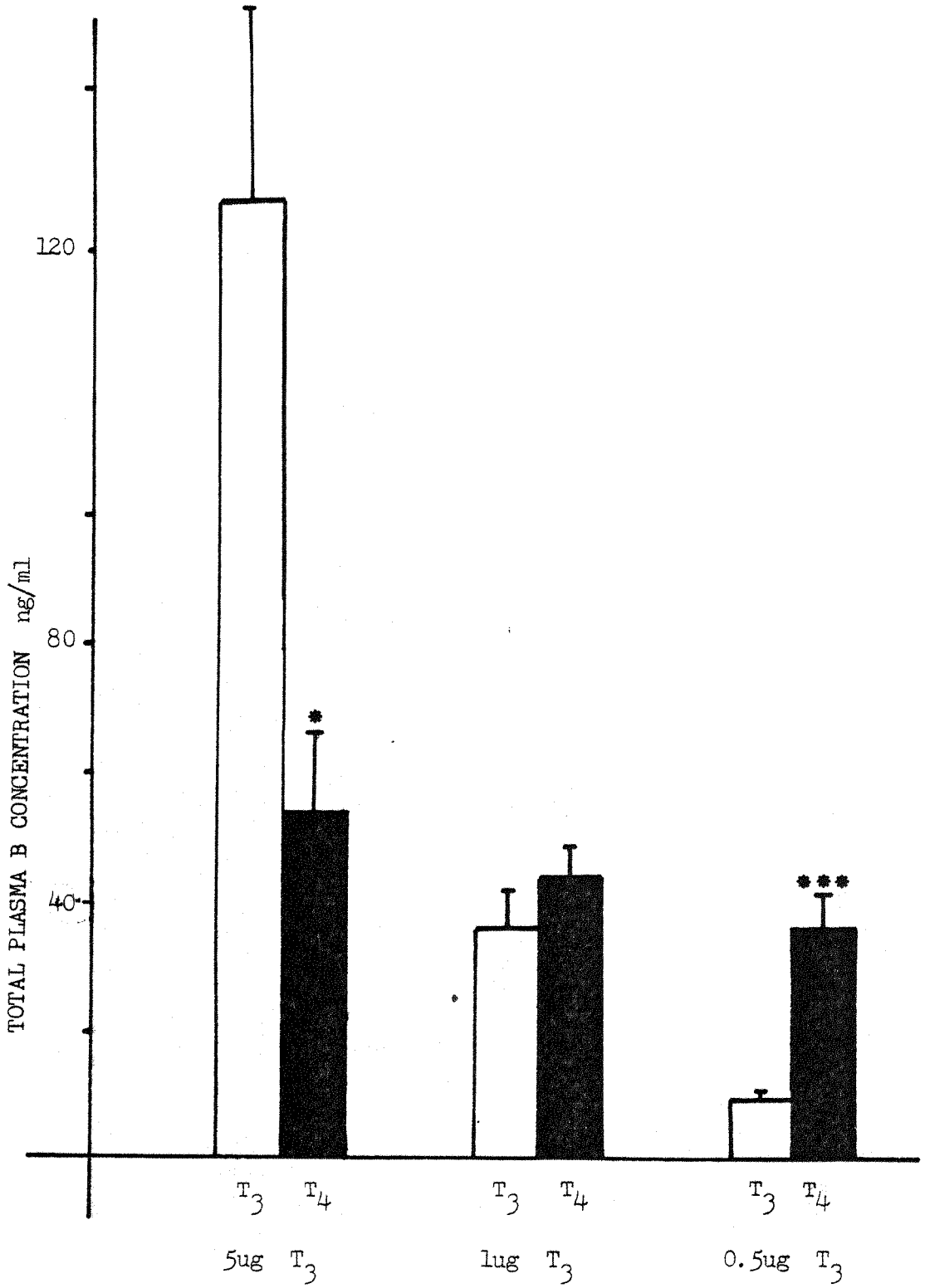


Fig. 7.3 The changes in total plasma B concentration in response to T_3 administration compared with the response to 10ug T_4/rat.

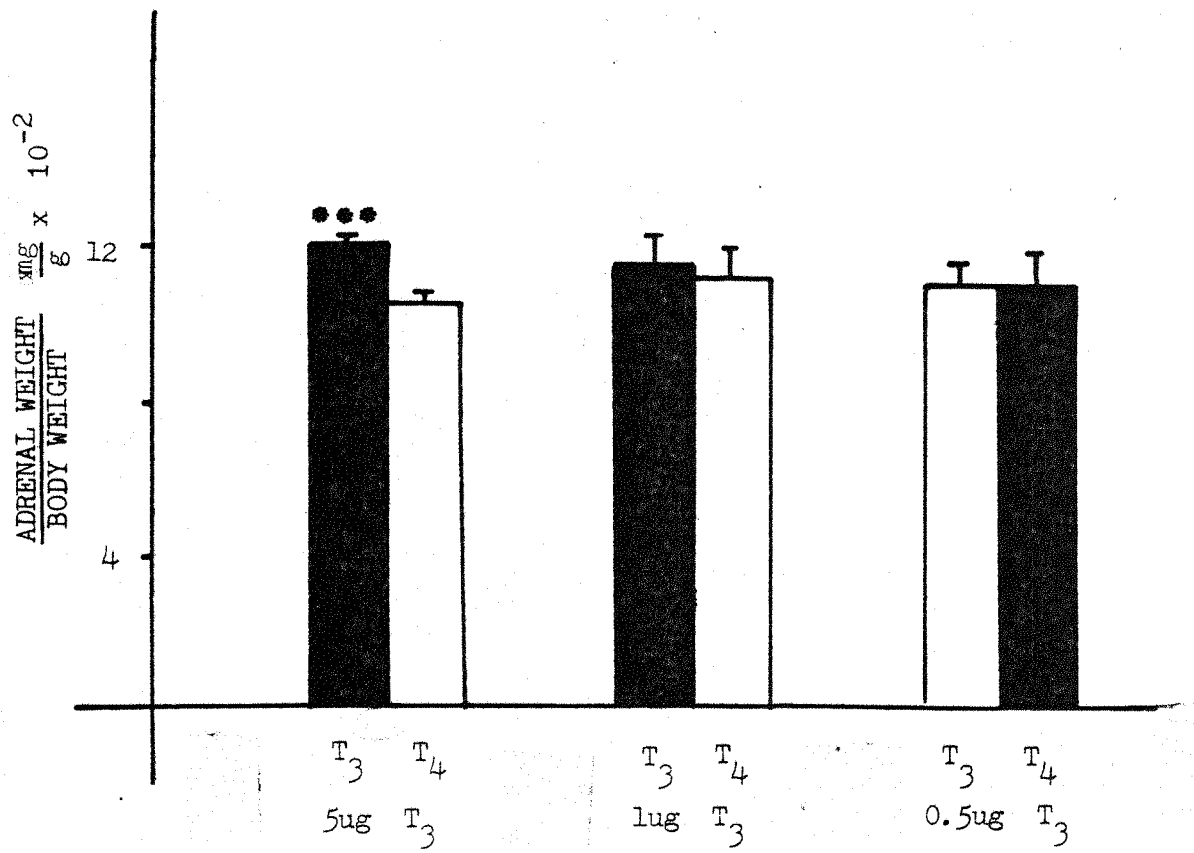


Fig. 7.4 The changes in adrenal to body weight ratio in response to T_3 administration compared with the response to 10ug T_4/rat. (see text for details).

The action of rT_3

Table 7.5: The Action of rT_3 in the 9 Day Old Rat or % Free B

See Fig. 7.5

	n	% Free B	
saline	6	72.0 ± 8.2	NS
rT_3	7	78.1 ± 8.1	

There was no significant difference between saline and rT_3 treated rats at day 9.

Table 7.6: The Action of rT_3 in the 19 Day Old Rat or % Free B

See Fig. 7.5

	n	% Free B	
saline	10	39.3 ± 3.7	NS
rT_3	10	48.1 ± 4.0	

There was no significant difference between rT_3 treated rats and saline treated controls at day 19.

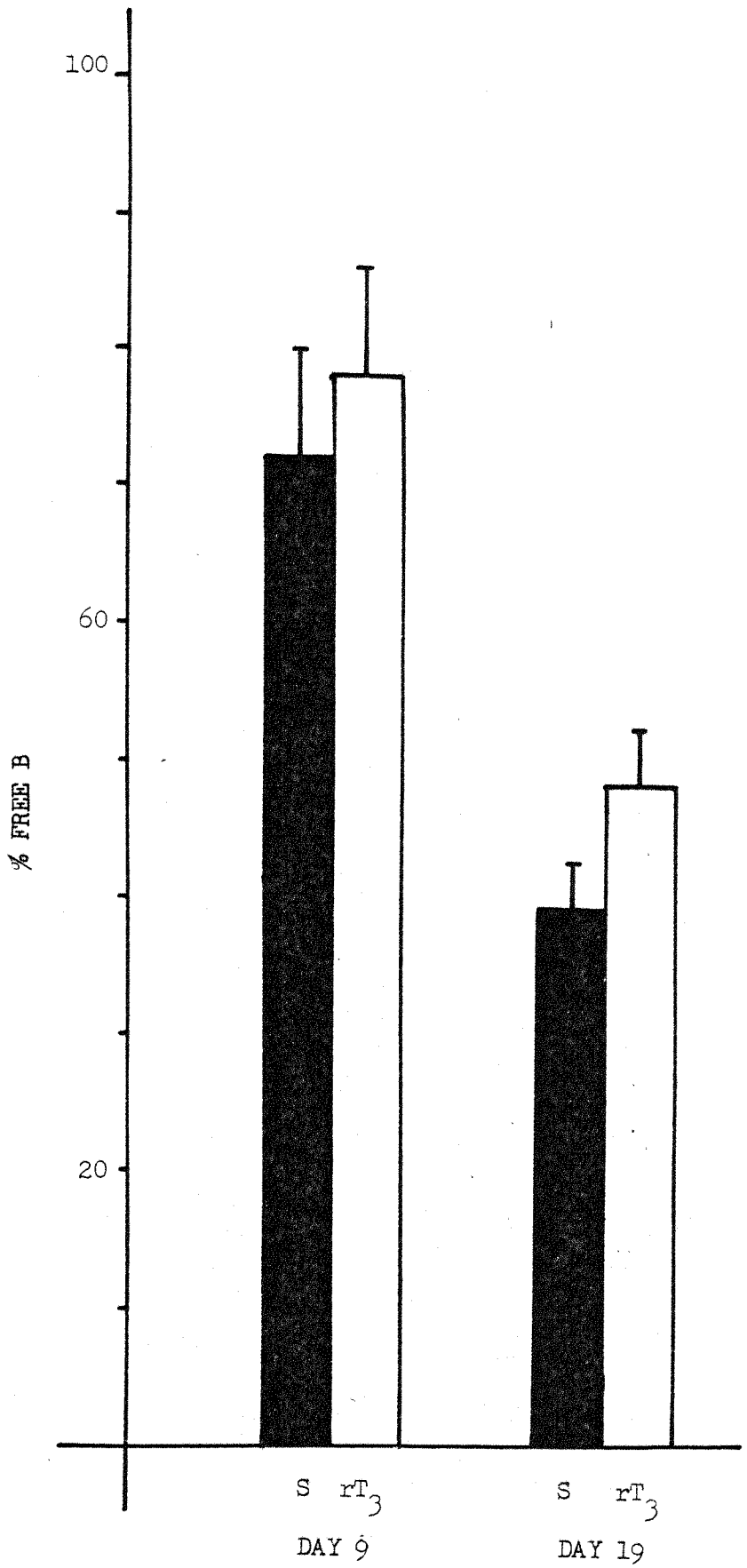


Fig. 7.5 The effect of rT_3 on % free B in 9 and 19 day old rats.

Table 7.7: The Action of rT_3 on the T_4 Response in the 9 Day Old Rat

See Fig. 7.6

	n	% Free B	
T_4	11	24.8 ± 2.5	
$T_4 + rT_3$	12	37.2 ± 3.6	$p < 0.05$

rT_3 was able to cause a significant inhibition of the T_4 response on % free B in the 9 day old rat.

Table 7.8: The Action of rT_3 on the T_3 Response in the 9 Day Old Rat

See Fig. 7.7

	n	% Free B	
T_3	7	15.5 ± 1.4	
$T_3 + rT_3$	8	12.3 ± 2.0	NS

rT_3 had no effect on the T_3 response in the 9 day old rat.

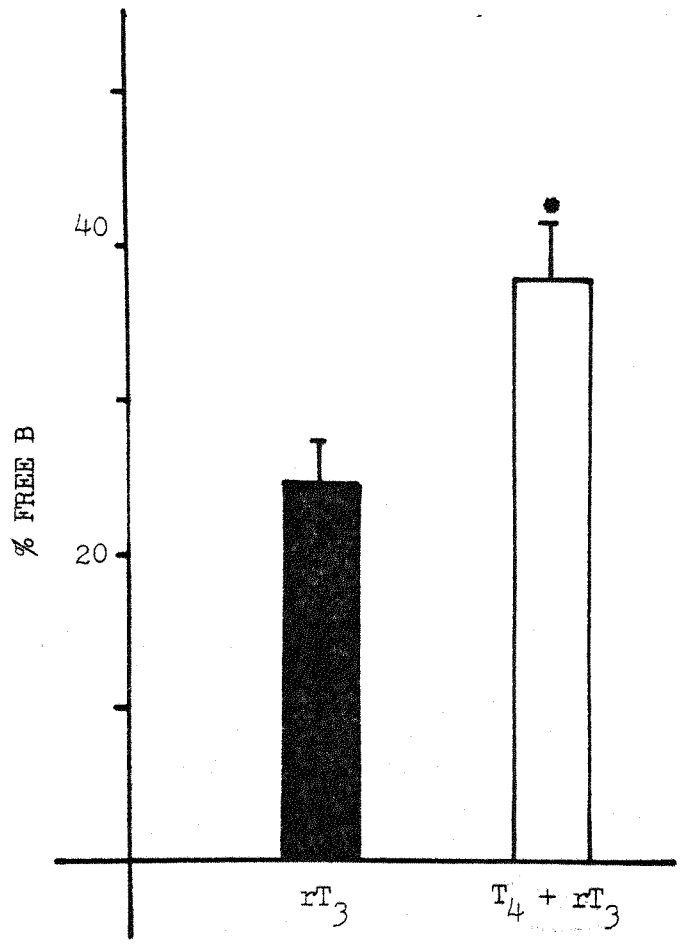


Fig. 7.6 The effect of rT_3 on the % free B response to T_4 in 9 day old rats.

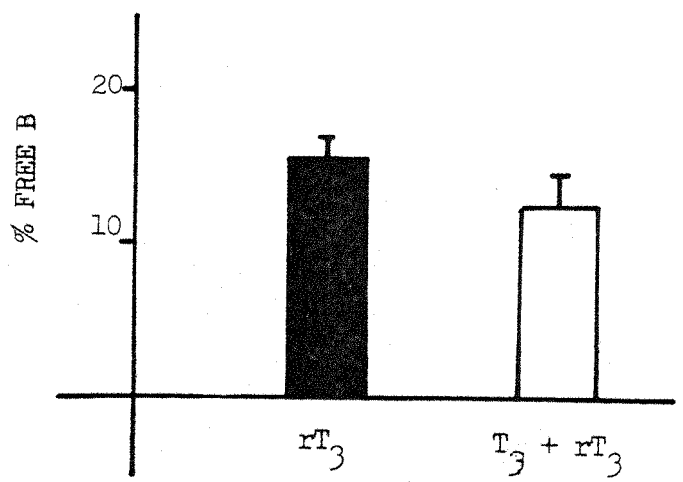


Fig. 7.7 The effect of rT_3 on the % free B response to T_3 in 9 day old rats.

Comment

Gross and Pitt-Rivers (1952) showed that T_3 was more potent than T_4 at preventing goitre formation in rats. Most investigators of the metabolic actions of the hormones have suggested T_3 to be 3 to 5 times more potent than T_4. The results from this study, where a specific action of the hormone has been measured more directly, indicate that T_3 is approximately 10 times more potent than T_4 at stimulating CBG synthesis and causing a premature fall in % free B in the 9 day old rat. In young rats the rate of hepatic generation of T_3 from T_4 is low (Harris, Fang, Prosky, Braverman and Vagenakis, 1978). CBG synthesis is a hepatic function and it might therefore be expected that the effectiveness of T_4 would be reduced if conversion to T3 is a prerequisite for action thus increasing the apparent potency of T_3.

This study demonstrates that rT_3 was able to inhibit the effect of T_4 but not the effect of T_3 on % free B in the 9 day old rat. rT_3 may act by inhibiting the enzyme β deiodinase which converts T_4 to T_3. In young rats hepatic generation of T_3 from T_4 is low (Harris et al., 1978) so the half life of rT_3, which is normally removed by β deiodination, is increased. rT_3 is therefore able to inhibit T_4 to T_3 conversion *in vivo* in 9 day old rats. This is in agreement with Coiro, Harris, Goodman, Vagenakis and Braverman (1980) who demonstrated that an infusion of 100 ug/100 gms body weight for 4 hours caused a 50% inhibition of the *in vitro* hepatic T_4 to T_3 conversion.

rT_3 has no action of its own in the 9 day old rat because there is little T_4 in animals of this age (Dussault and Labrie, 1975). rT_3 still has no action at day 19 although T_4 is present and PTU is able to inhibit thyroid function and prevent the fall in % free B normally seen by this age. However, hepatic generation of T_3 from T_4 has reached adult values by day 19 (Harris et al., 1978) and therefore rT_3 can be cleared very quickly from the plasma by β deiodination. This is confirmed by Balsam, Eisenstein, Sexton and Ingbar (1978) who showed that the metabolic clearance rate (MCR) of rT_3 was 15 times higher than the MCR of T_3 in adult rats. Thus rT_3 has no effect in 19 day old rats because it is cleared from the plasma very quickly.

SECTION 8

Introduction

T_4 treatment *in vivo* appears to increase the amount of B secreted per mg of adrenal tissue (see Section 4). This could be caused by an increase in the secreting capacity of the adrenal tissue itself or by an inhibition of B metabolism. Therefore, an *in vitro* preparation was used to see if T_4 treated adrenals secreted more B in response to ACTH, using the method of Richardson and Schulster (1972).

Experimental Procedure

3 coeval litters were taken, and each litter was divided into two groups. The experimental group received 10 ug T_4/rat in ethanolic saline and the control group received ethanolic saline only for 5 days from day 4 to day 8 post-partum. On day 9, the litters were divided into their two groups. The animals were killed by decapitation, the adrenals removed and cleared of fat and connective tissue. Then, they were quartered and placed in 5% collengasae(Sigma) in Krebs Ringer Bicarbonate containing 0.5% BSA and 0.2% glucose (KRBAG). All the adrenals from the saline treated animals were pooled together in one flask and all the adrenals from the T_4 treated animals were pooled together in another flask. The 2 flasks were then incubated, with shaking, at 37°C for 1 hour in an atmosphere of 95% O_2:5% CO_2 in the collengase solution in a volume of 5 ml. The adrenals were physically disrupted using a 5 ml syringe fitted with a 40 x 10 mm size needle during the incubation. When the cells were fully dispersed, they were filtered through nylon gauze (64 u) and centrifuged at 2000 g for 10 minutes. They were then resuspended in 1 ml of cold KRBAG. This washing process was repeated 3 times. The DNA content of the 2 cell suspensions was determined using the method of Lebarea and Paigen (1980). The volume of KRBAG was then adjusted so that both the control and the T_4 treated cell suspensions contained the same amount of DNA. Incubation of the cell suspensions were carried out in KRBAG at 37°C under an atmosphere of 95% O_2:5% CO_2 for 1 hour

in a shaking water bath. 50 ul of the cell suspension were incubated in a final volume of 300 ul in the presence or absence of ACTH (10^{-3} IU/ml incubation). When the incubation was complete the samples were stored at -20°C until the B concentration was determined.

Results

Table 8.1: B Production from the Incubations

Pretreatment		n	ng B Produced/ 50 ul Incubation Volume
saline	baseline	5	0.82 ± 0.29
	ACTH	5	1.31 ± 0.24
T_4	baseline	4	0.57 ± 0.24
	ACTH	5	2.54 ± 0.98

ACTH caused a 60% increase in B production from the cell suspension of the saline treated rats and 345% increase in B production from the cell suspension of T_4 treated rats.

COMMENT

This suggests that T_4 administration causes an increase in the adrenal's response to ACTH. However these results need careful interpretation. Although both cell suspensions contained the same amount of DNA and therefore the same number of cells, the proportion of cells which produced B need not be identical. It is therefore not clear whether the increase in response seen after T_4 treatment is caused by an increase in the numbers of cells producing B or by an increase in the responsiveness of individual cells to ACTH.

SECTION 9

Introduction

The profiles of rat plasma binding of ^{3}HB were determined at day 3, day 9, day 21 and at day 9 after T_4 treatment using a Sephadex G-100 gel filtration as described in Methods 10. The profiles of day 3 and day 21 rat plasma with ^{3}HB and ^{14}C oestradiol (E_2) were also determined.

Experimental Procedure

Trunk blood was collected from rats at the appropriate ages by decapitation. The samples were centrifuged, the plasma removed and stored at -20°C until required. 5 days of 10 ug T_4/rat was administered as described in Section 5. 300 ul of ^{3}HB (10 uCi/ml) in ethanol and, if required, 150 ul of ^{14}C E_2 (5 uCi/ml) in ethanol were evaporated to dryness. 600 ul of plasma and 600 ul of column buffer were added. The mixture was allowed to equilibrate at 0°C for 1 hour before it was applied to the top of the column. 2 ml fractions were collected and a 0.5 ml aliquot was used to assess the amount of radioactivity present. Aliquots from every 3rd fraction were counted.

Results

Fig. 9.1 shows a profile of day 3 rat plasma with ^{3}HB. This shows both a bound peak (the first peak) and an unbound peak (second peak).

Fig. 9.2 shows a profile of day 21 rat plasma with ^{3}HB showing only an unbound peak.

Fig. 9.3 shows a profile of day 21 rat plasma with ^{3}HB showing both a bound and an unbound peak.

Fig. 9.4 shows a profile of day 9 rat plasma with ^{3}HB after 5 days of T_4 treatment. This shows both an unbound and a bound peak.

Fig. 9.5 shows a profile of day 3 and day 21 rat plasma with ^{14}C E_2 and ^{3}HB. This shows that the bound peak of ^{3}HB runs in the same place at day 3 and day 21 and the ^{14}C E_2 peak runs a little after.

Fig. 9.6 shows a profile of day 3 rat plasma with ^{14}C E_2 and ^{3}HB at room temperature. $^{14}C_2$ gives two peaks and ^{3}HB only one peak.

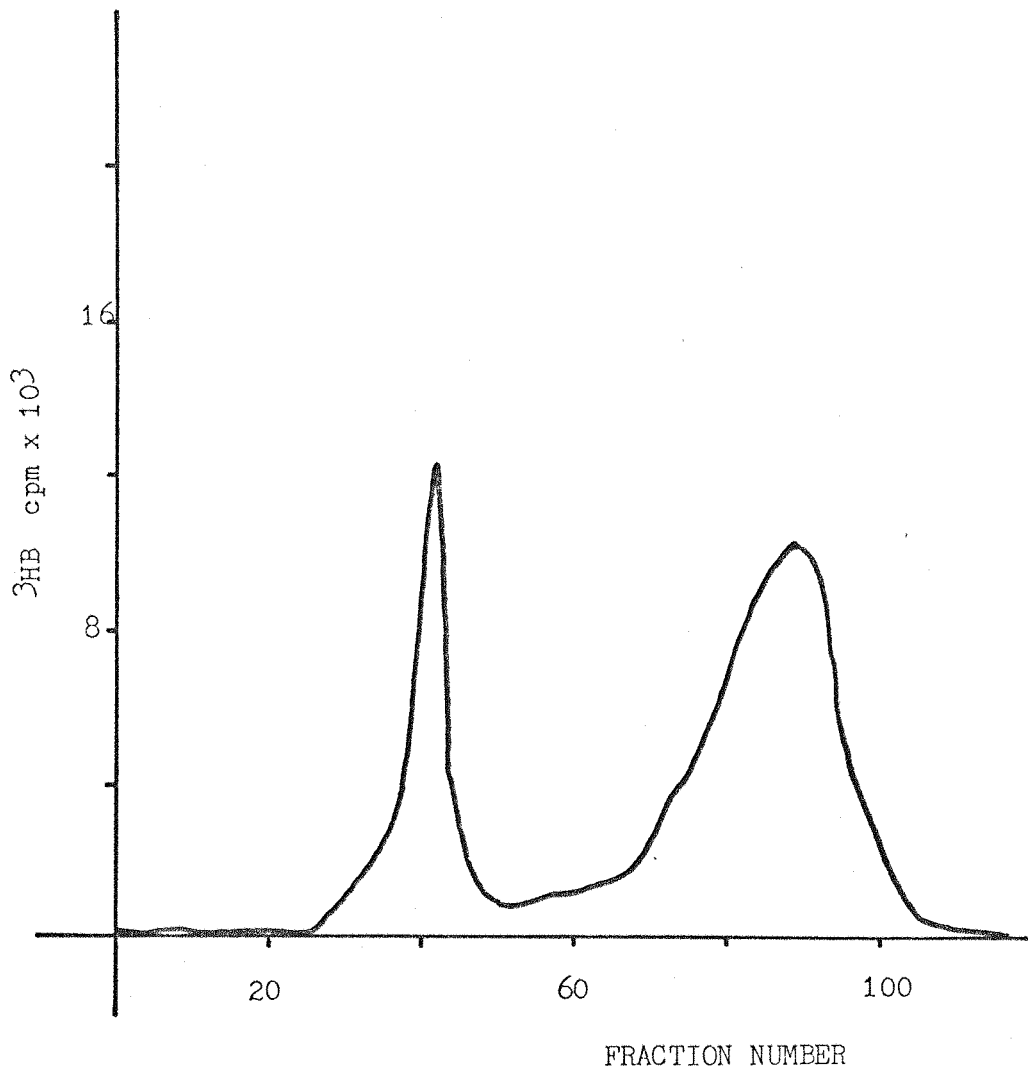


Fig. 9.1 A profile of rat plasma from 3 day old rats with ^{3}HB.

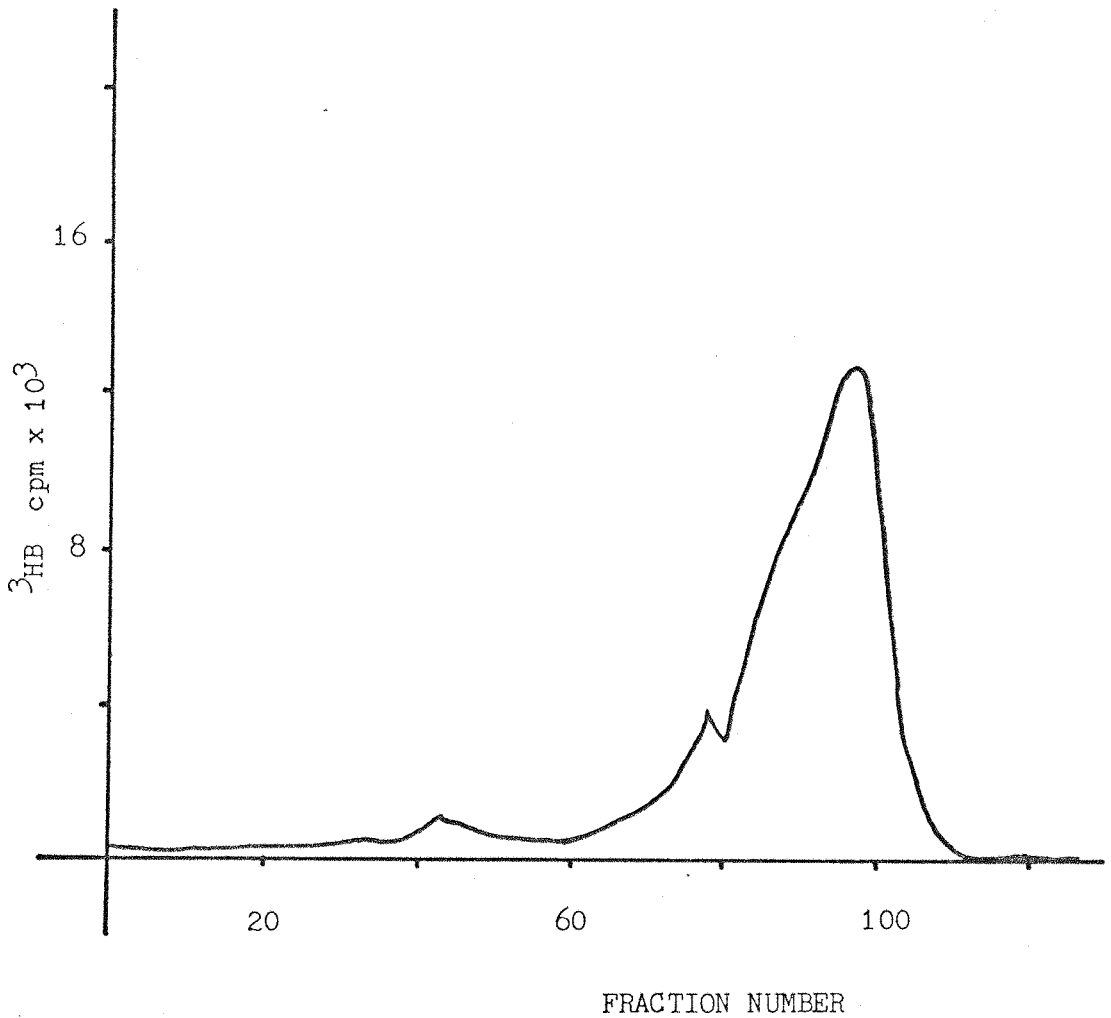


Fig. 9.2 A profile of rat plasma from 9 day old rats with ^{3}HB.

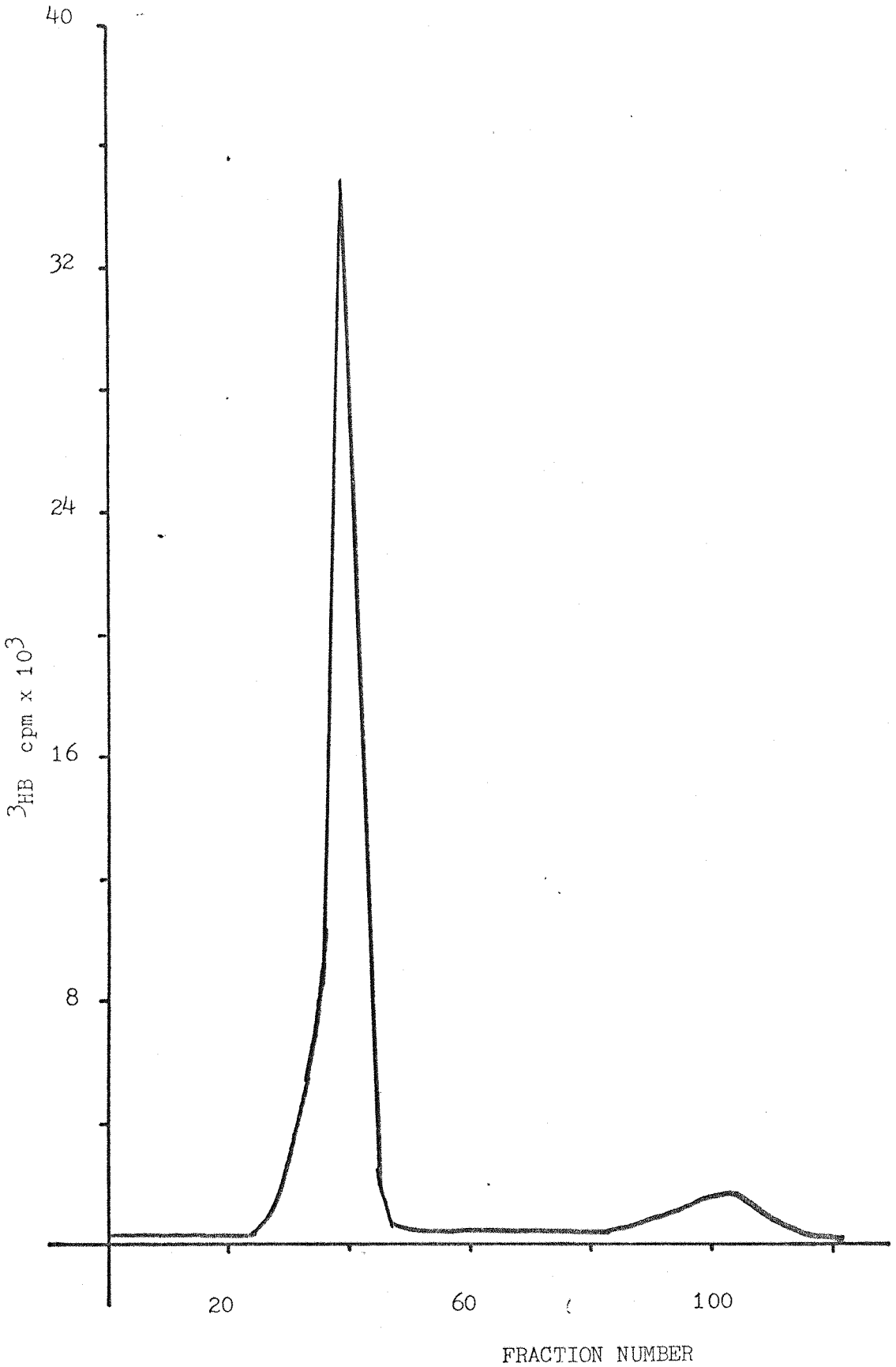


Fig. 9.3 A profile of rat plasma from 21 day old rats with ^{3}HB.

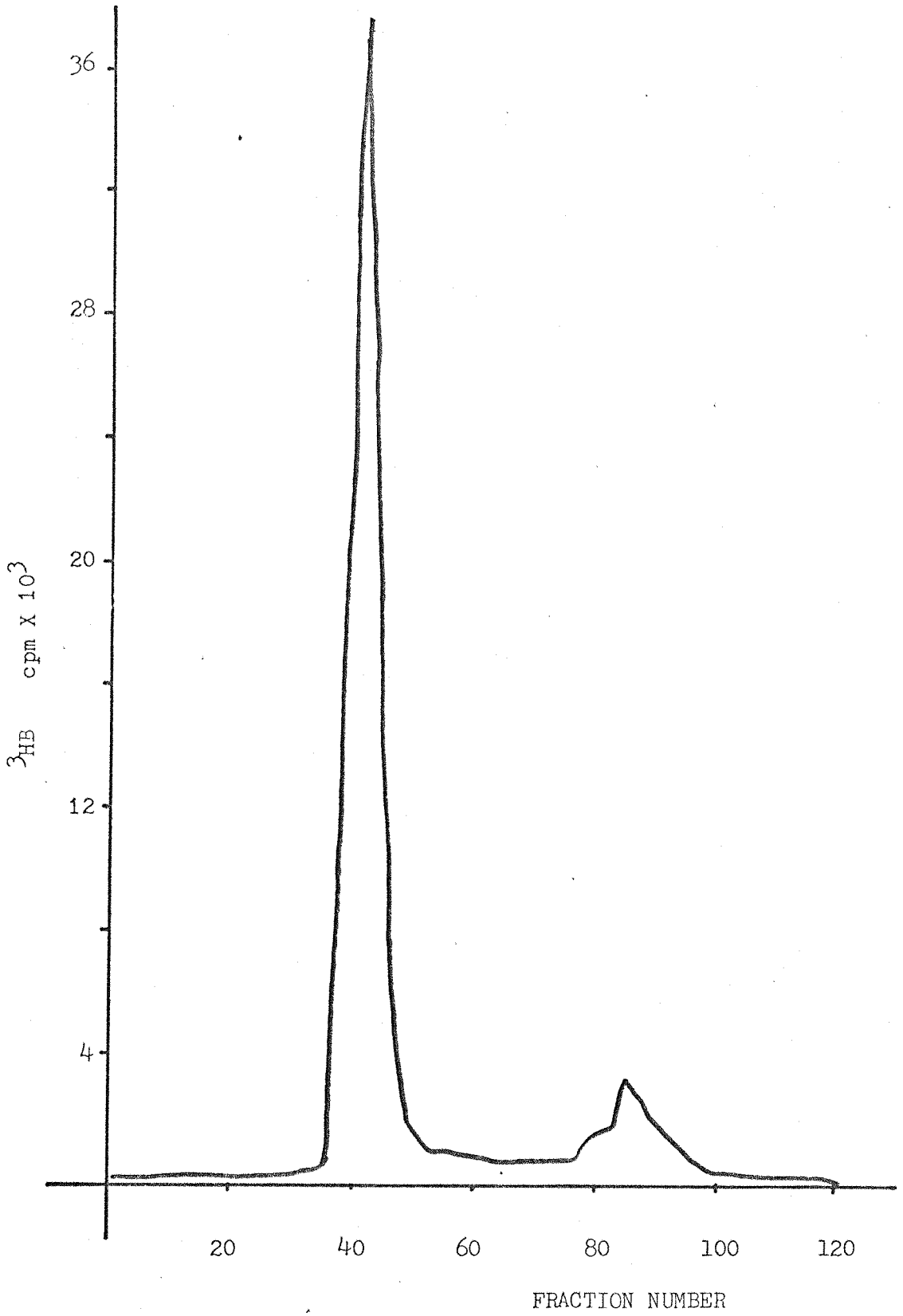


Fig. 9.4 A profile of rat plasma from T_4 treated 9 day old rats with ^{3}HB.

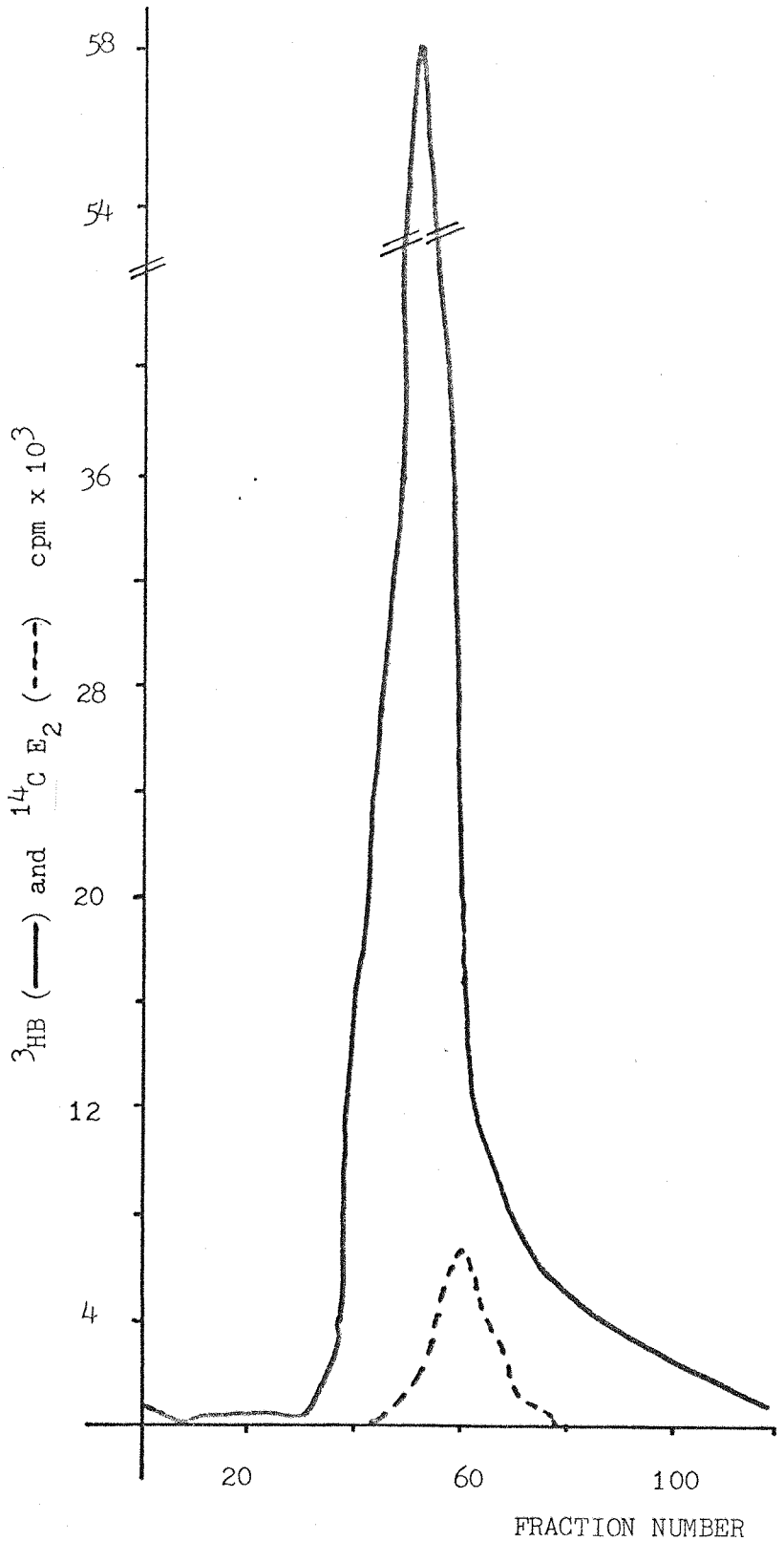


Fig. 9.5 A profile of rat plasma from 3 and 21 day old rats with ^{3}HB and ^{14}C E_2.

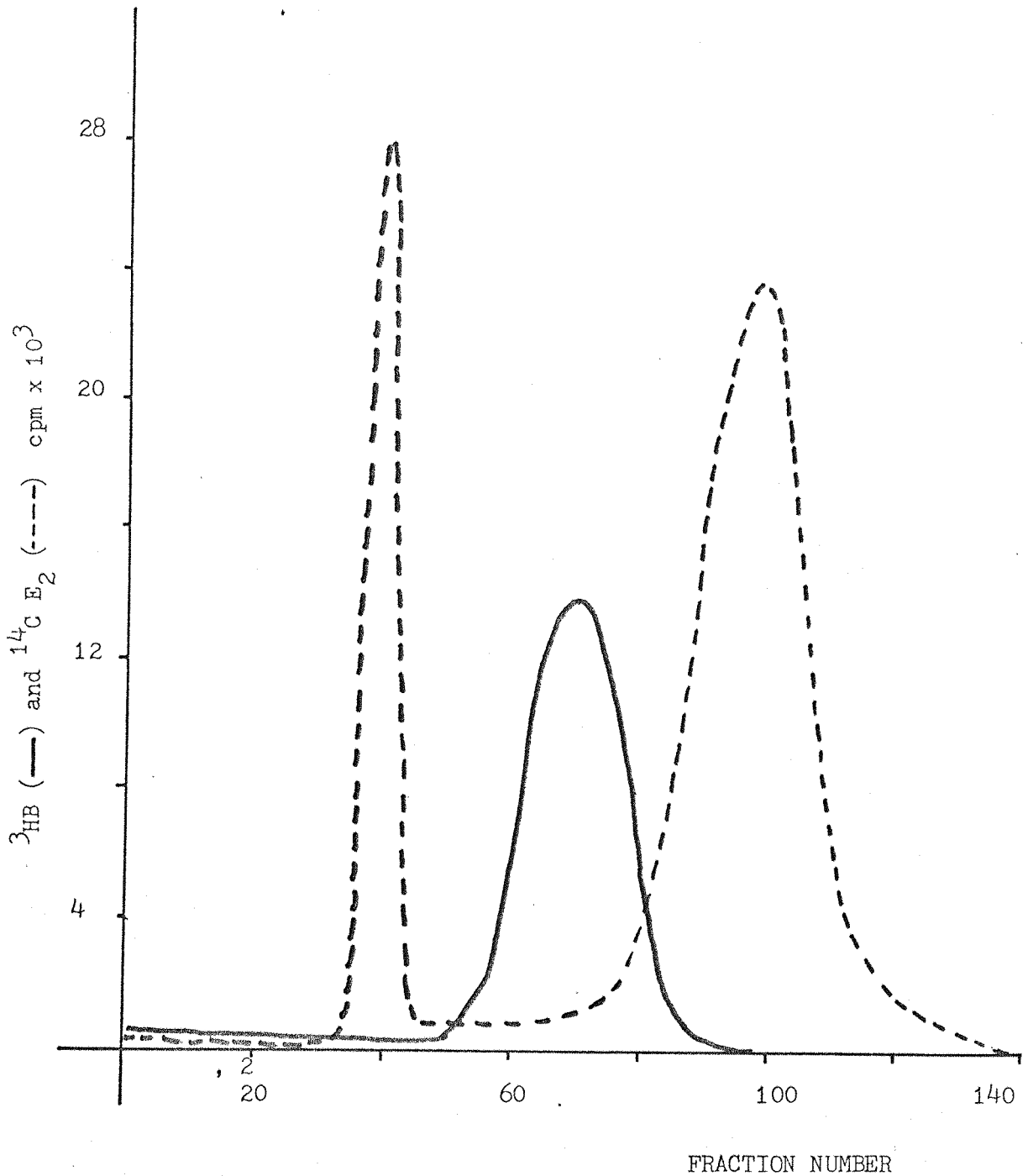


Fig. 9.6 A profile of rat plasma from 3 day old rats with ^{3}HB and ^{14}C E_2 at room temperature

Comment

The results from the gel filtration studies presented in this section provide qualitative support, by more conventional methods, for the binding measurement presented earlier using the modified Pearlman and Crepy method. A peak corresponding to binding of ^{3}HB is clearly visible at day 3 and day 21 but is very much less prominent at day 9 (Figs 9.1, 9.2 and 9.3). T_4 treatment of rats prior to day 9 results in the appearance of a bound peak (Fig. 9.4). The bound peak appears at the same position in each run indicating that it is likely that the same protein is involved in each case. This is more clearly evident from Fig. 9.5 where a mixture of day 3 and day 21 plasmas show only one sharp ^{3}HB bound peak. In this experiment ^{14}C labelled E_2 has been included. The bound peak for E_2 is separated from that for ^{3}HB indicating no interaction between CBG binding of glucocorticoids and the binding of androgens or oestrogens by their specific binding proteins which also show considerable changes in concentration over this period. At room temperature a bound peak for ^{3}HB is not discernible but the ^{14}C peak is more resistant to the increased temperature at day 3 (Fig. 9.6).

SECTION 10

Introduction

In Section 10 the isolation of CBG from human plasma and subsequent experiments using the isolated CBG are described.

Isolation of CBG from Human Plasma by Affinity Chromatography

Preparation of the Affinity Resin

This involved coupling the diaminocompound 3,3' diamino-dipropylamine (Eastman Chemicals) to the cynanogen bromide activated Sepharose 4B (CNBr Sepharose, Pharmacia) and then forming an amide between the cortisol hemisuccinate (Sigma) and the free amino groups on the Sepharose. The procedure adopted was a modification of the method of Rosner and Bradlow, 1974.

The diamino compound, (20 m moles per 10 mls Sepharose), was diluted with water to a volume of 10 mls. The pH was adjusted to 10 with concentrated HCL. The solution was cooled to 4°C. The freeze-dried CNBr Sepharose was washed with HCl, pH 2-3, for 15 minutes (200 mls per g) to activate it. 3.5 mls of washed swollen gel was generated from 1 g of freeze-dried CNBr Sepharose. Finally the activated gel was washed with 50 mls of ice cold water per 10 g of gel under suction. The previously prepared diamino compound was added immediately, the contents transferred to a flask and stirred very gently at 4°C overnight. The amino Sepharose was washed with water (at least 200 mls per 10 mls of gel) and stored with a bacterial retardant (0.01% azide) at pH 4-5. The success of the reaction was assessed using 2, 4, 6 trinitobenzene sulphonate.

The next step was couple the amino Sepharose to the cortisol hemisuccinate using dicyclohexylcarbodiimide (Aldrich Chemicals). The amino Sepharose was washed with 100 mls water, 100 mls of 75% water: 25% dioxane, 100 mls of 50% water: 50% dioxane, 100 mls of 25% water: 75 dioxane and 200 mls of 100% dioxane per 20 g amino Sepharose. The gel was poured into a beaker, allowed to settle, and as much of the dioxane as possible was removed. In separate

containers, 1 m mole of cortisol hemisuccinate and 1 m mole of dicyclohexylcarboiimide per 10 mls of amino Sepharose, were dissolved in a minimum volume of dioxane. Both reagents were added to the amino Sepharose and stirred at room temperature for 3 hours. The total volume of the reaction mixture was not allowed to exceed 25 mls per 10 mls of amino Sepharose. After 3 hours the reaction mixture was poured into a glass sintered funnel and washed thoroughly with 500 mls dioxane, 200 mls 75% dioxane: 25% water, 200 mls 50% dioxane: 50% water, 200 mls 25% dioxane: 75% water and finally 500 mls of water per 20 g of cortisol-Sepharose. The cortisol-Sepharose was incubated with 2% BSA for 1 hour to remove any unreacted cortisol. It was then washed and treated with 6 M urea for a further 0.5 hours, and then washed with 1 litre of water per 10 mls of gel.

The binding capacity of the gel was checked by incubating 50 ul of the gel with different volumes of charcoal stripped plasma, pH 4-5 for 1.5 hours. The volumes of plasma used were 2.5 mls, 5.0 mls and 7.5 mls. After 1.5 hours the contents of the flasks were allowed to settle and an aliquot was removed. The pH was adjusted to 7.0 and the sample was assayed for CBG activity (see Methods 12). From this a ratio of serum to cortisol-Sepharose was chosen which would result in 50-70% of the CBG activity present being bound.

Preparation of the Plasma

The plasma was obtained from the Wessex Regional Transfusion Centre. Firstly it was treated with solid $CaCl_2$ to give a final concentration of 20 mM and the clot that formed was removed. Next, 230 g/litre of solid ammonium sulphate was added slowly and the plasma stirred at 4°C for 30 minutes. The CBG, which is soluble at this concentration of ammonium sulphate, was harvested by centrifugation at 16 000 g for 30 minutes at 4°C. The endogenous steroid was removed by stirring with 10 g/litre of Norit A charcoal for 30 minutes at room temperature. The charcoal was removed by centrifugation and the pH of CBG solution (serum) was adjusted to 5.5 with 3 M HCl. The ratio of serum to cortisol-Sepharose was determined as described previously.

Adsorption and Elution of the CBG from the Cortisol-Sepharose

The serum was added to freshly washed cortisol-Sepharose (500 mls 80% methanol and 100 mls water per 20 mls of gel) and stirred gently at 4°C for 1.5 hours. The serum cortisol-Sepharose slurry was transferred to a glass sinter and most of the plasma was removed by suction. The gel was washed further under suction with 750 mls per 20 mls of gel of ice cold 0.05 M sodium phosphate:0.2 M sodium chloride pH 7.0. Finally, the gel was washed with 200 mls per 20 mls of gel of cold sodium phosphate buffer pH 7.0, 0.001 M. 20 mls per 20 mls of gel, of sodium phosphate buffer pH 6.5, 0.05 M:0.1M sodium chloride 24°C containing cortisol (200 ug/ml) was added to the gel. The column was allowed to equilibrate at room temperature for 1 hour before the CBG was eluted from the column with the pH 6.5 sodium phosphate buffer containing cortisol. Most of the CBG appeared in the first 50 mls of elute for a 20 ml gel volume. 5 x 20 ml fractions were collected from the 20 mls of resin. The binding activity was assessed using the Sephadex method.

The 2.4.6 Trinitrobenzenesulphonate Test (Cuatrecasas, 1970)

This simple colour test is a means of checking the success of the coupling of the diaminodiproplyamine to CNBr-Sepharose.

1 ml of a saturated sodium borate solution was added to the slurry (0.2 ml in distilled water) of reacted or untreated Sepharose. Three drops of a 3% aqueous solution of sodium 2.4.6 trinitrobenzenesulphonate was added. The colour reaction was complete in 2 hours. A yellow colour indicated unreacted Sepharose and an orange colour indicated amino Sepharose.

Concentration of CBG Solution (For Prep. 3 Only)

The CBG solution was placed in a round-bottomed flask and shell frozen before it was freeze-dried to 10% of its original volume.

3 preparations of CBG from human plasma were carried out using the method described previously. Table 10.1 gives the details of each preparation.

Table 10.1:

	Prep. 1	Prep. 2	Prep. 3
Volume of gel used	5 mls	15 mls	15 mls
Volume of plasma used	500 mls	1500 mls	1500 mls
Fraction size	5 x 10 mls	5 x 20 mls	1 x 100 mls
B binding using Sephadex method			
Plasma	47% bound	-	-
Fraction 1	39% bound	89% bound	16% bound
Fraction 2	0% bound	78% bound	
Fraction 3	0% bound	93% bound	
Fraction 4	0% bound	66% bound	
Fraction 5	0% bound	50% bound	
B binding after concentration	-	-	0% bound

Fraction 1 was the only fraction used from preparation 1. Fractions 1, 2 and 3 were used from preparation 2. Freeze-drying of the CBG solution in preparation 3 resulted in a complete loss of activity.

Experiments with the Isolated CBG Solution

Experimental Procedure

Litters were taken and divided into 2 groups, one the experimental, the other the control. The control group received 0.05 M sodium phosphate:0.02 M sodium chloride pH 6.5 whenever the experimental group received a dose of CBG solution. On day 9 the litters were killed by decapitation as quickly as possible and trunk blood was collected. After centrifugation and separation the plasma was stored at -20°C until assay. The % free B and the total plasma B concentration were determined for each sample.

Experiment 1

Animals were given 0.1 mls of CBG solution (Prep. 1 fraction 1) or 0.1 mls of the vehicle sc for 5 days from day 4. Rats were killed on day 9.

Table 10.2: % Free B and Total Plasma B Conctration After Buffer or CBG Treatment

	n	% Free B		n	Total Plasma B Concentration ng/ml	
Buffer	6	86.3 ± 4.5	NS	8	19.2 ± 1.7	NS
CBG	4	70.8 ± 6.1		6	18.8 ± 1.9	

Experiment 2

The same procedure was followed as for Experiment 1 using the CBG solution from Prep. 2 fractions 1, 2 and 3 pooled together.

Table 10.3: % Free B and Total Plasma B Concentration After Buffer or CBG Treatment

	n	% Free B		n	Total Plasma B Concentration ng/ml	
Buffer	7	78.3 ± 6.4	NS	7	7.4 ± 1.5	NS
CBG	6	72.8 ± 7.1		7	7.0 ± 1.8	

Experiment 3

The effect of administering the CBG solution orally or by ip injection.

Table 10.4: % Free B After Administering the CBG Solution (Preparation 2) Orally or ip Injection

		% Free B
By Mouth	Buffer	100
		100
	CBG	100
		100
ip	Buffer	82
		99
	CBG	93
		100

COMMENT

The results presented in the other sections have strongly suggested that the change in % free B has very important consequences on the activity of the adrenal axis during the postnatal period. T_4 administration has been shown to cause a fall in % free B in the 9 day old rat with an increase in total plasma B concentration and relative adrenal weight. T_4 has also caused an increase in the responsiveness of the adrenal axis to ACTH, ADH and insulin. However, as T_4 has other actions in the young rat, particularly on brain development (Eayrs, 1961) to be certain that this effect of T_4 is caused through its effect on CBG synthesis it has been attempted to produce a change in binding without using T_4. CBG can be isolated from human plasma using affinity chromatography. This was attempted and proved successful. This solution was then injected sc into young rats. No change in % free B was observed suggesting that the solution of CBG had not been absorbed. This preparation of CBG had a low binding activity so the lack of response may have been because too little CBG was injected. Preparation 2 gave a CBG solution of much greater activity but no change in % free B was observed when it was given to young rats. One reason for the lack of effect is that the CBG solution may not be absorbed using this route of administration. In Experiment 3, 2 other routes of administration were tried, ip and feeding the solution by mouth. Feeding the CBG solution to young rats was a possible route of absorption as intact protein can be absorbed across the gut wall in rats of this age. However the results of this experiment proved negative. As it was possible that the quantity of CBG given was insufficient another preparation of CBG was carried out and it was attempted to concentrate the preparation using a freeze-drying technique. However this resulted in a complete loss of activity. The reasons for the inability of administered CBG to produce a decrease in % free B in the 9 day old rat may be:-

1. The CBG solution given was too dilute
2. The CBG solution was not absorbed
3. The CBG solution was absorbed but because it was of human origin it was broken down rapidly. This explanation is the most likely because of the recent evidence of

Hossner and Billiar (1981) who showed that in the rat intact rat CBG had a 5 fold longer half life than human CBG. They suggested that the different half lives between the human and rat CBG were caused by differences in the metabolism of the two types of CBG by endogenous neuroaminidases. Thus, the experiments need to be repeated using CBG isolated from rat plasma.

DISCUSSION

DISCUSSION

Relative adrenal weight is high at birth in the young rat, decreases rapidly during the first week of life to reach minimum values by day 6, remains at a low value until day 13 and then increases to reach adult values by day 21 post-partum (Fig. 1.1). These changes in relative adrenal weight show similar trends to those observed but not explained by Bartova (1968) and Milkovic, Peruzovic, Romic, Paunovic and Pope (1977). Histological evidence of Van Dorp and Deane (1950) indicates that the changes in relative adrenal weight are caused primarily through changes in the relative weight of the adrenal cortex and not the adrenal medulla. Thus, suggesting that there is very little cortical growth in the newborn rat adrenal until after day 9 post-partum. Cohen (1963) demonstrated that the fall in relative adrenal weight began from day 19 of gestation (term 21-22 days in the rat), and then continued to fall more rapidly after birth. The specific activity of the cholesterol side-chain cleavage enzyme, the rate-limiting step in B synthesis (Gower, 1979) shows a similar trend to the change in relative adrenal weight (Doering and Clayton, 1969), thus when the adrenals are small they secrete less hormone than when they are larger. This suggests that the change in relative adrenal weight is associated with a decreased ability to synthesise steroid. The present study indicates that relative adrenal weight may be an important factor in the increase in plasma B concentration seen from day 13 to day 21 post-partum, (Fig. 1.3). The pattern of total plasma B concentration during the postnatal period in untreated rats shows a similar trend to the relative adrenal weight, in that total plasma B concentration is high at birth, decreases during the first week, and remains low, and increases to day 21 post-partum (Fig. 1.2). This shows a similar pattern to the composite findings of others (Daniels, Hardy, Malinowska and Nathanielsz(1972); Malinowska, Hardy and Nathanielsz (1972) and Henning, 1978. The latter measurements have been made using modifications of the competitive protein binding assay (CPB assay) of Murphy (1967) rather than a more specific radioimmunoassay (RIA). Both these methods give 5-10 times lower values for plasma B concentration than other methods such as fluorimetry. In recent years RIA has

proved to be the most sensitive, reliable and widely used technique for measuring steroids in microvolumes of plasma (Buster, 1980). Other methods include, CPB assay, spectrophotometry, fluorimetry, gas liquid chromatography and the double isotope method.

Spectrophotometry utilises an ultraviolet spectrophotometric detection procedure. This technique requires large volumes of plasma and cumbersome purification procedures to measure the diversity of relevant steroids. Fluorimetry is a similar type of technique to spectrophotometry. Steroids, like many other molecules, have the characteristic property of absorbing light and then emitting light at a longer, less energetic wavelength. A specific wavelength of light is used to excite the molecule (λ excitation) which is to be measured and the emission at a longer specific wavelength (λ emission) is measured. Fluorimetric methods frequently give much higher values than other methods. For example, Haltmeyer, Denenberg, Thatcher and Zarrow (1966) report a value of 60 ng/ml for plasma B concentration at day 9 compared with values measured by CPB assay of 15 ng/ml (Daniels et al., 1972) and of 5 ng/ml by RIA (Fig. 1.2). Gas liquid chromatography is able to separate steroids and each steroid can be identified by its elution profile and quantified by various detection devices. This method again is cumbersome and can not be applied to the microvolumes of plasma it is possible to obtain from neonatal rats. The double isotope derivative method involves the formation of a labelled derivative of the steroid using e.g. ^{14}C acetate. The derivative is isolated chromatographically and counted. Knowing the specific activity of the acetate used, it is possible to calculate the amount of derivative formed and hence the amount of steroid present in the original sample. Some pure ^{3}H labelled steroid is added to the original sample to allow a correction to be made for the inevitable losses in the various stages of the process - hence the term double isotope derivative. This technique is specific and is good for providing reference measurements, however it is a very lengthy, laborious process and is therefore not practical for a large number of samples. CPB assays are based on the competition between a steroid hormone with a radioactive label and the same, unlabelled hormone for a limited amount of binding sites on a protein binding agent. For example plasma B concentrations can be measured by CBP assay using human plasma as a source of binding

protein. Such an assay gives a plasma B concentration of 15 ng/ml at day 9 (Daniels et al., 1972) compared with 5 ng/ml by RIA (Fig. 1.2). CPB assays tend to give higher values for plasma steroid concentration because these assays measure any compound which will bind to the binding agent. For example when human plasma is used as the source of binding protein for measuring plasma B concentration, F and progesterone will also bind to the CBG thus tending to give an overestimation of the plasma B concentration. However CPB assay can be made more specific, e.g. Henning (1978) using rat plasma as the source of binding protein measured very similar plasma B concentrations to those measured by RIA in this study.

RIA is based on the reversible interaction between the haptenic sites of the antigen and the antigen binding sites of the antibody. The hapten groups which react with the antibody binding sites are small (about the size of a tetrapeptide). Steroids are small molecules of MW below 1000 (B MW = 348) and therefore must be bound to a protein carrier such as BSA before they become immunogenic. In the assay, a limited amount of specific antibody reacts with the hormone and corresponding hormone labelled with radioisotope (usually ^{3}H or ^{125}I). A standard curve can be generated by increasing the amount of cold hormone added to the system and thus decreasing the fraction of labelled hormone which is bound to the antibody. After a suitable incubation period the bound and the free hormone can then be separated by a variety of means including, adsorbing the free steroid (with e.g. dextran coated charcoal or Florisil); precipitating the steroid-antibody complex using a second antibody or using a solid phase separation system. The radioactivity can be assessed in the bound or the free fractions or both. A standard curve can then be constructed and unknown samples measured. RIA is an improvement on the other techniques already described, providing that the assay system is validated properly so only the desired steroid is measured. In this study 2 antibodies were used to measure total plasma B concentration. In both cases the phenomen of parallelism of the standard curve with extracted plasma was shown (Figs M.5 and 6). This indicates that the assays are measuring only B. The recovery of the assay for both antisera was high (Fig. M.7) showing that the extraction procedure was adequate. Similarly, parallelism was shown for the F assay (Fig. M.17) and the recovery of 98% indicated that this assay

system was specific for F and the extraction procedure was efficient. Although the values for total plasma B concentration using CPB (Henning, 1978) are not very different from the values obtained by RIA in this study, RIA has an important advantage in that it is more sensitive and therefore a smaller plasma sample is required, e.g. 50 ul plasma samples were used, at day 9 in this study compared with 100 ul samples in the Henning (1978) study. This is particularly useful in this study as only small samples can be collected from young rats without stressing them. The importance of a sensitive, specific assay for total plasma B concentration in the young rat is further confirmed by the measurement, by specific RIA, of a significant plasma F concentration (Fig. 1.2).

In terms of the relative adrenal weight and the total plasma B concentration, the pituitary adrenal axis appears to lose activity during the first postnatal week regaining activity during the third week. A similar pattern of activity is observed for the responsiveness of the axis during the postnatal period (see Section 2). In this study fixed challenges of ACTH, ADH and insulin were used to test the responsiveness of the adrenal axis. Some workers have studied the responsiveness of the adrenal axis during the postnatal period to a variety of noxious stimuli such as ether (Allen and Kendall, 1967; Levine, Glick and Nathane, 1967; Schoenfield, Leathem and Rabbi, 1980); histamine (Cote and Yasumura, 1975); hot and cold exposure (Zarrow, Haltmeyer, Denenberg and Thatcher, 1966) and convulsive electric shock treatment (Schapiro, Geller and Eiduson, 1962). Other workers have used these and other agents to study the response of rats at individual ages, Tang and Phillips (1977) used ether to study the stress response of 7 day old rats and Jailer (1950) used adrenaline and ADH to study the response of 4 day old rats. These workers assessed the stress-response either by measuring adrenal ascorbate depletion (Jailer, 1950) or plasma B concentration by fluorimetry (Zarrow et al., 1966; Levine et al., 1967; Allen and Kendal, 1967)or CPB assay (Schoenfield, Leathem and Rabii, 1980; Cote and Yasumura, 1975; Tang and Philips, 1977). The use of agents and conditions such as histamine, adrenaline, ether, hot and cold exposure or electric shock treatment have the disadvantages that they are very irritant and in the cases of the latter three are difficult to quantify. Insulin, however, by inducing hypoglycaemia

which is a potent physiological stimulus to the adrenal axis provides a closer representation to a physiological stress relevant to the young rat. It is also possible to quantify the dose and the degree of hypoglycaemia achieved. Insulin tests the responsiveness of the entire adrenal axis (Karteszi, Dallman, Makara and Stark, 1982). It is also possible to quantify the dose of ACTH and ADH used and important information is gained on the functioning of the different components of the axis. ACTH administration tests the response of the adrenal directly. ADH acts directly at the pituitary to cause the secretion of ACTH (Rivier, Vale and Guilleman, 1973), and therefore investigates the pituitary release of ACTH without interaction with the hypothalamus.

When examining the responsiveness of the pituitary adrenal axis, it is important to use the proper controls. Firstly, controls must be saline-injected animals as opposed to simply baseline uninjected animals. The significance of this is illustrated clearly in Section 2 when saline injection alone caused an increase in total plasma B concentration at day 21 but not at day 3 or day 9. Thus, at day 3 and day 9 the response would be similar comparing ACTH treated rats with either baseline values or saline injected controls but at day 21 there would be a considerable difference. Secondly, littermate controls must be used whenever possible because there is much between litter variation. Fig. D.1 represents data not previously presented in this thesis and illustrates this point. The responsiveness of day 3 rats to pyrogen or saline injection was tested. There was no significant difference between the saline treated groups and the pyrogen treated groups but there was a large standard deviation. However, there was a strong correlation between the two groups' individual litters. Thus the pyrogen-treated animals from individual litters had similar values for total plasma B concentration to the saline treated animals from the same litter.

At day 3 rats were very responsive to an acute ACTH challenge but this response was lost by day 9 (Fig. 2.1). At day 21 the effect of saline injection alone produced a significant increment in total plasma B concentration so the response to ACTH could only be observed in rats which were pretreated with dexamethasone, a synthetic glucocorticoid which not only inhibits the endogenous secretion of ACTH but does not cross-react in the B assay (Fig. 2.4). Various workers

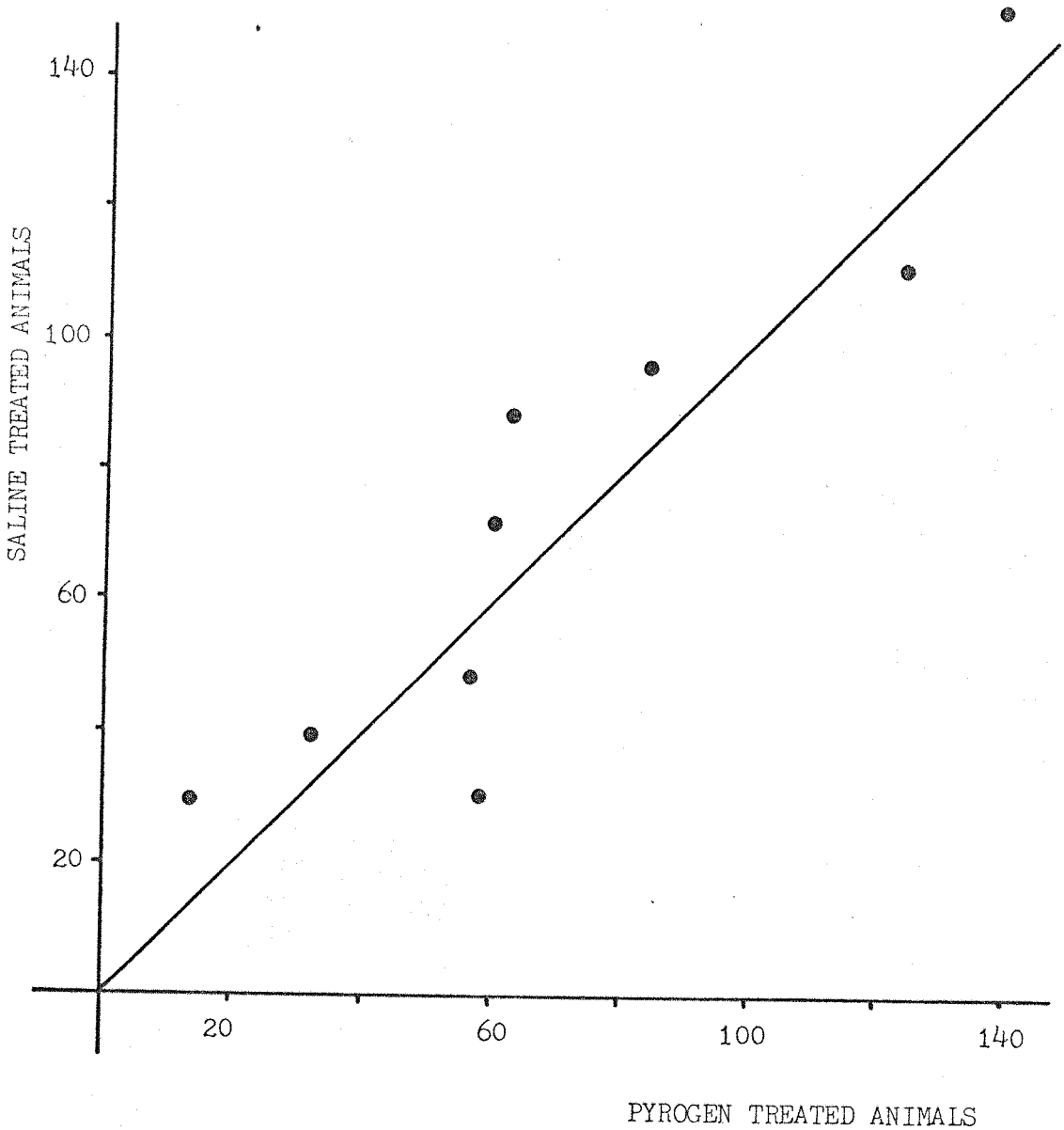


Fig. D.1 Total plasma B concentration after saline treatment plotted against total plasma B concentration after pyrogen treatment of litter-mates.

have studied the response of total plasma B concentration in young rats to exogenous ACTH and have found similar trends (Allen and Kendall, 1967; Bartova, 1967; Levine, Glick and Nakane, 1967; Cote and Yasumura, 1975; Guillet, Saffran and Michaelson, 1980; Schoenfield, Leathem and Rabii, 1980) although these workers have used larger doses of ACTH and have killed the animals at different intervals after the injections. A period of 30 minutes was chosen for the PRESENT study as it is used clinically in the short Synacthen test of adrenal function. The PREVIOUS groups have not expressed their results as an increment in total plasma B concentration after ACTH, (ACTH treated values - saline treated values). Since the baseline secretion following saline injection varies during development the increment must be calculated to observe the changes in the amount of hormone produced. The small response at day 21 after ACTH contradicts the findings of other groups but this may be because they used much larger doses than the one used here. However the significant effect of saline alone has also been observed by Schoenfield et al. (1980).

Schapiro, Geller and Eiduson (1962) observed that young rats did not respond to ADH until after day 14 post-partum. This DIFFERS FROM the findings in Section 2 (Fig. 2.2) where day 3 rats responded to an acute ADH challenge and day 9 and day 21 rats did not. At day 21 again there was a large increment in plasma B concentration caused by saline alone but dexamethasone could not be used to block the release of endogenous ACTH caused by the injection as the ADH induced secretion would also be blocked. The difference between the results of the present study and those of Schapiro et al. (1962) may be due to their use of fluorimetry or to the fact that they took their measurements after one hour. Zarrow, Haltmeyer, Denenberg and Thatcher (1966) found that 2 and 9 day old rat adrenals had a maximum response 15 to 30 minutes after a stress was induced and that the plasma B concentration had returned to control values after 1 hour. Therefore Schapiro et al. (1962) would not have been able to observe a stress response after 1 hour. The pattern of response in the PRESENT study is very similar for ADH and ACTH. This indicates that there is no change in the ADH induced pituitary secretion of ACTH during the postnatal period and that the change in response is caused by a change in the responsiveness of the adrenal itself.

The effect of insulin was studied after 1 hour of administration since in preliminary experiments using 21 day old rats 20-30 minutes elapsed before hypoglycaemia developed. A further 30 minutes were allowed before the effects of the hypoglycaemia were examined. Insulin induced hypoglycaemia at day 3, day 9 and day 21 but was significantly less effective at day 3. This suggests that there may be an immature response to insulin at day 3. The pattern of total increment in total plasma B concentration caused by the insulin induced hypoglycaemia was similar to the response caused by both ACTH and ADH (Fig. 2.3). This again suggests that lack of response at day 9 is caused by a change in the adrenal itself. This IS CONTRARY TO the findings of Hiroshige, Sato and Abe (1971) who studied the changes in hypothalamic content of CRF after exposure to noxious stimuli in adult and 2 day old rats. They found a biphasic response in adults but a slower monophasic response in the neonates and suggested that there may be a hypothalamic immaturity in the 2 day old rats. However, changes in content of a tissue are difficult to interpret and they used leg fracture under ether anaesthesia as the stress and ether anaesthesia as the control. It is likely therefore that their "control" animals were already highly stressed. Thus, when the relative adrenal weight and the total plasma B concentration are high the response of the adrenal axis to stimulation is high and when the relative adrenal weight and the total plasma B concentration are low the adrenal axis is unresponsive in terms of the total plasma B concentration. However, the proportion of the hormone which is free and active (Fig. 1.4) changes over the postnatal period.

Measurement of % free B steroid in plasma is a considerable technical problem. The estimation is made by incubating a known amount of radioactive steroid with a plasma sample. The bound and the free steroid may then be separated by a variety of means including:- equilibrium dialysis, flow analysis, ultrafiltration through a dialysis membrane, zonal chromatography, steady-state gel filtration and charcoal absorption. All these methods have one or more disadvantages and, with the exception of equilibrium dialysis, do not measure a true equilibrium state. Equilibrium dialysis, by combining the incubation step and the separation step, measures the true equilibrium.

However, this still has the disadvantages that large plasma volumes are required, the estimation of the plasma dilution can provide up to a 20% error (Moll and Rosenfield, 1978) and a long incubation period is required at relatively high temperatures which allows time for bacterial growth (Chopra, Abraham, Chopra, Solomon and Odell, 1972). In this study the % free B has been measured using a modification of the equilibrium method described by Pearlman and Crepy (1967) for testosterone which uses the cross-linked dextran, Sephadex G-25, in place of the dialysis membrane as the means of separating the bound and free steroid. This decreased the time required for equilibrium to be reached and thus reduced the common disadvantage of conventional dialysis. This method, because of the large surface area provided by the beads, means that the total volume of buffer can be very much reduced and therefore a smaller plasma sample is required. This low sample volume is a particular advantage in this study as only small volumes of plasma can be collected from young rats without causing stress or altering hormone values. Stress of young rats must be kept to a minimum as this will lead to activation of the adrenal axis thus preventing the study of the baseline secretion of the axis.

The % free B is low on day 1, increases to a maximum value by day 6, remains high until day 13 and then decreases to day 21 (Fig. 1.4). These values show similar trends to those reported by Gala and Westphal (1965) using equilibrium dialysis and those of Koch, Mialhe-Voloss and Stutinsky (1967a) and Henning (1978) by charcoal adsorption. The exact values are different through the differences in the methodology and BECAUSE these groups do not appear to have removed the endogenous steroid before doing the assay. As the total plasma B concentration varies during the postnatal period the effect of endogenous steroid will not be constant and the best way to avoid this source of error is to remove the endogenous steroid completely. The results are further confirmed by the gel filtration experiments in Section 9 which show that there is ^{3}HB bound to protein at day 3 and day 21 but that this bound peak is very much reduced at day 9. Martin, Cake, Hartmann and Cook (1977) observed that the % free B began to increase from day 19 of gestation as the total plasma B concentration decreased.

In the present study the assessment of % free B was carried out in diluted plasma but % free B will not change in a linear way with dilution. However the ratio of unbound to bound steroid (Su/Sb) does change in a linear manner with dilution since:-

$$[Su] + [P] \rightleftharpoons [Sb]$$

where:-

$$\frac{Su}{Sb} \propto \frac{1}{[P]}$$

P = protein
Su = unbound steroid
Sb = bound steroid

Thus Su/Sb can be used to gain an indication of the free B concentration in undiluted plasma. This calculation only gives an indication of the free B concentration because no corrections have been made for the temperature difference between the assay (room temperature) and the rat (37°C) and the small contribution to the total binding activity which albumin makes. This latter contribution is probably constant since the total protein concentration does not alter during the postnatal period (Table 1.4).

From day 3 to day 9 there is a 16 fold increase in Su/Sb with only a 3 fold decrease in total plasma B concentration. These changes suggest that in terms of the free active B concentration there is a considerable increase in activity. From day 9 to day 21 there was a 14 fold decrease in Su/Sb with a 14 fold increase in total plasma B concentration. These changes suggest that from day 9 to day 21 there is very little change in free active plasma B concentration. These results also show the importance of quantifying the protein binding of a hormone and not just the total plasma hormone concentration as the degree of binding can have such an important consequence for the activity of the axis.

The fetal rat adrenal is very active and capable of secreting large amounts of B, sufficient to cause the rise in maternal plasma B concentration seen before birth and to maintain maternal plasma B concentration after maternal adrenalectomy (Kamoun, 1970). Chatelain, Dupony and Allaume (1980) showed that plasma ACTH concentration was very high in the fetus in late gestation. These results need careful interpretation as it will be difficult to obtain blood in order to measure ACTH in the fetus without causing additional stress.

Immediately after birth, the total plasma B concentration is high (Cohen, 1976; Eguchi, Arishima, Morikawa and Hashimoto, 1977 and Corbier and Roffi, 1978), owing to the stresses of birth and a decrease in the volume of distribution as a result of separation from the mother. The plasma B concentration will be increased when the volume of distribution is decreased since:-

$$\text{Steroid Production} = MCR \times B \quad \text{or} \quad VD \times k \times B$$

(i.e. $MCR = VD \times k$)

$$B \propto \frac{1}{VD}$$

provided the production rate and k stay constant

where:-

MCR = metabolic clearance rate

k = fractional turnover rate

VD = volume of distribution

Prolonged, raised plasma B concentration causes a marked inhibition of ACTH secretion (Yates and Maran, 1975). Thus, after the stresses of delivery have subsided the plasma ACTH concentration will fall and adrenal growth and secretion will be suppressed.

In the following days there is a decrease in total plasma B concentration and relative adrenal weight. The rise in % free B from birth to day 9 exacerbates the situation by making an increasing proportion of the plasma B concentration free and therefore active. Thus, despite the falling of total plasma B concentration a strong negative inhibition will be maintained on the pituitary and will continue until the % free B begins to fall at the end of the second postnatal week. The fall in % free B at the same time may be able to reduce the inhibition on the hypothalamic-pituitary system with a result that ACTH secretion will increase and adrenal growth and secretion will be stimulated. The linear correlation between % free B and relative adrenal weight from day 5 to day 21 indicates the importance of % free B in determining relative adrenal weight during the postnatal period (Fig. 1.5).

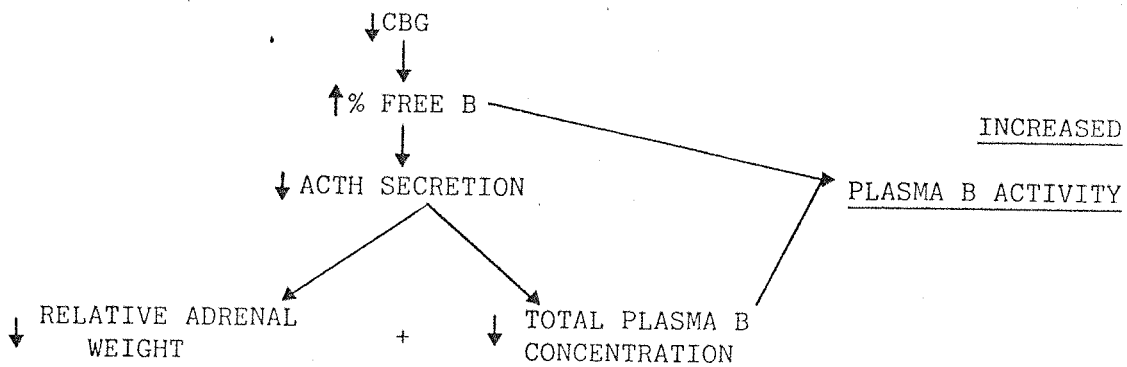
The change in % free B also has important consequences for the responsiveness of the adrenal in terms of the increment in the free active B concentration. From day 3 to day 9 there was an 8

fold fall in the secretion of total plasma B in response to an ACTH challenge with a 16 fold increase in Su/Sb. Thus, the response of the adrenal axis in terms of the increment in free active hormone has been maintained or even increased from day 3 to day 9 despite the smaller amount of hormone secreted at day 9. From day 9 to day 21 there was a 17 fold increase in the increment of total plasma B concentration in response to ACTH but there was a 14 fold decrease in Su/Sb. Thus, although the total amount of hormone secreted increased, the increment in the free active B concentration in response to ACTH was constant.

Therefore, the responsiveness of the adrenal axis increases from birth to day 9 and this increased response is maintained through the postnatal period. The high active B concentration may act by suppressing ACTH secretion leading to a decrease in the responsiveness of the adrenal in terms of the total amount of hormone secreted causing a decrease in adrenal growth and secreting capacity. These changes are summarised in Fig. D.2.

Plasma ACTH concentration was measured using an Amersham Immunoassay kit. This kit uses an antibody raised to the 1-24 fragment to which biological activity is attributed. This assay has been designed for clinical use where low sensitivity is not a problem since large volumes of plasma samples (2-8 mls) can be collected and resting plasma ACTH concentrations are greater than those in the young rat. However sensitivity is a problem in this study and blood from whole litters of the 3 and 9 day old rats had to be pooled together to get sufficient plasma to assay. The measurements of plasma ACTH concentration show that at day 3 and day 9 the plasma ACTH concentration was lower than at day 21 (Fig. 3.1). This is consistent with plasma ACTH concentration first being suppressed as a result of high % free B and then increasing as the % free B decreases. Guillet and Michaelson (1978) reported similar values for plasma ACTH concentration during the first 2 week post-partum but failed to see an increase by day 21 post-partum. ACTH secretion was not increased 5 minutes after ether inhalation at day 1 or day 7 which reflects the strong inhibition on pituitary secretion at this time. High glucocorticoid concentrations are known to inhibit ACTH secretion in response to stress (Dallman, 1979). These single measurements of plasma ACTH concentration are of only limited significance

From day 3 to day 9



From day 9 to day 21

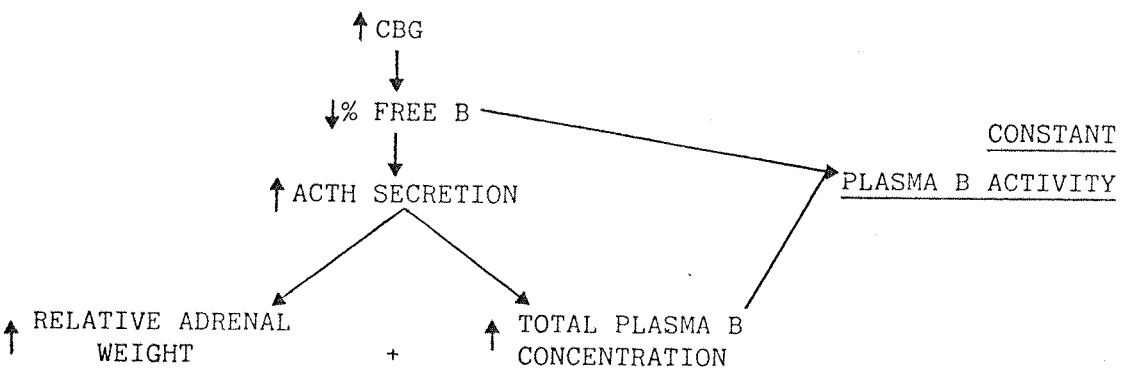


Fig. D.2: A Summary of the Changes Taking Place in the Adrenal Axis in the Neonatal Period in the Rat

since ACTH is secreted in distinct pulses and therefore ideally one should take frequent serial measurements as a single measurement cannot give a full picture of any change in the pattern of secretion (Rees, Jack, Thomas and Nathanielsz, 1975).

Holmes, Neto and Field (1980) demonstrated that hypophysectomy decreased the B responsiveness to ACTH stimulation in vitro in adult rats. A lack of ACTH secretion will therefore lead to a loss of adrenal responsiveness in terms of total plasma B concentration to ACTH as is observed from day 3 to day 9. When plasma ACTH concentration was increased, using depot ACTH, then there was a partial return of responsiveness in the 9 day old rat (Fig. 3.2). The effect of depot ACTH is not complete since administration of the hormone in a large dose in depot form leads to a slow release of ACTH. This slow release was non-pulsatile and it is the pulsatile secretion of a trophic hormone which is thought to maintain target tissue responsiveness (Bergquist, Nillius and Wide, 1979). Work by Philpott, Zarrow and Denenberg (1969a) and Guillet, Saffran and Michaelson (1980) showed that ACTH pretreatment made 7 day old rats more responsive in terms of total plasma B concentration thus confirming the results presented here.

The change in % free B has important consequences for the activity of the adrenal axis in the newborn rat. Although it appears that in terms of the total plasma B concentration the adrenal is active at birth, becomes inactive during the first week post-partum and regains activity in the third week post-partum, as the proportion of the hormone which is free changes the pattern of activity of the free active B concentration increases from birth to day 9 and this greater activity is then maintained until day 21. The total secretion of the adrenal per mg of tissue changes over this period so a constant response of the free active B concentration is maintained. The increment in total plasma B concentration can be expressed as a fraction of the adrenal to body weight ratio. This gives an indication of the total amount of B secreted by the gland and makes an allowance for the difference in body weight. It assumes a constant volume of distribution per g body weight.

i.e.

total hormone secreted = increment x volume of distribution (VD)

If one assumes VD $\propto$ body weight

Then:- total hormone secreted $\propto$ increment x B.wt

Therefore total secretion per mg of adrenal tissue =

$$\frac{\text{Increment in total plasma B concentration x body weight}}{\text{Adrenal Weight}}$$

=

$$\frac{\text{Increment in total plasma B concentration}}{\text{Adrenal Weight/Body Weight}}$$

=

$$\frac{\text{Increment in total plasma B concentration}}{\text{Relative Adrenal Weight}}$$

At day 3 the value is 3.6, at day 9 it is 1.1 and at day 21 it is 5.6. These values suggest that the adrenal axis responds to the increasing active hormone concentration by reducing the total amount of B secreted per mg of adrenal tissue as well as a decrease in relative adrenal size. The decrease in relative adrenal weight is caused mainly by changes in the relative adrenal weight of the adrenal cortex (Van Dorp and Deane, 1950). The in vitro superfusion experiments by Guillet, Saffran and Michaelson (1980) confirm that the adrenal becomes less sensitive to ACTH during the first week post-partum. However, Guillet et al. (1980) did not observe an increase in adrenal sensitivity to ACTH from day 7 to day 21 in vitro.

The high plasma B concentration maintains a strong inhibition on the pituitary. This is supported by the work of Butte, Kakihana, Farnham and Noble (1973) who measured the total brain content of B in the young rat from day 1 to day 21 post-partum. They found that the brain content of B was maximal at day 9. This high brain content of B will be able to suppress pituitary ACTH secretion and suggests a high active plasma B concentration.

From day 1 to day 9 the activity of some enzymes such as glucose-6-phosphatase, which are induced by glucocorticoids increase in tissues which respond to the free unbound hormone concentration, e.g. the kidney. In contrast in tissues such as the liver which are more protein permeable and therefore tend to respond to the total plasma hormone concentration (Keller, Richardson and Yates, 1969) the activity of glucose-6-phosphatase decreases from birth to day 9 (Greenard, 1975). There are two main phases of glucocorticoid dependent changes, one is around the time of birth and one is around the time of weaning. It was easy to interpret the changes as occurring at the time of high total plasma B concentration, however this is more difficult in the light of the changes in the free active B concentration. Many of the changes which occur in the second phase of development also require the presence of thyroid hormones. Therefore, development changes appropriate to weaning do not occur earlier when the plasma B concentration is high since thyroid hormone concentration only begins to increase from day 7 post-partum (Dussault and Labrie, 1975). T_4 has been shown to increase the number of glucocorticoid receptors in the lung of young rats (Morishige, 1982). T_4 action on the 'closure' in the gut is dependent on the presence of the adrenal cortex (Malinowska, Chan, Nathanielsz and Hardy, 1974) and thus this effect of T_4 on glucocorticoid receptors may be a general one. However, this situation is complicated by the finding of Henning, Ballard and Kretchmer (1975) who showed the specific binding of ^3H dexamethasone to intestinal slices from the young rat is maximal at day 10. This is a little early for T_4 to be effective. The lack of response may be caused by the low amount of non-protein sulphydryl groups in the young rat (Harris, Fang, Hinerfeld, Braverman & Vagenakis, 1979) which are known to increase stability of glucocorticoid receptors (Granberg and Ballard, 1977).

Plasma F concentration was less than 11 ng/ml during the first two weeks post-partum (Fig. 1.2). These values are similar to those reported by Daniels, Hardy, Malinowska and Nathanielsz (1972) using the CPB method. Philpott, Zarrow and Denenberg (1969b)suggest that there was no F in the plasma of a 1 day old rat. However, Kalavsky (1971) demonstrated that the adrenal content of F was very high in the fetal rat and showed that the ratio of B/F was 0.98 in the fetal rat adrenal compared with 9.0 in the adult rat adrenal.

The present results (see Section 1) show that when the total plasma B concentration is at its lowest the ratio of B/F was low. In the 9 day old rat plasma there is more F present than B whilst at day 3 and day 21 the situation is reversed. This means that at day 9 there is a significant contribution to the total glucocorticoid activity from F. F may also be bound with a lower affinity to the plasma binding proteins. Thus, when the free B concentration is high there is also a higher free F concentration. This situation is similar to the fetal and neonatal rabbit where there is more F present in the plasma than B (Malinowska, Hardy and Nathanielsz, 1972). Some glucocorticoid dependent changes can be induced prematurely by glucocorticoid administration, with F having a greater potency than B at a time when there is naturally more F present than B (e.g. gut closure; Daniels, Hardy, Malinowska and Nathanielsz, 1972). The high concentration of glucocorticoids may be important in the suppression of the thymus and immune system. Thymus function is under the control of the adrenal axis in the fetal rat as decapitation leads to an increase in thymus size which is prevented by glucocorticoid treatment (Jost and Picon, 1970). The suppression of the immune system may be advantageous in the young rat where the gut is still 'open' and therefore is absorbing intact maternal protein.

Van Baelen, Vandoren and De Moor (1977) using an immunodiffusion method to detect CBG specifically, produced evidence for a fall in plasma CBG concentration from day 1 to day 9 and an increase in CBG concentration from day 9 to day 21. Thus the increase in % free B from birth to day 9 presumably results specifically from this decrease in CBG concentration and does not reflect a general fall in plasma protein concentration as the total plasma protein concentration remains constant over this period (Table 1.4). Fig. 9.5 also demonstrates that the B binding at day 3 is not caused by the high levels of E_2 binding protein seen at this age. The decrease in plasma CBG concentration may be caused in a number of ways. Firstly, it may be caused by the disappearance of CBG of placental or maternal origin. The placenta is a very active organ involved in many metabolic processes and many proteins are synthesised by the placenta, e.g. human placental lactogen (Horne and Nisbet, 1979). Therefore synthesis of CBG by the placenta is not unlikely. Maternal CBG may be able to cross the placenta into the fetus as Brambell

and Halliday (1956) demonstrated that large γ globulins were able to cross the rat placenta via the yolk sac. A second possibility for the decrease in plasma CBG concentration in the neonate is the loss of maternal thyroid hormones. Maternal thyroid hormones are able to cross the placenta (Knobil and Josimovich, 1959) and may be able to stimulate CBG synthesis in the fetal liver. Roti, Fang, Braverman and Emerson (1982) demonstrated that the rat placenta was an active site of inner ring deiodination, i.e. T_4 is converted to rT_3. Amniotic rT_3 concentration is high through conversion of T_4 in the placenta (Roti, Braverman, Fang, Alex and Emerson, 1982) and therefore it seems unlikely that maternal T_4 will be able to stimulate fetal CBG synthesis, as any T_4 crossing the placenta will be broken down to rT_3 on the fetal side. A third possibility is that the fetal liver synthesises a CBG-like molecule which is under different control from the adult and the stimulation to synthesis is lost from day 19 of gestation onwards leading to a reduction in plasma CBG concentration.

The consequence of any one of these explanations is that after birth the plasma CBG concentration falls so that the % free B increases. The % free B begins to fall in the second postnatal week owing to an increase in CBG concentration (Van Baalen, Vandoren and De Moor, 1977). D'Agostino and Henning (1982) examined the *in vitro* hepatic production of CBG in the young rat and produced evidence for the increase in CBG concentration in the neonate being the result of *de novo* synthesis in the liver. At the same time as the % free B falls there is an increase in plasma thyroid concentration (Dussault and Labrie, 1975; Nathanielsz, 1976). The results presented here support the hypothesis that thyroid hormones are the physiological stimulus for the fall in % free B in the young rat. The results show that T_4 administration caused a premature fall in % free B in the 9 day old rat (Section 4). These results are in agreement with other workers (Koch, Mialhe-Voloss; Stutinsky, 1967b and D'Agostino and Henning, 1981). These workers began injecting T_4 earlier than day 4 when the % free B is high which may explain why D'Agostino and Henning (1981) saw an effect with a tenth of the dose that was effective in this study. However, these workers have not studied the time course of the response. The present study

showed that a single dose of 10 ug T_4/rat was sufficient to switch on CBG synthesis. There was no significant difference in the effect on % free B between groups injected with 10 ug T_4 on day 4 (Fig. 4.8) and groups receiving 10 ug T_4 for 5 days (Fig. 4.3). The magnitude of the fall in % free B depended on the timing of the initial injection. It was only after 5 days of 10 ug T_4 that there was a significant change in total plasma B concentration and relative adrenal weight. It is difficult to see why 10 ug on day 4, which produced a decrease in % free B in the 9 day old rat, caused no increase in total plasma B concentration and relative adrenal weight. Injecting 50 ug T_4 for 5 days was no more effective than injecting 10 ug T_4 suggesting that the changes produced were maximal. 5 days of 10 ug T_4/rat produced a plasma T_4 concentration which was 5 times higher than saline-treated controls. The value of plasma T_4 concentration for the saline-treated controls was very similar to the baseline values reported by Nathanielsz (1976) using the same method.

To evaluate the changes in free plasma B concentration the Su/Sb was again used. After T_4 treatment there was an 11 fold increase in the total plasma B concentration with a 9 fold decrease in Su/Sb. This suggests that there was very little change in the free active B concentration although there was a larger amount of hormone secreted and there was a greater amount of binding activity. After T_4 treatment the % free B falls and will reduce the strong negative feedback normally present in the 9 day old rat. Pituitary ACTH secretion will therefore increase, stimulating adrenal growth and secretion and consequently the total plasma B concentration increases, maintaining a constant free B concentration in the presence of the greater binding activity.

In addition to the action of T_4 on the static aspects of adrenal function, increases in the response of the adrenal axis in terms of the total plasma B concentration to the acute challenges of ACTH, ADH and insulin are observed. 10 ug T_4 on day 4 caused a decrease in % free B with no change in relative adrenal weight, and this led to an increase in the increment in total plasma B concentration caused by ACTH from 7.3 to 19.8 ng/ml (Fig. 5.1). This result is supported by the work of Meserve and Leathem (1974) who by feeding desicated thyroid to mothers during pregnancy and lactation demonstrated that 12 day old rats responded more to exogenous ACTH than untreated

litters. The effect of a daily dose of 10 ug T_4 for 5 days was to produce a fall in % free B with a small increase in relative adrenal weight and this led to a dramatic increase in the increment in total plasma B concentration in response to ACTH from 8 ng/ml to 93 ng/ml (Fig. 5.2). After 5 days of T_4 treatment, when both the % free B and the relative adrenal weight have changed, the 9 day old rat is very much more responsive to ACTH than after 1 day of T_4 treatment on day 4.

The change in the increment in the free B concentration can, as before, be studied using Su/Sb. After 5 days of 10 ug/rat T_4 there was a 9 fold decrease in Su/Sb with a 13 fold increase in the increment in total plasma B concentration. Thus, the change in the increment in the free active hormone concentration after T_4 treatment was small. The adrenal axis responds to the greater amount of binding activity with an increased total B output thus maintaining a constant level of response.

5 days of T_4/rat had a similar effect on the response to both ADH and insulin. This suggests that the increase in the responsiveness of the entire adrenal axis was caused through a change in the responsiveness of the adrenal itself. The similarity in the effect of T_4 on the response to ACTH and ADH suggest that T_4 is not altering pituitary ACTH secretion in response to ADH.

The adrenal increased its output of B per mg of adrenal tissue in response to ACTH after T_4 treatment. This was shown both *in vivo* and *in vitro*. *In vivo* (Section 5) it can be observed if the increment in total plasma B concentration is expressed as a fraction of the relative adrenal weight. After 5 days of T_4 treatment this value was 10 compared with 1.1 for saline-treated controls. The effect of this dose of T_4 on relative adrenal weight was small (16%) compared with the increase normally seen from day 9 to day 21 (40%). The apparent increased production per mg of adrenal tissue could be caused by a direct action of T_4 on adrenal B production. Alternatively it could be caused by a decreased volume of distribution and hence MCR or a decrease in peripheral metabolism caused by the greater binding activity. The *in vitro* incubation of adrenal tissue from 9 day old rats showed that T_4 treatment caused an increase in B production, thus confirming the increase in output per mg of adrenal tissue observed *in vivo* was a direct action. However, it

is not possible to say whether this is due to an increase in response of individual cells or whether there is an increase in the number of B producing cells.

Both T_4 and depot ACTH administration caused an increase in adrenal responsiveness to ACTH in terms of the total plasma B concentration. Depot ACTH caused a large increase in relative adrenal weight with no effect on % free B. The effect of T_4 was to cause a decrease in % free B with a small increase in relative adrenal weight. The increment in total plasma B concentration was greater after T_4 treatment than after depot ACTH. The production of B per mg of adrenal tissue is much greater after T_4 treatment (the increment as a fraction of relative adrenal weight is 2.6 after depot ACTH compared with 10 after 5 days of T_4). The reason for the difference is probably that T_4 is acting to increase the endogenous ACTH secretion from the pituitary. This secretion is pulsatile and it is the pulsatile secretion of ACTH which maintains adrenal responsiveness. When the pulsatile secretion of a trophic hormone is lost, as in the case of a long-acting analogue, the axis down-regulates (Bergquist, Nillius and Wide, 1979). So after the large dose of depot ACTH, the adrenal may well have lost its response to exogenous ACTH owing to down-regulation.

After depot ACTH administration there is an increment in total plasma B concentration in response to saline alone (Table 3.6). This indicates that the hypothalamic-pituitary system of the 9 day old rat does not fail to secrete ACTH in response to noxious stimuli because the young rat fails to recognise them as such. The absence of an increment in total plasma B concentration observed normally at this age (Table 2.4) therefore results from the small, unresponsive adrenals caused by the high free plasma B concentration.

If the rise in thyroid hormone concentrations are the physiological stimulus for the fall in % free B from day 9 onwards it should be possible to delay these changes using antithyroid drugs. PTU is a partial inhibitor of thyroid function. It reduces iodine uptake into the thyroid, T_4 secretion from the thyroid and the peripheral conversion of T_4 to T_3. PTU has been shown to be effective in reducing plasma T_4 concentration in the neonatal rat (see Results Section 6). Its administration reduced the fall in % free B (Fig. 6.1) normally seen by day 21. This was associated with a lower total

plasma B concentration (Fig. 6.2) and a decreased responsiveness to exogenous ACTH (Table 6.4). This shows again the importance of the % free B in determining the total plasma B concentration and adrenal responsiveness during the postnatal period. The decreased responsiveness to ACTH in terms of total plasma B concentration confirmed work by Meserve and Leathem (1981) who showed that thiouracil treatment of the mother reduced the response of day 21 rats to ACTH. The effect of PTU is not complete as it does not totally abolish thyroid function probably because the hypothroidism was not long enough to cause depletion of both the intracellular and extracellular stores of T_4 (Thomas and Nathanielsz, 1983). However, the action of PTU, be it only partial, supports the hypothesis that the rise in plasma thyroid hormone concentration is the physiological stimulus for the fall in % free B normally seen in the third postnatal week.

The thyroidal control of neonatal CBG synthesis provides a simple unique method to study the activity of other potential thyroid hormones in vivo. Most other systems rely on the measurement of changes in oxygen consumption, which are not always very accurate, or the prevention of goitre formation which really assesses the effect on TSH secretion rather than hormone action (Astwood, 1945). This study has shown that T_3 is ten times more potent than T_4 at causing a decrease in % free B in the 9 day old rat, along with an increase in total plasma B concentration and relative adrenal weight (Figs 7.2, 3 and 4). Most workers since Gross and Pitt-Rivers (1952), measuring the metabolic actions of thyroid hormones, have found T_3 to be 3-5 times more potent. The difference between the results presented here and other studies is probably caused because the measurements in this study have been of a specific function and the rate of hepatic β deiodination is low during the first week post-partum (Harris, Fang, Prosky, Braverman and Vagenakis, 1978). As CBG synthesis is a hepatic function the effectiveness of T_4 may be reduced if conversion to T_3 is a prerequisite for action. rT_3 is formed from α deiodination rather than β deiodination. Recently there has been much interest in its potential physiological actions, whether it has any direct stimulatory action of its own in vivo or whether it inhibits or stimulates T_4 to T_3 conversion thus giving rT_3 a peripheral regulatory role in thyroid hormone action. The present study (see Section 7) suggests that in the

19 day old rat where hepatic β deiodination is fully functioning, rT_3 has no significant action at physiological doses, probably because it is cleared quickly. However, in the 9 day old rat where the rate of hepatic β deiodination is slow, rT_3 was able to inhibit the action of T_4 with no effect on T_3 action. This would suggest that rT_3 was acting to inhibit T_4 to T_3 conversion. rT_3 has no stimulatory action of its own in the 9 day old rat, indicating the low level of endogenous thyroid hormone activity at this age. This system provides a novel and potentially useful way of assessing endogenous thyroid hormone activity.

In 1962 Schapiro, Geller and Eiduson defined the period in the young rat, where no response to stimulation was observed as the stress non-responsive period (SNR period). They attributed the lack of response to brain immaturity and a lack of hypothalamic neural connections. The results from this study provide a strong case for the SNR period being caused by a decrease in the activity of the adrenal itself and not a lack of hypothalamic-pituitary activity. The changes caused by T_4 here have been attributed to its effect on the % free B, however T_4 has many actions during development, particularly on the brain (see Introduction) and therefore ideally one should examine the effects of a change in plasma B binding activity brought about by means other than T_4 treatment.

Section 10 describes the isolation of CBG from human plasma by affinity chromatography using a modification of the method of Rosner and Bradlow (1974). The principle of the method is that F is chemically coupled to amino-Sepharose 4B to form an affinity resin which will bind CBG specifically. The plasma is first treated with calcium chloride, then ammonium sulphate to precipitate some of the unwanted proteins. The endogenous steroid is removed using charcoal as it will prevent the CBG binding to the affinity resin effectively. The pH of the plasma is then adjusted to 5.5 to prevent hydrolysis of the amine groups on the Sepharose thus rendering it inactive. A preliminary incubation with 50 ul of the resin and different volumes of plasma was made. A plasma to resin ratio giving 50% of the CBG present was used. If this ratio is exceeded CBG removal from the resin would be more difficult. The plasma and affinity resin are incubated together, with gentle stirring, for 1½ hours before the resin is washed with a low molarity phosphate

buffer containing a high salt concentration at 0°C. The CBG is eluted from the resin with a high salt concentration phosphate buffer containing an excess of F, at room temperature. 3 preparations of CBG were made successfully using this method. The first preparation was injected s.c. into young rats and the rats were killed at day 9. No change in % free B or total plasma B concentration was observed (Table 10.2). As the activity of the CBG solution was quite low a second preparation of CBG was made. This preparation had a much higher activity, but still there was no change in % free B in 9 day old rats after 5 days of treatment with the CBG solution s.c. Two different routes of administration were tried, ip and by mouth, but both these routes of administration were unsuccessful at causing a decrease in % free B in the 9 day old rat. This suggests that the CBG solution was not absorbed by any of the tried routes of administration or that the amount of CBG administered was too little. Thus, a third preparation was carried out and the solution was concentrated by freeze-drying. This lead to a complete loss of activity, so a different method of concentrating the CBG solution is required such as using a pressure cell. A third possibility for the loss of activity may be caused by the human source of the CBG being unable to produce a fall in % free B. Hossner and Billiar (1981) showed that human CBG was broken down 5 times more quickly in the rat compared with rat CBG. This seems the most likely explanation for injected CBG having no effect on % free B in the 9 day old rat and a preparation of CBG from the rat may prove more successful.

Thus, the most important further experiment to come from this study is to attempt to isolate CBG from rat plasma and see if this can cause a premature fall in % free B in the 9 day old rat with accompanying changes into total plasma B concentration and relative adrenal weight. It still remains to be shown what is causing the initial fall in % free B, relative adrenal weight and total plasma B concentration from day 19 of gestation.

BIBLIOGRAPHY

Allen, C. and Kendall, J. W. (1967)
Maturation of the circadian rhythm of plasma B in the rat.
Endocrinology, 80, 926-930

Anderson, H. J. (1960)
X-ray changes in children with hypothyroidism.
Acta paediatrica Supplement, 125, 83-101

Andrews, P. (1966)
Molecular-Sieve Chromatography.
British Medical Bulletin, 22, 109-114

Asrwood, E. B. (1945)
Chemotherapy of hyperthyroidism.
Harvey Lectures, 40, 195-234

Balfour, W. E. (1962)
The adrenal cortex in domestic animals.
British Medical Bulletin, 18, 114-116

Balfour, W. E. (1969)
Inhibition of thyrotropin secretion and deiodination of thyroxine.
Journal of Physiology, 200, 48p

Balsam, A., Eisenstein, Z., Sexton, F., Ingbar, S. (1978)
Studies on the peripheral metabolism of tri-iodothyronine and reverse tri-iodothyronine in the rat. Comparison of extraction anc chromatographic methods of analysis.
Endocrinology, 102, 1247-1253

Barker, S. B. and Klitgaard, H. M. (1952)
Metabolism of tissue excised from thyroxine-injected rats.
American Journal of Physiology, 170, 81-86

Barnes, R. J., Comline, R. S., Silver, M. (1978)
Effect of cortisol on liver glycogen concentrations in hypothectomised, adrenalectomised and normal foetal lambs during late and prolonged gestation.
Journal of Physiology, 275, 567-579

Barton, R. N., Little, R. A. (1978)
Effects of inhibition of adrenal steroidogenesis on compensation of fluid loss and on survival after limb ischaemia in the rat.
Journal of Endocrinology, 76, 293-302

Bartova, A. (1968)
Functioning of the hypothalamo-pituitary-adrenal system during the postnatal period.
General and Comparative Endocrinology, 10, 235-239

Baudry, M., Clot, J. P., Bouhnik, J., Michel, O., Michel, R. (1975)
Thyroid hormone action on electrophoresis patterns of proteins of the rat liver and muscle mitochondria.
Seventh International Thyroid Conference, Boston, Mass., Abstract 118

Bergquist, C., Nillius, S. J., Wide, L. (1979)
Intranasal Gonadotropin-releasing hormone agonist as a contraceptive agent.
Lancet, ii, 215-216

Bernal, J. and Refetoff, S. (1977)
Thyroid hormone action.
Clinical Endocrinology, 6, 227-249

Bradford, M. (1976)
A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
Analytical Biochemistry, 72, 248-254

Brambell, F. W. R. and Halliday, R. (1956)
The route which passive immunity is transmitted from the mother to the foetus in the rat.
Proceedings of the Royal Society (London), B, 145, 170-198

Bressler, R. and Wittels, B. (1966)
The effect of T_4 on lipid and carbohydrate metabolism in the heart.
Journal of Clinical Investigation, 45, 1326-1333

Brewer, D. B. (1957)
Congenital Absence of the pituitary gland and its consequences.
Journal of Pathology and Bacteriology, 73, 59-67

Buchanan, J. L., Primack, M. T., Tapley, D. F. (1971)
Effect of inhibition of mitochondrial protein synthesis in vitro upon T_4 stimulation of oxygen consumption.
Endocrinology, 89, 534-537

Buckingham, J. C., and Leach, J. H. (1980)
Hypothalamo-pituitary-adrenocortical function in rats with inherited diabetes insipidus.
Journal of Physiology, 305, 397-404

Buckingham, S., McNary, W. F., Sommers, S. C., Rothschild, J. (1968)
Is lung an analogue of Moog's developing intestine? I Phosphatases and pulmonary alveolar differentiation in foetal rabbits.
Federation Proceedings, 27, 328, Abstract No. 638

Buster, J. E. (1980)

Steroid radioimmunoassays applied to the study of animals models in Animal models in Fetal Medicine, p.314-362, Editor: Nathanielsz, P. W. Publishers: Elsevier/North-Holland Biomedical Press

Butte, J. C., Kakihana, R., Farnham, M. L., Noble, E. P. (1973)

The relationship between brain and plasma B stress response in developing rats.

Endocrinology, 92, 1775-1779

Carpenter, E. and Rondon-Tarchetti, T. (1957)

Differentiation of the embryonic rat thyroid in vivo and in vitro.

Journal of Experimental Zoology, 136, 393-405

Chan, W. S., Daniels, V. G., Thomas, A. L. (1973)

Premature cessation of macromolecule uptake by the young rat intestine following thyroxine administration.

Journal of Physiology, 231, 112p

Chapman, R. E., Hopkins, P. S., Thorburn, G. D. (1974)

The effects of fetal thyroidectomy and thyroxine administration on the development of the skin and wool follicles of sheep fetuses.

Journal of Anatomy, 117, 419-432

Chatelain, A., Dupony, J. P., Allaume, P. (1980)

Fetal-maternal ACTH and B relationships in the rat: Effect of maternal adrenalectomy.

Endocrinology, 106, 1297-1303

Chopra, I. J., Abraham, G. E., Chopra, U., Solomon, D., Odell, W. D. (1972)

Alterations of circulating E_2 17β in male patients with Grave's disease.

New England Journal of Medicine, 286, 124-129

Chopra, I. J., Williams, D. E., Orgiazzi, J., Solomon, D. (1975a)

Opposite effects of dexamethasone on serum concentration of 3,3'5' tri-iodothyronine (rT_3) and 3,3',5 tri-iodothyronine (T_3).

Journal of Clinical Endocrinology and Metabolism, 41, 911-920

Chopra, I., Chopra, U., Smith, S., Reza, M., Solomon, D. (1975b)

Reciprocal changes in serum concentrations of rT_3 and T_3 in systemic illness.

Journal of Clinical Endocrinology and Metabolism, 41, 1043-1049

Chopra, I. (1976)

An assessment of daily production and significance of thyroidal secretion of rT_3 in man.

Journal of Clinical Investigation, 58, 32-40

Cohen, A. (1963)

Correlations entre l'hypophyse et le cortex surrenal chez de le foetus de rat et le cortex surrenal du nouveau-ne.

Part 1 Etude ponderale des surrenales foetal et neo-nates du rat.

Achives D'Anatomie Microscopique et De Morphologie Experimentale, 52, 294-343

Cohen, A. (1976)

Adrenal and plasma B levels on the pregnant, fetal and neonatal rat during the perinatal period.

Hormone and Metabolic Research, 8, 474-478

Coiro, V., Harris, A., Goodman, H. M., Vagenakis, A., Braverman, L. (1980)

Effect of pharmacological quantities of infused 3,3',5' tri-iodothyronine on thyroxine monode-iodination to 3,3',5-tri-iodothyronine.

Endocrinology, 106, 68-75

Comline, R. S. and Silver, M. (1972)

The composition of foetal and maternal blood during parturition in the ewe.

Journal of Physiology, 222, 233-256

Corbier, P. and Roffi, J. (1978)

Pituitary Adrenocortical response to stress during the first day of postnatal life in the rat.

Biology of the Neonate, 34, 105-111

Cote, T. and Yasumura, S. (1975)

Effect of ACTH and Histamine stress on serum B and adrenal cyclic AMP levels in the immature rat.

Endocrinology, 96, 1044-1047

Cuatrecasas, P. (1970)

Protein Purification by affinity chromatography. Derivatization of agarose and polyacrylamide beads.

Journal of Biological Chemistry, 245, 3059-3065

D'Agostino, J. and Henning, S. J. (1981)

Hormonal control of postnatal development of corticosteroid binding globulin.

American Journal of Physiology, 240, E 402-406

D'Agostino, J. and Henning, S. J. (1982)

Postnatal Development of CBG. Effect of T_4.

Endocrinology, 111, 1476-1482

Dallman, M. (1979)
Adrenal feedback on stress-induced corticoliberin (CRF) and corticotrophin (ACTH) secretion.
in Interactions within the Brain- Pituitary-Adrenocortical System, p.149-162. Editors Jones, M. T., Gillman, B., Dallman, M. F., Chattopadhyay, S. Publishers Academic Press, London, New York, San Francisco

Daniels, V. G., Hardy, R. N., Malinowska, K. W., Nathanielsz, P. W. (1972)
Adrenocortical hormones and absorption of macromolecules by the small intestine of the young rat.
Journal of Endocrinology, 52, 405-406

Daniels, V. G., Hardy, R. N., Malinowska, K. W., Nathanielsz, P. W. (1973)
The influence of exogenous steroid on macromolecule uptake by the small intestine of the newborn rat.
Journal of Physiology, 229, 681-695

Desbal, B., Desbal, P., Agid, R. (1970)
Pituitary-adrenal control of fat mobilisation in rabbits
in Adipose Tissue: Regulation and metabolic functions
Editors: Jeanrenaud, B., Hepp, B., New York Academic Press

Doell, R. G. and Kretchmer, N. (1964)
Intestine invertase: precocious development of activity after injection of hydrocortisone.
Science, 143, 42-44

Doering, C. H. and Clayton, R. B. (1969)
Cholesterol side-chain cleavage activity in the adrenal gland of the young rat: Development and Responsiveness to ACTH.
Endocrinology, 85, 500-511

Donovan, B. T. (1970)
The Brain and ACTH secretion.
In Mammalian Neuroendocrinology, p.66-72, McGraw-Hill

Dussault, J. and Labrie, F. (1975)
Development of Hypothalamic-pituitary-thyroid axis in neonatal rat.
Endocrinology, 97, 1321-1324

Eayrs, J. T. (1955)
Cerebral cortex of normal and hypothyroid rats.
Acta Anatomica, 25, 160-183

Eayrs, J. T. (1961)
Age as a factor determining the severity and reversibility of the effects of thyroid deprivation in the rat.
Journal of Endocrinology, 22, 409-419

Edelman, I. S. and Ismail-Beigi, F. (1974)
Thyroid thermogenesis and active sodium transport.
Recent Progress in Hormone and Metabolic Research, 30, 235-257

Edmonds, C. J. (1978)
Aldosterone Secretion and its clinical disorders: i Control of Aldosterone Secretion, p.565-576
in General, Comparative and Clinical Endocrinology of the Adrenal Cortex, Part 2. Editors: Chester-Jones, I. and Henderson, I. W.
Publishers Academic Press, London, New York, San Francisco

Edwards, A. V., Hardy, R. N., Malinowska, K. W. (1974)
The effects of infusions of synthetic ACTH in the conscious calf.
Journal of Physiology, 239, 477-498

Eguchi, Y. (1961)
Atrophy of the fetal mouse adrenal following decapitation in utero.
Endocrinology, 68, 716-718

Eguchi, Y., Arishima, Morikawa, Y., Hashimoto, Y. (1977)
Rise in plasma B concentration in rats immediately before and after birth and in fetal rats after ligation of maternal uterine blood vessels or of the umbilical cord.
Endocrinology, 100, 1443-1447

Elgee, N. J. and Williams, R. H. (1955)
Effects of thyroid function on insulin degradation.
American Journal of Physiology, 180, 13-20

Elkholm, R. and Wollman, S. H. (1975)
Site of iodination in the rat thyroid gland deduced from electron microscopic autoradiographs.
Endocrinology, 97, 1432-1444

Fahmy, D., Reed, G., Hillier, S. (1975)
Some observations on the determination of cortisol in human plasma by radioimmunoassay using antisera against cortisol - 3- BSA.
Steroids, 26, 267-280

Feldman, D., Mondon, C. E., Horner, J. A., Weiser, J. N. (1979)
Glucocorticoid and estrogen regulation of CBG production by rat liver.
American Journal of Physiology, 237, E493-499

Fiegelson, P. and Killewich, L. A. (1979)
Hormonal and Developmental Modulation of tryptophan oxygenase mRNA.
In Glucocorticoid Hormone Action, p.245-252.
Editors Baxter, J. D. and Rousseau, G. G. Publishers: Springer-Verlag

Fleischer, N. and Vale, W. (1968)
Inhibition of vasopressin-induced ACTH release from the pituitary by glucocorticoids in vitro.
Endocrinology, 83, 1232-1236

Foster, P. S., Goldie, R. G., Paterson, J. W. (1983)
Effects of steroids on β -adenoceptor-mediated relaxation of pig bronchus.
British Journal of Pharmacology, 78, 441-445

Frankel, A., Cook, B., Graber, J. W., Nalbandov, A. V. (1967)
Determination of B in plasma by fluorometric techniques.
Endocrinology, 80, 181-194

Friedman, Y., Lang, M., Burke, G. (1977)
Inhibition of thyroid adenlate cyclase by thyroid hormone: A possible locus for the short-loop negative feedback phenomenon.
Endocrinology, 101, 858-868

Furihata, C., Kawachi, T., Sugimura, T. (1972)
Premature induction of pesinogen in developing rat gastric mucosca by hormones.
Biochemical, Biophysical Research Communications, 47, 705-711

Gala, R. and Westphal, U. (1965)
CBG in the rat - Studies on sex difference.
Endocrinology, 77, 841-851

Gala, R. and Westphal, U. (1966)
Further studies on the CBG in the rat: Proposed endocrine control.
Endocrinology, 79, 67-76

Gambert, S. R., Garthwaithe, T. L., Pontzer, C. H., Hagen, T. C. (1980)
Thyroid hormone regulation of CNS Beta-Endophin and ACTH.
Hormone and Metabolic Research, 12, 345-346

Geel, S. E., Valcana, T., Timiras, P. S. (1967)
Effect of neonatal hypothyroidism and of thyroxine on L-(^{14}C) leucine incorporation into protein in vivo and the relationship to ionic levels in the developing rat brain.
Brain Research, 4, 143-150

Geloso, J. P., Hemon, P., Legrand, J. Legrand, C., Jost, A. (1968)
Some aspects of thyroid physiology during the perinatal period.
General and Comparative Endocrinology, 10, 191-197

Gillies, G. and Lowry, P. (1979)
The relationship between vasopressin and CRF.
In Interactions within the Brain-pituitary-adrenocortical system, p.51-61
Editors: Jones, M. T., Gillman, B., Dallman, M. F., Chattopadhyay, S.
Publishers: Academic Press, London, New York, San Francisco

Gillies, G., Linton, E. A., Lowry, P. (1982)
Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin.
Nature, 299, 355-356

Gomez, C. J. (1971)
Hormonal influence on the biochemical differentiation of the rat cortex.
in Hormones and Development, 417-436.
Editors; Hamburgh, M. and Barrington, E. J. W.
Publishers: Appleton-Century-Crofts, New York

Gower, D. B. (1979)
Biosynthesis of corticosterone, androgens and oestrogens
in Steroid Hormones, p.31-44
Publishers: Croom Helm Ltd.

Granberg, J. P. and Ballard, P. L. (1977)
Role of sulphydryl groups in the binding of glucocorticoids by cytoplasmic receptors of the lung and other mammalian tissues.
Endocrinology, 100, 1160-1168

Greenard, O. and Dewey, H. K. (1971)
The prematurely evolved synthesis of liver tryptophan oxygenase.
Proceedings of the National Academy of Sciences, USA, 68, 1698-1701

Greenard, O. (1975)
Cortisol Treatment of neonatal rats. Effects on enzymes in the kidney, liver and heart.
Biology of the Neonate, 27, 352-360

Greer, M. A., Grim, Y., Studer, H. (1968)
Qualitative changes in the secretion of thyroid hormones induced by iodine deficiency.
Endocrinology, 83, 1193-1198

Gross, J. and Pitt-Rivers, R. (1952)
Physiological activity of 3,5,3'-L-tri-iodothyronine.
Lancet i, 593-594

Gryglewski, R. J. (1980)
The lung as a generator of prostacyclin.
in Ciba Foundation Symposium 78
Metabolic activities of the lung, p.147-150

Guilleman, R., Dear, W. E., Liebett, R. A. (1959)
Nychthemeral variation in plasma free corticosteroid levels in the rat.
Proceedings of the Society of Experimental Biology and Medicine, 101, 394-395

Guillet, R. and Michaelson, S. M. (1978)
Corticotrophin responsiveness in the neonatal rat.
Neuroendocrinology, 27, 119-125

Guillet, R., Saffran, M., Michaelson, S. (1980)
Pituitary-Adrenal response in neonatal rats.
Endocrinology, 106, 991-994

Halliday, R. (1955)
The absorption of antibodies from immune sera by the gut of the young rat.
Proceedings of the Royal Society, B 143, 408-413

Halmi, N. S. and Spirtos, B. N. (1955)
Analysis of the modifying effect of dietary iodine levels on the thyroidal response of hypophysectomised rats to TSH.
Endocrinology, 56, 157-160

Halmi, N. S., Granner, D. K., Albert, H., Doughman, D. J. (1959)
Inhibition of thyroidal iodide concentration by T_3: Evidence for an effect not due to depression of TSH but dependent on the pituitary.
Endocrinology, 65, 101-105

Haltmeyer, G. C., Denenberg, V. H., Thatcher, J., Zarrow, M. X. (1966)
Response of the adrenal cortex of the neonatal rat after subjection to stress.
Nature, 212, 1371-1373

Hamburgh, M., Lynn, E., Weiss, E. P. (1964)
Analysis of the influence of thyroid hormones on prenatal and postnatal maturation of the rat.
Anatomical Records, 150, 147-162

Harris, A., Fang, S., Prosky, Braverman, L., Vagenakis, A. (1978)
Decreased outer ring monodeiodination of thyroxine and reverse T_3 in the fetal and newborn rat.
Endocrinology, 103, 2216-2222

Harris, A., Fang, S., Hinerfeld, K., Braverman, L. E., Vagenakis, A. G. (1979)
The role of sulphydryl groups on the impaired hepatic 3',3,5, tri-iodothyronine generation from thyroxine in the hypothyroid, starved, fetal and neonatal rodent.
Journal of Clinical Investigation, 63, 516-524

Harris, G. W. (1948)
Neural control of the pituitary gland.
Physiological Reviews, 28, 139-179

Henning, S., Ballard, P., Kretchmer, D. (1975)
A study of cytoplasmic receptors for glucocorticoids in the intesine of pre/ post weaning rats.
Journal of Biological Chemistry, 250, 2073-2079

Henning, S. J. (1978)
Plasma concentration of total and free B during development in the rat.
American Journal of Physiology, 235, E451-456

Herd, P., Kaplay, S. S., Sanadi, D. R. (1974)
On the origin and mechanism of action of T_4 responsive protein.
Endocrinology, 94, 464-474

Hertzfeld, A. and Greenard, O. (1969)
Endocrine modification of the developmental formation of ornithine aminotransferase in rat tissues.
Journal of Biolgical Chemistry, 244,4894-4898

Hillier, A. P. (1971)
Human T_4 binding globulin and thyroxine-binding pre-albumin dissociation rates.
Journal of Physiology, 217, 625-634

Hiroshige,T., Sato, T., Abe, K. (1971)
Dynamic changes in hypothalamic content of CRF following noxious stimuli. Delayed response in early neonates in comparison with biphasic response in adult rats.
Endocrinology, 89, 1287-1294

Hoch, F. L. (1974)
Metabolic effects of thyroid hormones.
in Handbook of Physiology, Section 7 Endocrinology, Volume III Thyroid, p.391-412.
Executive Editor, Geiger, S. R.
Published by: American Physiological Society, Washington, D.C.

Hollander, C. S., Scott, R. L., Burgess, J. A., Rabinowitz, D., Merimee, T. J., Oppenheimer, J. H. (1967)
Free fatty acid; A possible regulator of free thyroid hormone levels in ma
Journal of Clinical Endocrinology and Metabolism, 27, 1219-1223

Holmes, S., Neto, F., Field, J. (1980)
Effect of in vivo treatment with ACTH and hypophysectomy on adrenal adenosine 3'5'-monophosphate and corticosterone responsiveness to ACTH stimulation in vitro.
Endocrinology, 107, 432-437

Hopkins, P. S. and Thorburn, G. D. (1972)
The effects of fetal thyroidectomy on the development of the ovine fetus.
Journal of Endocrinology, 54, 55-66

Horne, C. H. W. and Nisbet, A. D. (1979)
Pregnancy Proteins: A review.
Investigative Cell Pathology, 2, 217-231

Hossner, K. G. and Billiar, R. B. (1981)
Plasma clearance and organ distribution of native and desialylated rat and human transcortin: Species Specificity.
Endocrinology, 108, 1780-1786

Ichii, S. and Kobayashi, S. (1966)
Studies on the biosynthesis of sterol and corticosterone in the rat adrenal gland.
Endocrinologia Japonicia, 13, 39-45

Imura, H., Sparks, L. L., Grodsky, G. M., Forsham, P. H. (1965)
Immunologic Studies of ACTH. Dissociation of Biologic and Immunologic activities.
Journal of Clinical Endocrinology and Metabolism, 25, 1361-1369

Jacquot, R. (1971)
Some hormonally controlled events in liver differentiation in the perinatal period.
in Hormones and Development, p.587-600
Editors: Hamburgh, M. and Barrington, E. J. W.
Publishers: Appleton-Century-Crofts, New York

Jack, P. M. B. and Milner, R. D. G. (1975)
Adenocorticotrophin and the development of insulin secretion in the rabbit fetus.
Journal of Endocrinology, 64, 67-75

Jailer, J. W. (1950)
The maturation of the pituitary-adrenal axis in the newborn rat.
Endocrinology, 46, 420-425

Jambar, S. C. and Greenard, O. (1970)
Premature formation of glucokinase in developing rat liver.
Journal of Biological Chemistry, 245, 2779-2783

Jones, M. T., Brush, F. R., Neame, R. L. B. (1972)
Characteristics of Fast Feedback control of ACTH by corticosteroids.
Journal of Endocrinology, 55, 489-497

Jones, M. T., Gillman, B., Mahmoud, S., Holmes, M. C. (1979)
The characteristics and mechanism of action of corticosteroid negative feedback at the hypothalamus and anterior pituitary.
in Interactions within the Brain-Pituitary-Adrendocortical system, p.163-180
Editors: Jones, M. T., Gillman, B., Dallman, M., Chattopadhyay, S.
Publishers: Academic Press, London, New York, San Francisco

Josimovich, J. Ladman, A. J., Deane, H. W. (1954)
A histological study of the developing adrenal cortex of the rat during fetal and early postnatal stages.
Endocrinology, 54, 627-639

Jost, A. and Hatey, J. (1949)
Influence de la decapitation sur la teneur en glycogene due foie du foetus de lapin.
Comptes Rendus de la Societe de Biologie, 143, 146-147

Jost, A. and Picon, L. (1970)
Hormonal control of fetal development and metabolism.
Advances in metabolic disorders, 4, 123-184

Kalavsky, S. M. (1971)
Fetal rat adrenal steroidogenesis.
Biology of the neonate, 17, 427-435

Kaminski, T., Kohrle, J., Kodding, R., Hesch, R. (1981)
Autoregulation of 3,3',5' tri-iodothyronine production by rat liver microsomes.
Acta Endocrinologia, 98, 240-245

Kamoun, A. (1970)

Activite cortico-surrenal au cours de la gestation, de la lactation et du developpement pre et post-natal chez le rat.

Journal of Physiology (Paris), 62, 5-29

Kaplan, M. and Utiger, R. (1978)

Iodothyronine metabolism in rat liver homogenates.

Journal of Clinical Investigation, 61, 459-471

Karteszi, M., Dallman, M. E., Makara, G. B., Stark, E. (1982)

Regulation of the adrenocortical response to insulin-induced hypoglycaemia.

Endocrinology, 111, 535-539

Kekki, M. (1964)

Serum protein turnover in experimental hypo/hyperthyroidism.

Acta Endocrinologia Supplement, 91, 1-139

Keller, N., Sendelbeck, L. R., Richardson, U. I., Moore, C., Yates, F. E. (1966)

Protein binding of corticosteroids in undiluted rat plasma.

Endocrinology, 79, 884-906

Keller, N., Richardson, U. I., Yates, F. E. (1969)

Protein binding and the biological activity of corticosteroids. In vivo induction of hepatic and pancreatic alanine aminotransferase in normal and oestrogen treated rats.

Endocrinology, 84, 49-62

Kikkawa, B. W. Y., Orzalesi, M. M., Motoyama, E. K., Kaibara, M., Zigas, C. J., Cook, C. D. (1973)

The effect of T_4 on the maturation of fetal rabbit lungs.

Biology of the neonate, 22, 161-168

Kimura, T. (1969)

Effects of hypophysectomy and ACTH administration on the level of adrenal cholesterol side-chain cleavage demolase.

Endocrinology, 85, 492-499

Knobil, E. and Josimovich, J. B. (1959)

Placental transfer of thyrotrophic, thyroxine, tri-iodothyronine and insulin in the rat.

Annals of New York Academy of Sciences, 75, 895-904

Koch, B., Mialhe-Voloss, C., Stutinsky, F. (1967a)
Fractions liees ar libre de la corticosterone plasmatique et cours du developpment postnatal chez de rat.
Comptes Rendus, herbonnadaires des Seances de L' Academie des Science, D 264, 2497-2500

Koch, B., Miahle-Voloss, C., Stutinsky, F. (1967b)
Capacite de liason de la transcortine et reponse a l'agression pendant le periode post-natal chez le rat.
Comtes Rendus, herbonndaires des Sciences de l'academie des Science, D 266, 808-810

Krieger, D. (1979)
Plasma ACTH and corticosteroids.
in Endocrinology
Editors: Degroot, L., Cahill, Jr, G. F., Odell, W., Martini, L., Potts, Jr, J. T., Nelson, D. H., Steinberger, E., Winegrad, A. I.
Publishers: Grune and Stratton Ltd.

Kumegawa, M., Maeda, N., Yajima, T., Takuma, T., Ikeda, E., Hanai, H. (1980)
Permissive role of L-thyroxine in the induction of pancreatic amylase by cortisol in neonatal rats.
Journal of Endocrinology, 86, 497-500

Kumegawa, M., Maeda, N., Yajima, T., Takuma, T., Ikeda, E., Minamide, C. (1980)
L-thyroxine, cortisol and diet affect the level of amylase in the parotid gland of the developing rat.
Journal of Endocrinology, 87, 65-71

Labrie, F., Raynaud, J. P., Ducommun, P., Fortier, C. (1964)
Influence of thyroid activity on corticosterone binding by plasma proteins.
Proceedings of Canadian Federal Biology Society, 7, 44

L'Age, M., Gonzalez-Luque, A., Yates, F. E. (1970)
Adrenal blood flow dependence of cortisol secretion rate in unanaesthetised dogs.
American Journal Physiology, 219, 281-287

Lamberg, B. A. (1965)
Glucose metabolism in thyroid tissue.
Acta Medica Scandinavia, 178, 351-362

Landon, J., James, V. H. T., Cryer, R. J., Wynn, V., Frankland, A. W. (1964)

ACTH effects of a synthetic polypeptide β^{1-24}corticotropin in man.

Journal of Clinical Endocrinology and Metabolism, 24, 1206-1213

Leakey, J. and Dutton, G. J. (1975)

Precious development in vivo of UDP-glucuronyl-transferase and aniline hydroxylase by corticosteroids and ACTH using simple continuous flow technique.

Biochemical, Biophysical Research Communications, 66, 250-254

Lebarca, C. and Paigen, K. (1980)

A simple, rapid and sensitive DNA assay procedure.

Analytical Biochemistry, 102, 344-352

Lee, Y. P., Liu. C. Y., Hsu, H. T. (1970)

Effects of thyroid hormone on guinea pig.

Endocrinology, 86, 241-250

Levine, S., Glick, D., Nakane, P. (1967)

Adrenal and plasma B and vitamin A in rat adrenal glands during development.

Endocrinology, 80, 910-914

Liggins, G. C., and Kennedy, P. C. (1968)

Effects of electocoagulation of foetal lamb hypophysis on growth and development.

Journal of Endocrinology, 40, 371-381

Liggins, G. C. (1969)

Premature delivery of foetal lambs infused with glucocorticoids.

Journal of Endocrinology, 45, 515-523

Liggins, G. C., Fairclough, R. L., Grieves, S. A., Kendall, J. Z., Knox, B. S. (1973)

The mechanism of initiation of parturition in the ewe.

Recent Progress in Hormone Research, 29, 111-159

Liggins, G. C. (1976)

Adrenocortical-related maturational events in the fetus.

American Journal of Obstetrics and Gynacology, 126, 931-941

Loose, D. S., Do, Y. S., Chen, T. H., Feldman, D. L. (1980)

Demonstration of glucocorticoid receptors in the adrenal cortex; evidence for a direct dexamethasone suppressive effect on the rat adrenal gland.

Endocrinology, 107, 137-146

Lymangrover, J. R., Brodish, A. (1973)
Tissue CRF: an extra-hypothalamic CRF in the peripheral blood of stressed rats.
Neuroendocrinology, 12, 225-235

Malinowka, K. W., Hardy, R. N., Nathanielsz, P. W. (1972)
Plasma Adrenocorticosteroid concentration immediately after birth in the rat, rabbit and guinea pig.
Experentia, 28, 1366-1367

Malinowka, K. W., Chan, W. S., Nathanielsz, P. W., Hardy, R. N. (1974)
Plasma adreno-corticosteroid changes during T_4 induced accelerated maturation of neonatal rat intestine.
Experentia, 30, 61

Martin, C. E., Cake, M. H., Hartmann, P. E., Cook, I. (1977)
Relationship between foetal corticosteroids, maternal progesterone and parturition in the rat.
Acta, Endocrinologia, 84, 167-176

Melby, J. C. 1 (1979)
Anatomy of the adrenal cortex.
in Endocrinology
Editors: Degroot, L., Cahill, Jr, G. C., Odell, W., Martini, L., Potts, Jr, J. T., Nelson, D. H., Steinberger, E., Winegrad, A. I.
Publishers: Grune and Stratton Ltd.

Meserve, L. and Leathem, J. (1974)
Neonatal hyperthyroidism and the maturation of the rat hypothalamic-hypophyseal-adrenal-axis.
Proceedings of the Society of Experimental Biology and Medicine, 147, 510-512

Meserve, L. and Leathem, J. (1981)
Development of the hypothalamic-pituitary-adrenal response to stress in rats made hypothyroid by exposure to thiouracil from conception.
Journal of Endocrinology, 90, 403-409

Miettinen, T. A. (1968)
Mechanism of serum cholesterol reduction by thyroid hormones in hypothyroidism.
Journal of Laboratory and Clinical Medicine, 71, 537-547

Milkovic, S., and Milkovic, K. (1961)
Reactiveness of fetal pituitary to stressful stimuli. Does maternal ACTH cross the placenta.
Proceedings of the Society of Experimental Biology and Medicine, 107, 47-49

Milkovic, K., Peruzovic, M., Romic, P., Paunovic, J., Pope, K. (1977)
Differential effect of pre- and postnatal litter size reduction on body weight and the development of stress response in the rat.
Biology of the neonate, 32, 83-93

Moll, G. W. M., Rosenfield, R. L. (1978)
Some limitations of using equilibrium dialysis to study human serum albumin-testosterone interaction.
Journal of Clinical endocrinology and Metabolism, 46, 501-503

Moog, F. (1971)
Corticoids and the enzymic maturation of the intestinal surface: Alkaline phosphatase, leucyl naphthylamidase and sucrase.
in Hormones and development, p.143-160
Editors: Hamburgh, M., and Barrington, E. J. W.
Publishers: Appleton-Century-Crofts

Moog, F. (1979)
Endocrine influences on the functional differentiation of the small intestine.
Journal of Animal Science, 49, 239-249

Morishige, W. K., Joun, N. S., Guernsey, D. L. (1982)
Thyroidal influence on postnatal lung development in the rat.
Endocrinology, 110, 444-451

Morishige, W. K. (1982)
Thyroid influences on glucocorticoid receptor levels in the neonatal rat lung.
Endocrinology, 111, 1017-1018

Morreale de Escobar, G. and Escobar del Rey, F. (1962)
Influence of thiourea, potassium perclorate, thiocynate and graded doses of PTU on thyroid hormone metabolism in thyroidectomised rats; isotopically equilibrated with varying doses of exogenous hormone.
Endocrinology, 71, 906-913

Murphy, B. E. P. and Patte, C. J. (1964)

Determination of Thyroxine utilising the property of protein-binding.

Journal of Clinical Endocrinology and Metabolism, 24, 187-196

Murphy, B. E. P. (1967)

Some studies of protein-binding of steroids and their application to routine micro and ultramico measurements of various steroids in body fluids by competitive protein binding assay.

Journal of Clinical Endocrinology & Metabolism, 27, 973-990

Nathanielsz, P. W. (1976)

The fetal thyroid. II The thyroid in the rat, human and sub-human primate fetus.

in Fetal Endocrinology, An experimental approach, p.73-88 published by North-Holland publishing company, Amsterdam, New York, Oxford

Nichols, C. T. and Tyler, F. H. (1967)

Diurnal variation of adrenal cortical function.

Annual Reviews in Medicine, 18, 313-324

Osathanondh, R., Chopra, I. J., Tulchinsky, D. (1978)

Effects of dexamethasone on fetal and maternal thyroxine, tri-iodothyronine, reverse tri-iodothyronine and thyrotropin levels.

Journal of Clinical Endocrinology and Metabolism, 47, 1236-1239

Pankratz, D. S. (1931)

The development of the superadrenal gland in the albino rat, with consideration of its possible relation to origin of foetal movements.

Anatomical Records, 49, 31-49

Pasquini, J. M., Kaplun, B., Garcia-Argiz, C. A., Gomez, C. J. (1967)

Hormonal regulation of brain development. 1. The effect of neonatal thyroidectomy upon nucleic acids, protein and 2 enzymes in the developing cortex and cerebellum of the rat.

Brain Research, 6, 621-634

Pearlman, W. H., and Crepy, O. (1967)

Steroid-protein interactions with particular reference to testosterone binding by human serum.

Journal of Biological Chemistry, 242, 182-189

Philpott, J. E., Zarrow, M. X., Denenberg, V. H. (1969a)
Prevention of drop in adrenocortical activity in the 7 day old rat by pretreatment with ACTH.
Proceedings of the Society of Experimental Biology and Medicine, 131, 26-39

Philpott, J. E., Zarrow, M. X., Denenberg, V. H. (1969b)
The presence of adult type pattern of adrenal steroids in the one day old rat.
Steroids, 14, 21-31

Raiha, N. C. R. and Suihkonen, J. (1966)
The development of enzymes for urea biosynthesis in the rat and human liver.
Proceedings of the 36th Annual Meeting of the Society for Paediatric Research, Atlantic City, Paper No. 6.

Rall, L. B., Pictet, R. J., Githens, S., Rutte , W. J. (1977)
Glucocorticoids modulate the in vitro development of the embryonic rat pancreas.
Journal of Cell Biology, 75, 398-409

Rees, L. H., Jack, P. M. B., Thomas, A. L., Nathanielsz, P. W. (1975)
Role of adrenocorticotrophin during parturition in sheep.
Nature (London), 253, 274-275

Reichlin, S., Martin, J. B., Mitnick, M., Boshans, R., Grimm, Y., Bollinger, J., Gordon, J., Malacara, J. (1972)
The hypothalamus in pituitary-thyroid regulation.
Recent Progress in Hormone Research, 28, 229-286

Richardson, M. C., and Schulster, D. (1972)
Corticosteroidogenesis in isolated adrenal cells: Effect of adrenocorticotrophic hormone adenosine 3',5',-monophosphate and β^{1-24}adrenocorticotrophic hormone diazotised to polyacrylamide.
Journal of Endocrinology, 55, 127-139

Rivier, C., Vale, W., Guilleman, R. (1973)
An in vivo CRF assay based on plasma levels of radioimmunoassayable ACTH.
Proceedings of the Society of Experimental Biology and Medicine, 142, 842-845

Rodbard, D., and Lewald, J. E. (1970)
Computer analysis of radioligand assay and radioimmunoassay.
Karolinska Symposia on Research methods in Reproductive Endocrinology 2nd Symposium: Steroid assay by protein binding, 79-103

Roffi, J. (1971)

Mechanisms of action of corticosterone on physiological maturation of the foetal adrenal medulla.

in Hormones in Development, p.203-212

Editors: Hamburgh, M. and Barrington, E. J. W.

Publishers: Appleton-Century-Crofts, New York

Rosner, W. and Bradlow, H. L. (1974)

Isolation and Purification of Corticosteroid binding globulin from human plasma by affinity chromatography.

in Methods of enzymology, 36, Hormone Action, Part A, Steroid Hormones, p.104-109

Editors: O'Malley, B. W., Hardman, J. G.

Publishers: Academic Press, New York

Roti, E., Braverman, L. E., Fang, S., Alex, S., Emerson, C. (1982)

Ontogenesis of placental inner ring T_4 deiodinase and amniotic fluid 3,3',5'-T_3 concentration in the rat.

Endocrinology, 111, 959-965

Roti, E., Fang, S., Braverman, L., Emerson, C. (1982)

Rat placenta in an active site of inner ring deiodination of T_4 to rT_3.

Endocrinology, 110, 34-37

Samuels, L. T. and Nelson, D. H. (1975)

Biosynthesis of corticosteroids

in Handbook of Physiology, Section 7, Volume VI, Adrenal Gland

Executive editor: Geiger, S.

Published by American Physiological Society, Washington DC

Schapiro, S., Geller, E., Eiduson, S. (1962)

Neonatal Adrenal cortical responses to stress and vasopressin.

Proceedings of the Society of Experimental Biology and Medicine, 109, 937-941

Schapiro, S. and Norman, R. (1967)

Thyroxine: Effects of neonatal administration of maturation, development and behaviour.

Science, 155, 1279-1280

Schoenfield, N. M., Leathem, J. H., Rabii, J. (1980)

Maturation of adrenal stress responsiveness in the rat.

Neuroendocrinology, 31, 101-105

Schwartz, H. L., Surks, M. I., Oppenheimer, J. H. (1971)
Quantitation of extrathyroidal conversion of L-thyroxine to T_3 in the rat.
Journal of Clinical Investigation, 50, 1124-1130

Sereni, F. and Barnabei, O. (1967)
Nuclear RNA polymerase activity, rate of RNA synthesis and hydrolysis during development.
Advances in enzyme regulation, 5, 165-171

Sereni, F., Kenney, F. T., Kretchmer, N. (1959)
Factors influencing the development of tyrosine ∝ ketoglurate transaminase activity in the rat.
Journal of Biological Chemistry, 234, 609-615

Singer, P. A., and Nicoloff, J. T. (1972)
Estimation of T_3 secretion rate in euthyroid man.
Journal of Clinical Endocrinology and Metabolism, 35, 82-89

Steele, R. E. and Wekstein, D. R. (1972)
Influence of thyroid hormone on homeothermic development of the rat.
American Journal of Physiology, 222, 1528-1533

Steele, R. (1975)
Influence of corticosteroids on protein and carbohydrate metabolism.
in Handbook of Physiology Section 7 Endocrinology, Volume VI, Adrenal Gland
Executive Editor: Geiger, S.
Published by American Physiological Society, Washington DC

Sterling, K. and Milch, P. O. (1975)
Thyroid hormone binding by a component of mitochondrial membrane.
Proceedings of the National Academy of Sciences, USA, 72, 3225-3229

Stillman, M. A., Recht, L. D., Rosario, S. L., Seif, S. M., Robinson, A. G., Zimmerman, E. A. (1977)
The effects of adrenalectomy and glucocorticoid replacement of vasopressin and vasopressin-neurophysin in the zona externa of the medial eminence of the rat.
Endocrinology, 101, 42-49

Tang, F. and Phillips. (1977)
Pituitary-adrenal response to ether stress in neonatal rats.
Journal of Endocrinology, 75, 184-185

Tarentino, A. L., Richet, D. A., Westerfeld, W. W. (1966)
The concurrent induction of glycerophosphate dehydrogenase and malate dehydrogenase by thyroid hormone.
Biochemica, Biophysica Acta, 124, 295-309

Tatematsu, M., Takahashi, M., Tsuda, H., Hirose, M., Furihata, C., Sugimura, T. (1975)
Precious differentiation of immature chief cells in fundic musoca of infant rats by hydrocortisone.
Cell Differentiation, 4, 285-294

Thomas, A. L., Krane, E. J., Nathanielsz, P. W. (1978)
Changes in the fetal thyroid axis after induction of premature parturition by low dose continous intravascular cortisol infusion to the fetal sheep at 130 days of gestation.
Endocrinology, 103, 17-23

Thomas, A. L. and Nathanielsz, P. W. (1983)
The fetal thyroid
in Current Topics in Experimental Endocrinology
Editors: James, V. H. T. and Martini, L.
Publishers; Academic Press

Thorburn, G. D., Water, M. J., Young, I. R., Dolling, M., Buntine, D., Hopkins, P. S. (1981)
Epidermal growth factor: a critical factor in fetal maturation.
in Ciba Foundation Symposium 86. The fetus and dependent life.
Publishers; Pitman Press

Tice, L. W. and Wollman, S. H. (1974)
Ultrastructural localisation of peroxidase on pseudopods and other structures of the typical thyroid epithelial cell.
Endocrinology, 94 1555-1567

Trapp, G. A. and West, C. D. (1969)
Determination of corticosteroid-binding proteins by an absorption method.
Journal of Laboratory and Clinical Medicine, 73, 861-871

Upton, G. V., Corbin, A., Mabry, C. C. (1973)
Evidence for the Internal feedback phenomenon in Human subjects: Effects of ACTH on plasma CRF
Acta Endocrinologia (Kbh) 73, 437-441

Vagenakis, A. G., Burger, A., Portnay, G. I., Rudolph, M., O'Brian, J. T., Azizi, F., Arky, R. A., Nicod, P., Ingbar, S., Braverman, L. E. (1975)
Diversion of peripheral thyroxine metabolism from activating to inactivating pathways during complete fasting.
Journal of Clinical Endocrinology and Metabolism, 41, 191-194

Vale, W., Spiess, J., Rivier, C., Rivier, J. (1981)
Characterisation of a 41-residue ovine hypothalamic peptide that stimulates secretion of ACTH and β endorphin.
Science, 213, 1394-1397

Van Baelen, H. V., Vandoren, G., De Moor, P. (1977)
Concentration of transcortin in the pregnant rat and its foetuses.
Journal of Endocrinology, 75, 427-431

Van Dorp, A. W. R., and Deane, H. W. (1950)
A morphological and cytochemical study of the postnatal development of rat adrenal cortex.
Anatomical Records, 107, 265-281

Van Middlesworth, L. (1957)
Thyroxine secretion, a possible cause of goitre.
Endocrinology, 61, 570-573

Walkins, W. B., Schwabedal, P., Bock., P. (1974)
Immunohistochemical demonstration of a CRF associated neurophysin in the external zone of the rat median eminence.
Cell and Tissue Research, 152, 411-421

Walker, P., Weichsel, M. E., Fisher, D. A., Guo, S. M., Fisher, D. (1979)
Thyroxine increases NGF concentration in adult mouse brain.
Science, 204, 427-429

Walton, K. W., Scott, P. J., Dykes, P. W., Davies, J. W. L. (1965)
The significance of alterations in serum thyroid disfunction.II
Alterations in metabolism of ^{131}I low density lipoproteins in hypothyroidism and thyrotoxicasis.
Clinical Science, 29, 217-238

Wellman, K. F., and Volk, B. W. (1972)
Fine structural changes in the rabbit placenta induced by cortisone.
Archives of Pathology, 94, 147-157

Wells, L. J. (1947)

Progress of studies designed to determine whether the foetal hypophysis produces hormone that influence development.

Anatomical Records, 97, 409-421

Westgren, U., Melander, A., Wahlin, E., Lindgren, J. (1977)

Divergent effects of 6-propythoiuracil on T_3 and rT_3 serum levels in man.

Acta Endocrinologia, 85, 345-350

Wicks, W. D. (1968)

Induction of tyrosine ∝ ketoglutarate transaminase in fetal rat liver.

Journal of Biological Chemistry, 243, 900-906

Widdas, W. F. (1952)

Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer.

Journal of Physiology, 118, 23-39

Wiersinga, W. M., Modderman, P., Touber, J. L. (1980)

The effect of ∝ and β adrenoceptor agonists and antagonists on the in vivo conversion of thyroxine to tri-iodothyronine.

Hormone and Metabolic Research, 12, 346-347

Wilber, J. F., and Utiger, R. D. (1969)

The effect of glucocorticoids on thyrotrophin secretion.

Journal of Clinical Investigation, 48, 2096-2103

Wilkins,L. (1941)

Epiphysial dysgenesis associated with hypothyroidism.

American Journal of Diseases of Children, 61, 13-34

Wolf, G. and Rivlin, R. S. (1970)

Inhibition of thyroid hormone induction of mitochondrial ∝ glycerophosphate dehydrogenase in riboflavin deficiency.

Endocrinology, 86, 1347-1353

Wurtman, R. J., and Axetrod, J. (1966)

Control of enzymatic synthesis adrenaline in the adrenal medulla by adrenal cortical steroids.

Journal of Biological Chemistry, 241, 2301-2305

Wysocki, S. J. and Segal, W. (1972)

Influence of thyroid hormones on the enzyme activities of myelinating rat central nervous system.

European Journal of Biochemistry, 28, 183-189

Yamada, T. and Greer, M. A. (1959)
Studies on the mechanism of hypothalamic control of TSH secretion: Effect of T_4 injection into the hypothalamus or the pituitary on thyroid hormone release.
Endocrinology, 64, 559-566

Yates, F. E. and Maran, J. W. (1975)
Stimulation and inhibition of adrenocortical release.
in Handbook of Physiology, Section 7, Volume VI Adrenal Gland.
Executive Editor: Geiger, S.
Published by American Physiological Society, Washington, DC.

Zarrow, M. X., Haltmeyer, G. C., Denenberg, V. H., Thatcher, J. (1966)
Response of the infantile rat to stress.
Endocrinology, 79, 631-634